Dissection of the Mechanisms of Action of ES-62 in Inflammatory Disease

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A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

Faculty of Biomedical and Life Sciences

April 2006

Dedication

To an eternal optimist, my grandfather, Kendal Kean (1907-2005)

Summary

Filarial nematode parasite infections are endemic in developing countries of the Tropics. Collectively known as filariasis, these helminth parasite infections can occasionally induce gross inflammatory pathology in the infected host, however, more commonly, the host exhibits a somewhat suppressed non-inflammatory immune response to the parasite, permitting longevity of infection. Improved sanitation, increased use of antibiotics and vaccination has all but eradicated parasite infection in developed, Western society. Coincident with this improvement in hygiene, however, inflammatory autoimmune (e.g. rheumatoid arthritis) and allergic disease (e.g. asthma) has become more prevalent in these countries; a trend not observed in parasite-endemic countries. The hygiene hypothesis controversially proposes that these trends are directly associated, by predicting that insufficient exposure to pathogens in childhood results in development of an aberrant immune system that responds inappropriately to non-pathogenic stimuli as is manifested by allergy and/or autoimmunity. Thus, conversely, it has been proposed that exposure to parasite infection may prevent development of autoimmune and/or allergic diseases.

Filarial nematodes secrete immunomodulatory excretory-secretory (ES) products into the host bloodstream, which act to modulate inflammatory host immune responses and thus, protect the parasite from elimination. ES-62, an immunomodulatory glycoprotein product of rodent filarial nematode, *Acanthocheilonema viteae*, has previously been shown to modulate the responses of several immune cell types to promote an anti-inflammatory immune response *in vitro* and *in vivo*. Thus, it was hypothesised that ES-62 may exhibit anti-inflammatory potential in models of inflammatory disease prevalent in the Western world. Indeed, in a collagen-induced model of rheumatoid arthritis (CIA) ES-62 treatment induced amelioration of inflammation. Furthermore, this effect was mediated when ES-62 was administered before or after the onset of clinically detectable inflammation. In Chapter 3 of this thesis, progress towards dissection of the mechanisms of action of ES-62 in the CIA model was made, by analysing the role of phosphorylcholine (PC), a component of ES-62 and several other immunomodulatory pathogen-derived products. Indeed, the anti-inflammatory action of ES-62 in the CIA model was found to be PC-dependent, evidenced by reproduction of the anti-inflammatory effect when PC conjugated to albumin protein, OVA (OVA-PC) was used as a substitute and loss of the effect when PC-deficient recombinant ES-62 (rES-62) was used. However, not all of the immunomodulatory effects of ES-62 in this model were deemed PC-dependent. For example, the previously determined inhibitory effect of ES-62 on antigen-specific IgG2a production in the CIA

model was found to be PC-independent and thus, not directly associated with the anti-inflammatory action of ES-62. In addition, it was determined that ES-62 treatment *in vivo* or *in vitro* disrupted contact-dependent communication between T cells and macrophages, evidenced by inhibition of macrophage pro-inflammatory cytokine production. This method of cell-to-cell communication has been hypothesised as fundamental for progression and maintenance of synovial inflammation in RA, thus it was concluded that this may be a method by which ES-62 mediated therapeutic action in this model of autoimmune disease.

The anti-inflammatory action of ES-62 *in vivo* was not restricted to inhibition of TH1-mediated inflammation, such as that exhibited in CIA. Utilising a TH2-mediated ovalbumin (OVA)-induced model of allergic airway inflammation (such as that exhibited in asthma), it was determined that treatment with ES-62 during or after the onset of disease reduced peri-bronchial inflammation and airway eosinophilia (Chapter 4). This inhibitory action was associated with reduced production of antigen-specific TH2-type cytokines and antigen-specific IgE, a hallmark feature of allergic inflammation. Furthermore, examination of bone marrow dendritic cells (DC) derived from this model of airway inflammation revealed that ES-62 treatment resulted in differentiation of DC with a modulated phenotype, evidenced by enhanced expression of co-stimulatory molecules and production of TH1-promoting cytokine, IL-12. These findings suggested that such modulation of DC may be a mechanism of action by which ES-62 mediates anti-inflammatory action in this model.

In addition to the reduction of TH1-mediated inflammation and TH2-mediated inflammation in the CIA and OVA-induced airway inflammation models respectively, an anti-inflammatory effect of ES-62 was additionally demonstrated in the MRL/*lpr* model of autoimmune systemic lupus erythematosus (SLE). In Chapter 5, prophylactic ES-62 treatment of MRL/lpr mice was demonstrated to inhibit development of nephritis and articular inflammation, two pathologies commonly associated with SLE in humans. These actions of ES-62 were evidenced by a reduction of proteinuria levels and footpad swelling, respectively. The anti-inflammatory effects of ES-62 in this model were were not associated with modulation of lymphadenopathy, splenomegaly or hypergammaglobulinemia. However it was associated with a reduced inflammatory mediator response of lymph node cells and splenocytes to a mitogenic stimulus. Furthermore, DC derived from ES-62 treated MRL/lpr mice exhibited a reduced response to LPS stimulation, in terms of cytokine production, indicating that modulation of DC function may be a potential mechanism of action of ES-62 in this model of autoimmune inflammation.

Consistent with a role for PC as an immunomodulatory component of parasite-derived molecules such as ES-62, it was previously demonstrated that PC-containing glycosphingolipid (GSL) derived from the filarial nematode, *Ascaris suum*, exhibits immunomodulatory properties when used to treat macrophages, B cells and peripheral blood monocytes. To extend this study, it was demonstrated in Chapter 6 that *A. suum*-derived GSL inhibited the response of DC to LPS, evidenced by a reduction in LPS-induced cytokine production and upregulation of cell surface co-stimulatory molecules. Previously, such immunomodulatory action of *A. suum* GSL was demonstrated in macrophages to be PC-dependent. However the PC-dependency of GSL action was not apparent with DC. However, by examining synthetic mimetics of *A. suum* GSL PC was confirmed as an immunomodulatory constituent of GSL. Nevertheless, the presence of PC was per se found to be insufficient for immunomodulatory action on cells of the innate immune response (e.g. macrophages and DC). Indeed, it was shown that carbohydrate components of glycolipids are also necessary for such modulation of an immune response. Nevertheless, the findings presented in this chapter indicated that small PC-containing molecules with immunomodulatory activity can be synthesised in the laboratory and therefore, represent potential candidates for design of immunomodulatory drugs.

DC are the principle antigen-presenting cell type responsible for activation of T cells and hence, the ensuing antigen-specific immune response *in vivo*. Throughout the studies comprising this project it was clear that DC are often targeted by immunomodulators such as PC-containing ES products to facilitate manipulation of the immune system. Thus, it was speculated that allergenic substances might also employ manipulation of DC function for induction of an allergic immune response. Indeed, in Chapter 7, it was demonstrated that 2S seed albumins, SFA 8 and Ber e 1, which exhibited distinct allergenic properties also mediated distinct effects on DC functions *in vitro*. Exposure to SFA 8 (of ambiguous allergenicity), but not Ber e 1 (allergenic), resulted in production of TH1-promoting cytokines (IL-12 and TNFα) and enhanced expression of co-stimulatory and antigen-presentation molecules by DC. Moreover, using a transgenic OVA -specific TCR system, it was observed that pre-treatment of DC with Ber e 1, but not SFA 8 induced production of TH2-promoting cytokines, IL-4 and IL-5 by co-cultured OVA -specific T cells. This was reflected in such T cells by an enhanced expression of GATA-3, a TH2-type transcription factor. Thus, it appeared that the allergenicity of Ber e 1, compared with SFA 8, was associated with its ability to stimulate TH2-promoting cytokine production by DC. Therefore, it was concluded that allergenic proteins can also mediate their effects via modulation of DC in susceptible individuals.

In summary, the novel findings presented in this thesis support the theory that parasite infection may be protective against development of autoimmune and allergic inflammatory diseases prevalent in developed society. Furthermore, this protective effect may be facilitated by the immunomodulatory actions of parasite-derived, PC-containing ES products. Thus, via modulation of the function of innate antigen-presenting cells, such as dendritic cells, immunomodulatory molecules are capable of manipulating the action of immune system as a whole. This discovery draws attention to a highly evolved anti-inflammatory mechanism employed by parasites, which could potentially be exploited to design therapy for human inflammatory diseases of today.

Declaration

This work represents original work carried out by the author and has not been submitted in any form to any other University.

Dorothy Elizabeth Kean

April 2006

Acknowledgements

Firstly, I would like to thank my supervisor, Professor Maggie Harnett for her continued support and patience during the time I spent conducting laboratory research and in preparation of this manuscript. I began this project as a newcomer to the field of immunology. Therefore, by acting as a fountain of knowledge and skill, Maggie has helped me to learn the essentials of immunological research.

I would also like to thank Professor Billy Harnett and the members of his laboratory (including Ms Caitlin Egan and Dr Katrina Houston) in Strathclyde University for providing a constant supply of ES-62, essential for the majority of the experiments undertaken.

The research presented in this thesis extends across many topics of investigation, incorporating different techniques. Indeed, several of the investigations were made possible by the contributions of different collaborators; therefore I have many people to whom I am indebted for providing materials, equipment or helping with the running of disease models. I would like to thank Professor Iain McInnes, Dr Alastair Gracie, Dr Agnes Boitelle, Professor Rudolf Geyer, Dr Marcos Alcocer, Dr Charlie McSharry, Professor Mandy MacLean and members of their respective research teams.

In particular I would like to thank Drs Helen Goodridge and Fraser Marshall, who taught me the fundamentals of immunological research techniques and cell culture and Dr Ian Morecroft who trained me to use the myography technique. To the members of 'Lab 403', both past and present, thank you for creating an environment that was fun to work in and providing banter that will be hard to match.

"Some people think only intellect counts: knowing how to solve problems, knowing how to get by, knowing how to identify an advantage and seize it. But the functions of intellect are insufficient without courage, love, friendship, compassion and empathy" (Dean Koontz)

I am fortunate to be surrounded by people in my personal life who care a lot about me and I would like to take this opportunity to mention them. Especially to Eleanor, Claire, Veronika, Nat and the MacDonald clan who I have spent most of my time with outside of my research. Thank you for keeping a smile on my face and a drink in my hand (!).

Finally I would like to thank three very important people, who have helped to maintain my quality of life over the past four years, especially during preparation of this manuscript.

Mum and Pat, your constant encouragement has been a source of strength for me when the going got tough. Thank you for your unfaltering conviction in my abilities and for knowing me better than I know myself.

Last, but certainly not least, to David. You have been by my side throughout this project and never once has your faith in me wavered. I am very grateful for the daily, unconditional support and love you have provided me with. Thank you for halving my worries and giving me twice as many reasons to celebrate.

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Abbreviations

ALUM, aluminium hydroxide
APC, antigen presenting cell
BAL, broncho-alveolar lavage
BCR, B cell receptor
BM, bone marrow
BSA, bovine serum albumin,
CD, cluster of differentiation
CFA, complete Freund's adjuvant
CIA collagen-induced arthritis
Con A, Concanavalin A
CTL cytotoxic T lymphocyte
CTLA, cytotoxic T-lymphocyte associated antigen
DC, dendritic cell
DNA, deoxyribonucleic acid
EAE, experimental autoimmune encephalomyelitis
ELISA, enzyme-linked immunosorbance assay
ERK, extracellular regulated kinase
ES, excretory-secretory
FAM, 6-carboxy-fluorescin
FCS, foetal calf serum
FGF, fibroblast growth factor
FITC, fluorescein isothiocyanate
FSC, forward scatter
GRO, growth regulated oncogene
GM-CSF, granulocyte-macrophage colony-stimulating factor
GSL, glycosphingolipid
HLA, human leukocyte antigen
HPRT, hypoxanthine-guanine phosphoribosyltransferase
ICAM, intracellular adhesion molecule
IFN, interferon
Ig, immunoglobulin
IL, interleukin
i.n., intra-nasal
IP-10, interferon-inducible protein - 10
i.p., intra-peritoneal
i.v., intra-venous
JNK, c-Jun N-terminal kinase
KC, Keratinocyte cytokine
KCl, potassium chloride
kDa, kilo Daltons
KO, knock-out
LPS, lipopolysaccharide
M, molar
MAP, mitogen activated protein
MCh, methacholine
MF, microfilariae
MHC, major histocompatibility complex
MIG, monokine induced by gamma interferon
MIP, macrophage inflammatory protein
mRNA, messenger RNA
MS, multiple sclerosis
NF-κB, nuclear factor-κB
NK, natural killer
NO, nitric oxide
OD, optical density
OVA, Ovalbumin
PAMP, pathogen-associated molecular pattern

PBMC peripheral blood mononuclear cell

PBS, phosphate buffered saline

PC, phosphorylcholine

PCR, polymerase chain reaction

PE, phycoerythrin

PI, propidium iodide

PMA, Phorbol 12-myristate 13-acetate

PRR, pattern recognition receptor

r, recombinant

RA rheumatoid arthritis

RNA, ribonucleic acid

RT-PCR, reverse transcriptase polymerase chain reaction

s.c. subcutaneous

SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

SLE systemic lupus erythematosus

SSC, side scatter

STAT, signal transducer and activator of transription

T1DM, type I diabetes mellitus

TAMRA, 6-carboxy-tetramethyl-rhodamine

TCR, T cell receptor

TGF, transforming growth factor

TH, T helper

TLR, Toll-like receptor

TNF, tumour necrosis factor

T_{reg}, regulatory T cell

VEGF, vasoactive endothelial growth factor

1 General Introduction

1.1 The immune system

Analogous to the defence of a country against its enemies, the human body must protect itself against invasion by pathogens, which is the role of the highly evolved human immune system. To carry out its function the immune system forms two categories of defence. Firstly, forming the initial line of defence, the innate immune response and secondly, eliciting a specifically tailored method of protection, the adaptive immune response.

1.1.1 The innate immune system: providing a physical barrier to infection

The innate immune system is less highly evolved than the adaptive immune system and comprises the entire immune system in invertebrates. Primarily, the role of the innate immune system is to prevent external pathogens entering the body tissues. This is achieved by a variety of physical mechanisms including shedding of epithelial skin layers, secreting mucus to line vulnerable epithelial areas of access to the body (such as the airways and intestine) and the epithelial cilia that sweep away this mucus layer. All of these methods help to refresh the surfaces of the body that come into contact with potentially pathogenic substances from the external environment.

Following breach of the external barriers (e.g. breaking the skin) the human body tissues are vulnerable to invasion by harmful pathogens, such as bacteria. At this point, the biologically active aspect of the innate immune system is triggered and is responsible for recognition and disposal of the invading pathogen. This aspect of innate immunity comprises soluble proteins and bioactive molecules that are either constitutively present in biological fluids (such as complement proteins), or are released from activated cells. These include cytokines that regulate the function of innate cells such as TNFα and IFNα/β, lipid mediators (e.g. leukotrienes and prostaglandins) of inflammation and bioactive enzymes (including plasmin) that contribute to inflammation. Innate immune system cells are normally phagocytes (e.g. macrophages, dendritic cells and neutrophils) or granulocytes (e.g. mast cells, eosinophils) adept at consuming particulate substances and secreting the soluble mediators of inflammation. To be effective, the immune system must accurately recognise pathogens and pathogenic substances (and distinguish them from self). In addition, it must destroy and dispose of such substances and finally, there must be appropriate communication of these actions between cells of the immune system. Thus, when activated, innate immune cells normally phagocytose pathogens and produce pro-inflammatory cytokines to activate other nearby innate immune cells.

1.1.2 Recognition of pathogens by the innate immune system

Innate recognition of pathogens is achieved by activation of pattern recognition receptors (PRRs) expressed on the surface of innate immune cells, such as macrophages and dendritic cells (DC). These PRRs enable identification of pathogen-associated molecular patterns (PAMPs), exhibited by categories of common pathogens. Thus, ligation of PRRs activates mechanisms, which bring about destruction and disposal of the pathogen displaying the PAMP. Common examples of PAMPs include lipopolysaccharide (LPS), a component of Gram-negative bacterial cell wall, CpG DNA motifs (specific to bacteria), double stranded RNA (specific to viruses) and mannans, a component of fungi cell wall. To complement this range of PAMPs, there exists families of PRRs, for example, Toll-like receptors (TLRs), which are commonly expressed on the surface of several immune system cells. TLRs derive their name from their similarity to the protein *Toll*, involved in fungal defence by Drosophila [1]. These receptors are membrane bound and upon ligation by specific PAMP, they trigger an intracellular signaling pathway, which induces innate immune cell activation and responses. For example, TLR4, one of the first TLRs to be identified, is specific for LPS from Gram-negative bacteria [2].

1.1.3 Activation of the Adaptive Immune System

Of the cell types that comprise the innate immune response dendritic cells (DC) in particular have a wide-ranging, important role. As a professional antigen-presenting cell, one of the main functions of DC is to form a conduit between the innate and adaptive immune responses. Following exposure to 'danger' signals, such as pathogen products that signal via TLRs and pro-inflammatory cytokines, immature DC undergo a series of phenotypic changes. This results in the transition from a phenotype that is highly efficient at antigen-processing to a phenotype ideally suited for activating naïve $CD4^+$ T cells. Thus, when a pathogen invades the body it is recognised by DC, which phagocytoses the pathogen and, following processing, presents antigen at the cell membrane, complexed with a class II major histocompatability complex (MHCII) protein. Once activated, the expression of co-stimulatory and adhesion molecules is upregulated on the surface of the DC in preparation for activation of a naïve $CD4^+$ T cell (Figure 1.1) [3]. Naïve T cells express reciprocal surface proteins, which act as receptors for the co-stimulatory molecules expressed on the DC and help to ensure activation of the T cell in response to antigen recognition. Furthermore, the pattern of cytokines secreted by DC at the time of antigen-presentation helps to determine the phenotype of the adaptive immune response [4, 5].

1.1.4 Adaptive Immune System: initiation of the specific response

$CD4^+$ T cells are important for directing the nature of adaptive immune responses, which is achieved by secreting particular patterns of cytokines. This function normally

determines whether an antigen-specific immune response is mediated by antibodies (humoral) or cells (e.g. $CD8^+$ T cells). Thus, $CD4^+$ T helper cells generally direct ('help') the function of B cells, which are principally involved in secretion of antibodies. Antibodies act in an antigen-specific manner to promote pathogen elimination by targeting and activating the functions of cells of the innate immune system. In contrast, $CD8^+$ T cells or cytotoxic T lymphocytes (CTLs), as their name implies, are primarily involved in effector cell-mediated responses, where upon recognition of their specific antigen they directly kill target cells.

Lymphocytes excel at recognition of antigens and like cells of the innate immune system, T and B cells also express recognition receptors on their surface, known as the T cell receptor (TCR) and the B cell receptor (BCR), respectively. The TCR and BCR are put together from a combination of several genes in such a way that each lymphocyte displays a different specificity and as a result recognizes unique antigen. Therefore, these receptors are much more selective than those expressed by cells of the innate immune response. Importantly, however, unlike the innate immune system this random generation of antigen receptors by the adaptive immune system means it cannot decipher the nature of the antigen, and therefore can result in the recognition of self-antigen, which can lead to autoimmune disease. Therefore, prior to export into the periphery, T and B cells undergo rigorous selection processes in the thymus and bone marrow, respectively, leading to the generation of a population of mature cells that are tolerant to self-antigen but specific for foreign antigen. Furthermore, in the periphery $CD4^+$ T cells will normally only be activated by antigen if the antigen for which they are specific is presented to them and, furthermore, only if it presented to them in the context of MHCII, with accompanying co-stimulation (e.g. via CD40-CD40L interaction). Considering only antigen-presenting cells express MHCII and co-stimulatory molecules, this system helps to ensure that only antigens that have first been recognized as pathogenic by cells of the innate immune response are able to activate T cells (Figure 1.2). Thus, upon presentation and recognition of specific antigen a lymphocyte will be activated to proliferate, generating several copies of itself, all with identical specificity, known as a clone. The majority of the members of this clone will then differentiate and carry out their effector function, while others remain, known as memory cells. Thus, any subsequent response to the same antigen will be faster and result in more efficient removal of the pathogen.

1.1.5 Different types of adaptive immune response

As mentioned above, the pattern of cytokines secreted by antigen-presenting cells at the time of T cell activation help to define the nature of the adaptive immune response. Two main types of antigen-specific adaptive immune response were originally identified and characterized as TH1-type and TH2-type immune responses, based on the pattern of

cytokines secreted by TH1 and TH2 CD4+ T helper (TH) cells respectively [6]. TH1 immune responses are primarily cell-mediated involving the actions of CTLs and macrophages, whilst TH2 immune responses are generally antibody-mediated and involve the actions of mast cells and eosinophils (Figure 1.3). In terms of infection with pathogens, TH1-type immune responses are normally activated in response to intracellular (e.g. bacterial) infections and can be distinguished by production of IFNγ by TH1 cells, whilst TH2-type immune responses are activated by extracellular (e.g. helminth parasite) infection, characterised by production of TH2 cytokines, IL-4, IL-13 and IL-5 by $CD4^+$ TH2 cells [7, 8].

Generally the aspects required to execute an adaptive immune response can be divided into two categories: initiation mechanisms and effector mechanisms (Figure 1.4). Initiation of the adaptive immune response involves recognition of the activating antigen (e.g. pathogen) and activation of $CD4^+$ T cells, which then have the power to direct the cells of the adaptive immune response. Effector mechanisms of the immune response include the actions of the immune system following recognition of antigen, in an effort to destroy the antigen and rid the body of it. For example, B cell antibody production in conjunction with macrophage-mediated antigen phagocytosis or CTL-mediated cell destruction. Thus, initiation and effector mechanisms of the immune response generally involve cells of both the innate and adaptive immune responses.

1.2 Autoimmunity: the consequence of a dysfunctional immune system

The principle function of the immune system is to defend against infection by attacking and eliminating harmful pathogens, whilst not attacking the body's own cells and tissues. A dysfunctional immune system can therefore initiate development of chronic, disabling autoimmune diseases. Indeed, more than 80 clinically distinct autoimmune diseases have been discovered and diagnosed to date [9]. Several of these diseases are well known, for example, rheumatoid arthritis (RA), multiple sclerosis (MS), type I diabetes and systemic lupus erythematosus (SLE); however most are relatively unheard of, such as pemphigus and autoimmune inner ear disease. Collectively, autoimmune diseases afflict approximately 5-8% of the US population. This prevalence is greater than that of cancer (approximately 3%) and in the same range as heart disease (8%)[9]. All autoimmune diseases are chronic, disabling disorders that have the ability to inflict a poor quality of life. Furthermore, these diseases can be organ-specific, such as diabetes; or can affect several body systems simultaneously, such as in SLE.

1.2.1 T cells in autoimmunity

Prevention of the immune system targeting the body's own cells or molecules (known as autoreactivity) is mediated by a series of processes known as immune tolerance ([10-12]) and is conducted in two main sites. Immature autoreactive B and T cells naturally arising in the bone marrow and thymus are prevented from maturing and entering the peripheral circulation, as part of a process known as central tolerance. However, this process is not perfect and a small percentage of autoreactive lymphocytes escape to the circulation. In normal non-autoimmune people, these autoreactive cells are prevented from attacking self by mechanisms of peripheral tolerance. Peripheral tolerance can be maintained in several ways ([13-15]). For example autoreactive lymphocytes require a secondary, co-stimulatory signal to be present for adequate activation and initiation of an antigen-specific immune response. Thus, when an autoreactive cell recognises antigen in the absence of the secondary signal induced by inflammation they are eliminated from the circulation (by programmed cell death) or become functionally inactivated (anergic), preventing initiation of an inappropriate immune response. It has been suggested that this system is dysfunctional in autoimmune individuals, leading to activation of autoimmune responses. For example, it was demonstrated recently that development of inflammation in a model of MS was dependent upon expression of CD40, a co-stimulatory molecule that signals through CD40 ligand (CD154) on the surface of T cells [16]. Furthermore, it has been suggested that expression of co-stimulatory CD40 on DC acts to promote stimulation of an antigen-specific autoimmune response, whilst in the absence of CD40 tolerance to presented antigen is promoted [17]. Another mechanism of peripheral tolerance is through the action of a subset of T cells known as T regulatory cells (T_{regs}). By secreting anti-inflammatory cytokines, such as IL-10, or via cell-cell contact, T_{regs} suppress the activation of autoreactive cells in the local environment. Evidence to support this theory was obtained when it was demonstrated that inflammatory bowel disease (IBD) -like inflammation spontaneously developed in IL-10 deficient mice [18, 19] and a model of MS spontaneously arises upon depletion of $CD4^+CD25^+$ T cells [20].

1.2.2 B cells in autoimmunity

As described above, the major role of B cells in the immune system is to produce antibodies that help target immune cells to the pathogen requiring destruction e.g. bacteria. In some autoimmune diseases, B cells mediate production of autoantibodies that are specific for self-antigen on cells or tissues within the body, hence targeting the immune system-mediated destruction of body tissues. In particular, it has been demonstrated that autoantibodies are a major mechanism of pathogenesis in systemic lupus erythematosus (SLE) [21-23].

SLE is a prototypical inflammatory autoimmune disorder of heterogeneous pathology, affecting multiple organs and body systems, which can include lymph node enlargement, splenomegaly, vasculitis, glomerulonephritis and arthritis [21]. As a result of marked hypersecretion of autoantibodies and consequent inflammatory processes, tissue damage and end organ failure ensues in this chronic debilitating disease. The concentration of circulating anti-DNA autoantibodies is significantly elevated in SLE patients and has been shown to correlate with disease severity; highlighting its function in mediation of disease pathogenesis [24].

Similarly, the identification of rheumatoid factor (antibody that binds to the Fc portion of immunoglobulins) [25] implicated B cell-secreted autoantibodies in mediation of RA pathogenesis [26]. Furthermore, the TH1 cells involved in MS pathogenesis provide help to B cells, which in turn secrete autoantibodies specific for myelin [27]. However, the importance of B cells and autoantibodies in MS pathogenesis has been a matter of debate, nevertheless it has been suggested that the level of circulating anti-MOG autoantibodies is predictive of eventual disease diagnosis [28]. Likewise, autoantibodies specific for insulin and islet proteins can be detected in the bloodstream of eventual type 1 diabetics as early as *in utero*, demonstrating the humoral component of pathogenesis in this disease [29, 30].

1.2.3 Genetics of autoimmunity

It has been identified that pre-disposition to autoimmune disease also has a genetic component. Thus, animal models of disease have highlighted different groups of genes that are associated with incidence of (or protection from) certain autoimmune disorders [31]. This is not to say that autoimmune diseases are inherited disorders, such as cystic fibrosis (which is the result of a single gene mutation) [32]. Rather, as a result of inheriting multiple alleles of genes involved in the function of the immune system, the combined effect induces susceptibility to the disease [33, 34]. These findings have been supported by several studies. For example, particular autoimmune diseases disproportionately affect certain genders, races and ethnic groups [35]. Moreover, information gained from studies of twins revealed that monozygotic twins are more likely to suffer the same autoimmune disorder than dizygotic twins [36]. For example, approximately 25% of monozygotic twins are concordant for SLE, supporting a genetic component in susceptibility to this disease [37]. Furthermore, relatives of individuals suffering from autoimmune disease are more likely to develop the same or another autoimmune disorder, than non-relatives. Genes implicated in susceptibility to autoimmunity include the MHC family of genes, which are involved in immune system regulation and recognition of antigen. These genes have been associated with disposition to MS, type I diabetes, RA, SLE and different types of irritable bowel disease [34, 38-41]. For example, specific human leukocyte antigen (HLA)-DR

genes, which also reside in the major histocompatibility complex (MHC) and participate in antigen presentation, have been consistently implicated [42, 43]. However, the risk of developing RA is at most 50% determined by genetic factors [44].

1.2.4 Environmental exposures influencing the development of autoimmunity

Concordance for autoimmune disease in monozygotic twins is never 100%, indicating that environmental exposure also has a significant role to play in onset of disease. Thus, infectious agents, chemicals and toxins have been implicated in induction of autoimmunity. In particular, bacterial and viral infections have been associated with triggering subsequent autoimmunity [45, 46]. The mechanism of this is unclear, although it has been postulated that molecular mimicry is a potential method, where infection-induced expansion of effector T cells that cross-react with self antigen persist in their attack after the infection has been cleared [47]. Alternatively, autoreactive T cells may be activated in a bystander mechanism by cells of the innate immune response releasing pro-inflammatory cytokines during an immune response to infectious pathogens [48]. Nevertheless, although several viral and bacterial infections have also been implicated in RA [44, 49], none have been conclusively supported.

The potential effects of occupational exposure to chemicals and toxins has not been thoroughly investigated however, exposure to mercury and silica has been demonstrated to cause autoimmunity in humans and animals [50, 51]. In addition, it has been demonstrated that reactive oxygen species may be involved in the pathogenesis of SLE [52].

Nutrition and smoking have been implicated as important lifestyle factors that influence onset and progression of autoimmunity. For example, it is known that smoking increases the risk of RA and reduces the risk of ulcerative colitis [53, 54] [53, 55], however studies investigating other disorders, such as SLE, have been inconclusive [56]. Based on studies in NOD mice (a murine model of type 1 diabetes; T1DM), it has been suggested that avoidance of dietary gluten can prevent development of T1DM [57], however this has been disputed by human studies [58]. By contrast, consumption of high levels of essential fatty acids (omega-3 and omega-6) has been shown to correlate negatively with susceptibility to RA [59], although these studies have received minimal support.

Finally, the disproportionate prevalence of autoimmunity in women of childbearing age has implicated sex hormones in the pathogenesis of these diseases. Women with MS outnumber men by as much as 2:1 and whilst SLE afflicts approximately 100 per 100,000 [4], it is more common in women than men [60]. In an effort to explain this distribution, it is

known that oestrogen stimulates B cell growth and production of antibodies and cytokines [61]. Likewise, other hormones present in enhanced levels in females, such as prolactin and growth hormone, have been demonstrated to promote autoimmune disease [62, 63]. This remains an active topic of research.

In summary, autoimmune diseases are characterised by the presence of clonally expanded populations of auto-reactive lymphocytes [11] that have escaped negative selection during development or have failed to become deleted following activation in the periphery. Mechanisms must be in place for successful pathogenesis of autoimmunity, involving persistent and accessible antigen, available and present co-stimulation and inadequate tolerance mechanisms (Figure 1.5). Therefore, full understanding of all these processes will aid the development of improved strategies for prevention of autoimmune disease. The common denominator in the majority of autoimmune diseases is that they are TH1-mediated, indicating that pro-inflammatory TH1 cell-mediated immune processes are generally responsible for initiation. In addition, humoral (antibody-mediated) immunity is a common feature in autoimmune disease, implicating B cells in disease pathogenesis. Animal models of autoimmune diseases have permitted extensive analysis of pathology in several conditions and continue to help identify putative therapeutic targets for human disease. The prevalence of several autoimmune diseases (e.g. T1DM) has been observed as currently increasing, indicating that lifestyle and environmental factors (that can ultimately be controlled) could be changed to slow or even reverse this progression. This may involve modulation of exposure to common bacterial or viral infections, or modification of our modern diet.

1.3 Allergy and Allergic Diseases

Allergies and allergic diseases can induce a vast spectrum of debilitation, which ranges from mild and easily controlled to chronically disabling and potentially fatal, depending on the allergy and the allergic individual. In a similar manner to autoimmune disease, allergies are usually mediated by inappropriate activation of an immune response. The terms allergy and atopy are generally used interchangeably to mean IgE-mediated hypersensitivity to non-pathogenic substances [64]. The prevalence of atopic disease varies in different parts of the world, and has been quoted between 17 and 25% [65]. Frequently, individuals exhibit more than one type of allergy or allergic manifestation (e.g. dermatitis and rhinitis). These allergic individuals are said to exhibit an atopic phenotype.

However, in immunological terms, allergy and autoimmunity are quite distinct. Thus, allergies are generally characterised by activation of TH2-mediated immune responses and are associated with elevated production of IgE antibody. Immediate-type hypersensitivity reactions are characteristic of allergies and are induced in response to

IgE-mediated recognition of specific allergen. Allergic inflammation usually involves activation of innate immune cells such as mast cells [66], basophils and eosinophils by ligation of IgE expressed on their surface (Figure 1.6).

1.3.1 Allergens

When an allergic (or atopic) individual is exposed to normally harmless exogenous or foreign antigens (allergens), an allergic immune response is activated. Contact with allergen is via distinct routes, depending on the antigen. For example, contact with airborne allergens (e.g. house dust mite and pollen) occurs in the nasal and pulmonary mucosa, whilst contact with food allergens (e.g. nut or milk proteins) occurs via the mouth or gut mucosa. Inflammatory immune responses in allergic individuals are normally transient; i.e. they occur in response to allergen and can usually be prevented by avoiding contact with the allergen. For example, removal of food allergens from the diet or use of dust mite-impermeable mattress covers. However, avoidance is often not feasible to completely or satisfactorily avoid the allergen, e.g. tree and grass pollens [67].

The normal manifestation of allergy to inhaled allergens is allergic rhinitis, more commonly known as hayfever. Allergic rhinitis can be a seasonal or year-round affliction, depending on the allergen to which the allergic individual is sensitised. Similarly, food allergies are caused by an inappropriate inflammatory immune response to otherwise harmless food proteins, resulting in elevated production of allergen-specific IgE antibody. This must not be confused with non-immunological food intolerance. Foodstuffs commonly associated with allergy include cow's milk, eggs, wheat, fish, nuts, soya bean and citrus fruit. The most common food allergen is cow's milk protein [68]. Allergy to cow's milk protein often presents in the first few months of life [69], diagnosed by erythema of the skin and possible dermatitis. Nut and fish allergies are commonly characterised by severe, life-threatening allergic responses such as urticaria, angioedema, pharyngeal and laryngeal oedema [65]. Furthermore an allergic reaction to these foodstuffs can be fatal through development of anaphylactic shock and asphyxia [70]. Severe allergy to fish or nut proteins are usually persistent life-long conditions, however, milder egg and cow's milk allergies are normally transient afflictions that disappear after childhood [65]. It is common for atopic individuals sensitised to tree pollen to also be allergic to tree nuts, such as hazelnuts [71].

It has been suggested that allergies and allergic diseases are the result of a combination of multi-gene pre-disposition and environmental exposure to allergenic substances [72]. Exposure to food antigens in neonatal life has been implicated in establishment of allergic status in later life in susceptible individuals. However, support for this theory has been disputed. Whilst it has been demonstrated that exclusively breastfeeding an infant is

protective from development of allergy to cow's milk protein [73, 74], it has also been suggested that allergic sensitisation to other allergens may occur *in utero* or via the breastmilk. Therefore it has been advised that commonly allergenic substances should be omitted from the maternal diet during pregnancy and breastfeeding in suspected atopics [75]. Conflicting evidence concerning the prevention of allergy development in neonatal life continues to be published [76]. The best course of action for prevention of allergy in potentially atopic infants is currently unclear.

1.3.2 Treatment of allergies

Traditional pharmacological treatment of allergies (such as allergic rhinitis) has included oral anti-histamines and nasal corticosteroids. More recently, allergen immunotherapy has been investigated for use in the treatment of asthma and allergic rhinitis patients [77-80]. Allergen immunotherapy involves weekly subcutaneous administration of allergens at increasing doses until an optimal maintenance dose is attained. At this point, optimal doses are administered less frequently to maintain tolerance to the allergen. Beneficial effects of this type of therapy have been variable, however immunotherapy-induced reduction of symptoms have persisted after cessation of therapy in some patients. Nevertheless, the risk of systemic reactions during immunotherapy is substantial, as in a few rare cases, patients undergoing immunotherapy have died following onset of anaphylactic shock.

1.3.3 Allergic Diseases

Allergic diseases are inflammatory conditions associated with IgE-mediated inflammatory reactions and aberrant TH2-mediated immunity. In contrast to transiently manifested allergies, allergic diseases are chronic disabling afflictions that can be temporarily exacerbated by exposure to one or multiple allergens (e.g. asthma). Fatality as a result of allergic disease usually occurs during an episode of inflammatory exacerbation (e.g. an "asthma attack"). Asthma, allergic rhinitis (AR), and atopic dermatitis (AD) are considered the atopic triad [81].

1.3.3.1 Allergic Rhinitis

Allergic rhinitis (hay fever), affects approximately 10-20% of the population in developed countries [82] and is one of the most common chronic conditions for which medical care is sought. The variable inflammatory response exhibited in allergic rhinitis (including sneezing, watery rhinorrhea, nasal congestion, itchy palate and itchy, red, watery eyes) is initiated by ligation of allergen and IgE on the surface of mast cells in the airway [82, 83]. Sensitization to specific allergens can be identified by skin prick or *in vitro* tests for allergen-specific IgE antibody [67]. Inhaled allergens that normally cause allergic rhinitis include plant, animal, house dust mite and fungi-derived antigens, amongst others. Allergy

to plant pollen is seasonal and therefore can be easily distinguished from perennial allergies, which are usually caused by house dust mite or animal dander. It has been suggested that development of allergic rhinitis can precede development of allergic asthma, therefore adequate treatment of allergic rhinitis might prevent development of asthma.

Immunologically, allergic rhinitis is characterised by eosinophilia of the airways and enhanced expression of epithelial and endothelial cell adhesion molecules [82]. The epithelial cell layer is an active centre of cell infiltration and inflammatory mediator release and the nasal mucosa is continually inflamed during the period of exposure to allergen. TH2-type cytokines (IL-4 and IL-5) and chemokines, released by T cells and mast cells, are present in elevated quantities and are a hallmark of AR inflammation. Likewise, histamine, released by mast cells, is a major mediator of AR.

1.3.3.2 Asthma

Asthma is a common allergic disease that has doubled in prevalence since 1980 [84] and affects approximately 3 million people in the UK [85]. As a chronic inflammatory pulmonary disorder, it is characterised by reversible airflow obstruction, airway hyperresponsiveness to allergenic and non-allergenic substances, wheeze, cough or shortness of breath [84]. Furthermore, asthma is categorised as an allergic disease thus, exposure to allergen induces exacerbation of airway inflammation in sensitised asthmatics. Indeed, exposure to allergens derived from house dust mite, cockroach and spores of the fungus, *Alternaria,* elicit the strongest reactions in asthmatics [86-88]. Kinetically and immunologically, asthmatics experience two types of inflammatory response to antigen (or allergen) challenge. Inhalation of aerosolised antigen causes immediate acute bronchospasm, known as the *early response.* The *late phase response* is exhibited 4-6 hours after exposure and is characterised by more severe airway obstruction.

1.3.3.3 The immediate asthmatic response

Epidemiological and clinical studies have demonstrated a correlation between IgE levels and asthma severity [89]. It is understood that the early asthmatic response to antigen is mediated by the action of allergy-associated IgE antibody. To initiate IgE synthesis, allergen must previously encounter and activate pulmonary DC. Activated DC subsequently migrate to the lymph nodes and present processed allergen to T and B cells. TH2 cytokines IL-4 or IL-13, in combination with T cell help, induce B cell secretion of IgE isotype antibody [90]. Within the lungs, allergen-specific IgE binds to high-affinity IgE receptors (FcεR1) on the surface of mast cells or basophils and low-affinity FcεRII on the surface of lymphocytes [91]. Thus, ligation of IgE with allergen induces the immediate

response involving degranulation of mast cells and basophils resulting in release of inflammatory mediators including prostaglandins, leukotrienes, histamine and TH2 cytokines, IL-4 and IL-5, which act to recruit more mast cells and eosinophils to the site of inflammation.

1.3.3.4 The late asthmatic response is mediated by TH2-type immune response

The late phase response is brought about as a result of infiltration of several different inflammatory cell types (including eosinophils, macrophages, neutrophils, TH2 cells, mast cells and basophils) to the lungs. As mentioned previously, the asthmatic airway is a TH2-polarised environment exhibiting enhanced levels of TH2 cytokines, IL-4, IL-5, IL-13 and IL-9 [92]. In response to recognition of antigen in the presence of TH2-promoting cytokine, IL-4, naïve T cells are activated and differentiate into TH2 cells, able to trigger a TH2-mediated specific immune response. Initiated 4-6 hours after the immediate inflammatory response, the late phase response is characterised by prolonged, more severe airway constriction and obstruction, compared with that exhibited in the early phase, and airway hyperresponsiveness.

In contrast to the majority of 'simple' allergies, asthma is a progressive disease, which can lead to permanent lung function impairment and irreversible airflow obstruction [93, 94]. The irreversible aspect of asthmatic inflammation is brought about slowly over time, by repeated activation of the early and late phase responses. Inflammatory mediators released during these processes result in pulmonary tissue damage and permanent structural remodelling of the airways (Chapter 4).

1.4 The Hygiene Hypothesis

The prevalence of allergies, allergic diseases and several autoimmune disorders has progressively increased in developed countries during the past 2-3 decades [95-101]. This has coincided with improved sanitation and healthcare and eradication of multiple life-threatening infectious diseases in these parts of the world. It has been suggested that the increased prevalence of allergy in developed countries is related to a lack of exposure to such infectious agents in childhood, while the immune system matures [102]. This theory has been dubbed 'the hygiene hypothesis' and has been a topic of much debate and controversy. In support of the hygiene hypothesis, the incidence of allergic disease and allergy is reduced in developing countries [103], where improvements in medical care and sanitation and changes in lifestyle during the past few decades have not been as pronounced as in developed countries.

Independent of socioeconomic status of country, a pattern of increased allergic disease in urban areas and decreased allergic disease in rural areas has also been observed [104,

105]. The hygiene hypothesis proposes that these epidemiological observations are inextricably linked and advocates that exposure to bacterial or viral infections, in childhood, promotes development of a balanced immune system [106]. Without this neonatal 'training', the immune system behaves in an inappropriate manner in genetically pre-disposed individuals and the result is allergy [107]. A common outcome of changing lifestyles in developed countries is reduced family size. In a seminal study triggering formulation of the hygiene hypothesis [102], it was observed that the incidence of allergic rhinitis was inversely related to the number of children in the household. Furthermore, there was a higher incidence of allergic rhinitis and eczema in the oldest child of a family (who will have begun life with no siblings), than in younger children. It was proposed that increased numbers of siblings promotes increased exposure to infectious agents, e.g. rhinoviruses, (as a result of unhygienic contact with siblings) and protection from development of allergic disease. In developing countries lifestyle is more traditional and families are larger, supporting this relationship. However, the hygiene hypothesis, as described here, has been widely disputed [108, 109].

1.4.1 Opposition to the Hygiene Hypothesis

It has been suggested that TH1 and TH2 immune responses are counter-balancing. Immunologically, the hygiene hypothesis proposes that stimulation of Th1-mediated immune responses (normally elicited in response to bacterial and viral infections) dampens the TH2 bias of the neonatal immune system and prevents development of inappropriate TH2-mediated allergy to irrelevant environmental antigens [107]. The process of polarising the immune response towards TH1 and away from TH2 responses has been termed immune deviation. The model of TH1-TH2 counterbalance, implicated in the hygiene hypothesis offers an explanation for increasing TH2-mediated allergy in the developed world. However this theory is contradicted by the observation that autoimmune diseases are simultaneously increasing in these countries. Autoimmune diseases are usually mediated by aberrant TH1-type immune responses [109]; therefore increasing prevalence of TH1-mediated autoimmunity in countries with improved sanitation and reduced exposure to TH1-promoting infections is contradictory to the TH1-TH2 immune deviation mechanism of the hygiene hypothesis model.

Also in contradiction to the hygiene hypothesis, the majority of people in parasite endemic countries exhibit potent TH2-mediated immune responses to parasitic worms (helminths) [110]. This observation highlights the fact that children raised in these countries are not solely exposed to bacterial and viral infections that induce TH1-mediated immune responses. Despite displaying 'TH2-skewed' immune systems (including high levels of serum IgE and TH2 cytokines), parasite-infected individuals do not develop associated allergy to environmental allergen. This is in direct contradiction to the hygiene hypothesis,

which proposes that a TH2 biased immune system promotes development of allergy. In fact, *schistosome*-infected Gabonese children exhibited a 63% reduced risk of allergy to house dust mite [111] and Nigerian children infected with *Ascaris lumbricoides* displayed relatively little evidence of allergic inflammation to environmental allergens [112]. These observations indicate that TH2 skewing of the immune system is not sufficient for development of allergic disease. Interestingly, the severity of parasite infection is inversely related to the incidence of allergy. Helminth-infected children exhibiting high levels of IgE specific for a selection of allergens, displayed reduced skin-prick reactivity to house dust mite and other allergens when compared with children that had a reduced parasite load [113]. Furthermore, a study in Brazil demonstrated that schistosome-infected subjects were 7 times less likely to be allergic to inhaled allergens than non-infected subjects [114]. These studies support a role for helminth-mediated suppression of allergy that is not mediated by reduction of TH2 immunity. When helminth infections were cleared by medication, skin-prick test reactivity to house dust mite increased, implicating parasite infection as inhibitory to development of allergy. This was further supported by a decrease in skin-prick reactivity as worm burden increased in parasite-infected subjects [115]. However, this pattern is not clear-cut: a few studies have found no association between allergy and helminth infection, and some studies have observed increased allergy in helminth-infected subjects [116]. The results of these studies might have been influenced by chronicity and severity of parasite infection, however it is important to take them into account when formulating hypotheses.

1.4.2 Reduced incidence of allergy in parasite-infected individual is due to an enhanced anti-inflammatory regulatory network

Replacing the theory of Th1-TH2 counterbalancing and immune deviation, it has been suggested that an enhanced anti-inflammatory network within the immune system is induced by exposure to parasite infection in childhood [117, 118]. Therefore, under this revised model, allergy is prevented by regulatory mechanisms that inhibit inflammatory immune effector mechanisms. The anti-inflammatory component of the immune system is facilitated by the action of anti-inflammatory cytokines IL-10 [117] and TGFβ, secreted by a subset of $CD4^+$ T cells known as regulatory T cells. IL-10 induced by allergen is inversely associated with the size of the allergic skin reaction to house dust mite [119] and both IL-10 and TGFβ are capable of counter-regulating both TH1 and TH2 mediated inflammation [120-124]. In accordance with the theory of enhanced regulatory mechanisms in parasite-infected individuals, robust anti-inflammatory networks are developed to restrict parasite-Ag-induced tissue damage. This anti-inflammatory network might underlie the reduced prevalence of allergy in helminth-infected people. Indeed, PBMC isolated from filariasis patients display elevated IL-10 production [123, 125, 126] and the reduced proliferative responses of lymphocytes and immunosuppressed

phenotype exhibited in filariasis has been attributed to IL-10 and TGFβ [123]. Elimination of the parasite from the host often induces onset of parasite-induced inflammatory pathology, such as elephantiasis [127]. This observation provides further support for an enhanced anti-inflammatory network during active parasite infection. By direct inference, these findings suggest that atopic residents of developed countries have an underdeveloped anti-inflammatory aspect to their immune systems.

1.4.3 Reduced autoimmunity might also be explained by enhanced anti-inflammatory network in parasitized individuals.

A robust anti-inflammatory network in parasitised individuals might also explain the reduced prevalence of inflammatory autoimmune disease in parasite-endemic areas. Regulatory signals act in a blanket manner to reduce inflammation, independent of immunological phenotype; therefore, in addition to reduction of TH2-mediated allergic inflammation, a robust anti-inflammatory network could inhibit TH1-mediated autoimmune inflammation. It has been suggested that the increasing prevalence of autoimmunity in industrialised areas, in parallel with improved sanitation and decreased exposure to infections, is contradictory to the hygiene hypothesis. Paradoxically, in a few studies, the prevalence of asthma and allergic disease (i.e. allergic rhinitis) have been positively correlated with the prevalence of autoimmune disease, for example, type 1 diabetes, rheumatoid arthritis and celiac disease [128-130]. This correlation indicates that Th1-mediated autoimmune responses and TH2-mediated allergic responses are not mutually exclusive and that there may be an underlying thread connecting these two types of aberrant immune activation. Potentially, the hygiene hypothesis could be extended to include autoimmunity. However, direct evidence linking lack of infections in childhood and development of autoimmune disease has not been obtained. This might be explained by the relatively low prevalence of autoimmune diseases (compared with allergy and allergic diseases), making it difficult to conduct epidemiological studies with adequate numbers of subjects.

1.4.4 By what mechanisms do parasites reduce inflammatory signals?

It has been established that parasite infections promote anti-inflammatory immune signals capable of inhibiting allergic responses. How is this facilitated? It is known that polyclonal IgE (specific for many different antigens) is elevated in parasite infection. Elevated levels of serum IgE are also indicative of atopy. In an effort to explain this apparent paradox, it was suggested that in parasite infection, non-allergen specific IgE and allergen-specific IgE bind to FcεRI receptors on mast cells in a competitive manner. The excess of parasite-induced non-allergen specific IgE ensures that mast cell IgE receptors are occupied by many non-allergen specific IgE molecules and few allergen-specific IgE molecules. Mast cell degranulation requires binding of *two* allergen-specific IgE-FcεRI to

one antigen, therefore, the presence of many irrelevant IgE specificities will inhibit the allergic response to house dust mite [115, 131]. Whilst this hypothesis sounds plausible, it has not been universally supported.

An alternative explanation of the IgE paradox has been proposed. As mentioned above, TH2-type IgG4 antibody is also secreted in elevated quantities in parasite-infected people. IgG4 inhibits mast cell degranulation induced by IgE [132]. As mentioned previously, mast cell degranulation initiates release of inflammatory mediators and cytokines. Therefore, this action of IgG4 could block the immediate allergic response to allergen. In support of this IgE-blocking theory, It has been identified that successful immunotherapy of allergy in humans is associated with induction of allergen-specific IgG4 antibody, despite persistent presence of allergen-specific IgE [133]. Supporting a role for IgG4 in down-modulation of allergic responses, prolonged exposure to cat allergens induced elevated serum IgG4 and reduced atopy [134]. In summary, IgG4 has been considered an alternative TH2 antibody [134], inducing an augmented TH2 immune response that is not associated with allergic inflammation. Furthermore, it has been suggested that inflammation associated with autoimmunity could also be subverted by action of an augmented TH2 immune response such as that induced by IgG4 [135].

Clearly parasites can modulate the immune system in an intelligent way, so that the host maintains an appropriate degree of protection from the parasite, but the immune response is less inflammatory than that exhibited in response to other infectious agents. A mutual relationship ensues; the host does not eliminate the parasite and simultaneously, the parasite infection does not induce severe host tissue damage. With this knowledge, is it possible to exploit the anti-inflammatory effect of parasite infection for treatment of inflammatory diseases of the Western world? Recently, in a controversial study, Joel Weinstock treated volunteers with eggs of porcine whipworm *Trichuris suis* and discovered that active parasite infection induced amelioration of inflammatory bowel disease symptoms, normally mediated by a pro-inflammatory TH1 type immune response [136-138]. This was the first confirmatory study in humans of an effect frequently observed in murine models of autoimmune inflammation [32, 139, 140] and has been termed 'worm therapy' [138]. Following the success of Weinstock's study, treatment of MS (another TH1-mediated autoimmune disease) patients with *T. suis* eggs has been proposed [138]. This method of treating inflammatory conditions by direct administration of parasites, whilst clearly therapeutic, is relatively crude and not appealing for patients with atopic or autoimmune disease. To take this theme of research further, dissection of the mechanism of anti-inflammatory action by helminth parasites would provide a model for effective therapy of inflammation. Thus, pharmacologically mimicking the anti-inflammatory effect of

helminths without the associated risk and disadvantages of chronic infection would be more appropriate for viable treatment of allergic and autoimmune disease.

1.5 Filarial Nematode Infections

1.2 billion people worldwide are at risk of infection with filarial nematodes (WHO 2005). Collectively known as filariasis, infection with filarial nematode parasites can be categorised into different forms (lymphatic filariasis or onchocerciasis), depending on the infective filarial nematode species. In humans, lymphatic filariasis is the result of mosquito-borne infection with one of three species of filarial nematode, *Wuchereria bancrofti, Brugia malayi or Brugia timori.* Due to its severe inflammatory pathologies (such as gross genital hydrocoele and morbidly swollen limbs (lymphoedema)) in a small percentage of infected individuals, lymphatic filariasis is commonly known as 'elephantiasis'. Onchocerciasis is caused by infection with *Onchocerca volvulus*, which is transmitted by the blackfly. Pathologies commonly associated with onchocerciasis include blindness ('river-blindness') and inflammatory skin lesions. Lymphatic filariasis has been identified as the second (second to mood (affective) disorders) leading cause of permanent and long-term disability world-wide [141-143].

1.5.1 Pathology associated with filarial nematode infection

By means of a mosquito bite, infective L3 larvae of the filarial nematode enter the host and migrate to a lymphatic vessel. Here, they grow and mature to adulthood by approximately 30 days post-infection (*pi*). The adult worms mate and the female produces millions of microfilariae (MF) by day 60 *pi*. The MF pass into the circulation, where mosquitoes take up a small number as part of a blood meal. Within the mosquito muscles the MF mature into L3 larvae, in preparation for infection of a new host to complete the life cycle [127](Figure 1.7).

It has been established that people living in filariasis endemic areas can be classified into one of three categories [127, 144]. 'Asymptomatic microfilaraemia' is the most common classification exhibited in populations of endemic countries. This category of people are MF^+, but display no external symptoms or pathology to indicate the large numbers of microfilariae present in their circulation. Due to this apparent absence of immune response to MF, the infection can persist for decades within the host body. Individuals exhibiting chronic inflammatory pathology such as elephantiasis, lymphoedema and hydrocele comprise a second category. It is thought that eliminating the parasite infection from the host bloodstream induces chronic inflammation; therefore, these individuals are usually MF^-. 'Endemic normals' comprise the remainder of the population, consisting of those who have been exposed to filarial nematodes, but exhibit no evidence of disease, despite the presence of anti-filarial antibodies circulating in their blood. The existence of

'endemic normal' individuals is controversial and has been widely contested [127, 144]. Asymptomatic microfilaraemics can experience periods of acute inflammation, but how this change occurs is unclear. There is evidence to suggest that acute episodes of bacterial infection may be responsible for conversion from asymptomatic to chronic inflammation phenotype [145-147].

1.5.2 Immunology of filarial nematode infection

1.5.2.1 TH2-type immune response to parasites

Individuals harbouring MF exhibit markedly elevated levels of TH2-type IgG4 antibody and reduced levels other IgG subclasses (IgG1, IgG2 and IgG3) in their blood stream [148]. Furthermore, the levels of IgG4 have been shown to correlate with the severity of infection and MF counts [149, 150]. IgG4 does not fix complement unlike the other subclasses and binds weakly to Fc receptors, therefore it is thought that it binds filarial antigen without strong stimulation of effector cells.

IgE, a TH2-type immunoglobulin isotype often associated with inflammatory TH2-mediated allergic responses, is also induced in filaria-infected individuals. It has been suggested that IgE production may represent killing of worms in the host [151] and that IgE offers some protection from infection, because its levels are inversely associated with parasite load [152]. In support of this finding, IgE is exhibited at low concentrations in asymptomatic MF^+ patients and in much higher concentrations in infected individuals with chronic inflammatory pathology (who have allegedly eliminated the MF)[153]. Although several immunological parameters have been measured in filariasis and onchocerciasis patients, the mechanism underlying the pathology of microfilaraemia has not been completely elucidated.

In filariasis patients, serum IFNγ is reduced and IL-10 is increased. IL-10 is a regulatory cytokine that can down-regulate IFNγ, a key cytokine for promotion of TH1-mediated immunity [154]. In summary, filarial nematode infected individuals exhibit a mild, mainly humoral, TH2-mediated immune response to infection. This type of immune response is much less inflammatory, and causes much less tissue damage than pro-inflammatory TH1cell-mediated immune responses. Interestingly, the TH2-mediated immune response is mutually beneficial to the parasite and does not induce its elimination from the host.

1.5.2.2 Filarial nematode-infected individuals exhibit an immunosuppressed phenotype

In addition to exhibition of a TH2-mediated immune response, MF^+ patients appear somewhat immunosuppressed [155] hence, the normal immune response to inflammatory stimuli and subsequent (e.g. bacterial) infections is impaired in these individuals [156].

The degree of immunosuppression appears to correlate with parasite load. In addition to increased production of IL-10 and high levels of IgG4, reduced T cell proliferation is a common symptom of MF^+ infected individuals [157, 158]. Parasite infection-induced immunosuppression is normally passed from mother to child *in utero* [159]. Babies born of filaria-infected mothers thus display reduced inflammatory responses to infection and generally also exhibit increased parasite loads also. The suppressed host immune system is beneficial to the filarial nematodes and ensures longevity of the parasite infection in the host, because once microfilariae have been released into the host bloodstream, an inflammatory immune response is damaging to both host and parasite. It has been suggested that immunosuppression may also render the individual susceptible to further infection with other parasites [160], however these areas of research are unclear. It is important to note that host immunity is not reduced to such a degree that a subsequent bacterial infection would be fatal; death of the host would not benefit the parasite either. More simply, the normal immune response is dampened, in order that inflammatory immune signals are not circulating in enhanced levels.

It has been suggested that anti-inflammatory T regulatory cells (T_{regs}) are responsible for reduced inflammatory responses in parasite infected individuals [158]. T_{regs} are naturally occurring Ag-specific $CD4^+$ T cells that secrete IL-10 and TGFβ, cytokines that can mediate anti-inflammatory effects. T_{regs} are induced by adult female worms that produce MF and by MF themselves. In support of this hypothesis, T cells that secrete IL-10 in response to filarial antigen have been isolated from PBMC of onchocerciasis patients with high MF loads [158].

1.5.3 ES products

Excretory-secretory (ES) substances produced by filarial nematodes have been postulated as mediators of the immunosuppressed phenotype exhibited in filariasis [161]. ES products include proteinases, protease inhibitors, cystatins, anti-oxidants and a homologue of macrophage inflammatory factor (MIF) [162, 163]. For example, parasite-derived antigens have been demonstrated to display collagenase activity [164]. Furthermore, cystatins (cysteine protease inhibitors) isolated from *A. viteae, O. volvulus* and *B. malayi* have been studied in rodent models of infection. It has been demonstrated that after release from the nematodes, cystatins initiate down-regulatory mechanisms by inducing production of IL-10 [165] [166]. In support of this theory, immunosuppressed filariasis patients exhibit enhanced levels of circulating ES products, which correlate with parasite load [167]. Furthermore, the scale of immunosuppression exhibited in filariasis patients has been shown to correlate with the quantity of circulating parasite-derived antigen [168, 169].

In a rodent model of infection with filarial nematodes, *Acanthocheilonema viteae*, enhanced secretion of ES products has been detected prior to onset of patency [170] and similar results have been obtained in a rodent model of infection with human parasite, *B. malayi* [171]. Satisfactory evidence confirming the immunomodulatory nature of parasite-derived ES products was obtained when ES product isolated from MF of *W. bancrofti* directly inhibited migration of leukocytes in patients with clinical filariasis [172]. In support of these findings, ES products derived from adult worms and MF of *O. volvulus* impaired proliferation of lymphocytes isolated from onchocerciasis patients [173].

1.5.4 ES-62

ES-62 is the 62kDa glycoprotein ES product of rodent filarial nematode *Acanthocheilonema viteae* [170]. Homologues of ES-62 are produced by human filarial nematode *Brugia malayi* and feline filarial nematode *Brugia pahangi* [174-176]. ES-62 comprises more than 90% of the total protein secretions produced by female adult (stage L4) *A. viteae* worms [170]. Work from this laboratory has focused on elucidating the structure, properties and functions of ES-62 in relation to the immune system, with the aim of elucidating the role and mechanism of action of ES products in human filarial nematode infections. In our laboratory, the immunological actions of ES-62 have been intensively investigated and ES-62 has been shown to exhibit a plethora of immunomodulatory actions on cells of the immune system *in vitro and in vivo.*

1.5.4.1 The structure of ES-62

Native ES-62 is tetrameric in structure and has been shown to contain three distinct types of N-glycan and the immunomodulatory post-translational modification, phosphorylcholine (PC) [177, 178]. N-glycans and PC are common components of molecules derived from filarial nematodes [179]. Analysis of the protein coding sequence revealed the presence of four potential N-linked glycosylation sites [180]. It is thought that PC is attached to the ES-62 molecule by binding directly to an *N*-acetyl glucosamine residue contained within the N-glycans. Several of the immunomodulatory actions of ES-62 have been attributed to the PC component of the native molecule, but it has not yet been defined whether the non-PC components of ES-62 exhibit any immunomodulatory functions. Analysis of the amino acid sequence of the non-PC components of ES-62 revealed 37-39% homology with a family of carboxy- and aminopeptidases, indicating that ES-62 might exhibit aminopeptidase activity [181]. Aminopeptidases are proteins necessary for protein degradation, cell cycle control and hormone level regulation, amongst other functions [182]. Indeed, a mild aminopeptidase action of ES-62 was confirmed by successful hydrolysis of aminopeptidase substrate, Leu-NHMec [180]. It was postulated that this PC-independent action of ES-62 might represent a parasite-specific function, for example, in digestion and uptake of proteins in the adult worm gut. Nevertheless, this proteolytic

action of ES-62 was mild in comparison with its profound and widespread immunomodulatory activity.

1.5.4.2 The immunomodulatory effects of ES-62 *in vitro*

Treatment with relatively high concentrations of ES-62 (25-50μg/ml) induced low-level proliferation of murine splenic B cells *in vitro* [183]. However, this action was weak compared with the inhibitory effect of ES-62 (at low concentrations: 0.25-2μg/ml) on B cell proliferation in response to ligation of the BCR [183]. This disruption of B cell activation was mediated by selective down-regulation of protein kinase C (PKC) isotypes and uncoupling of mitogenic signalling pathways, downstream of the BCR [184, 185]. Furthermore, this action of ES-62 was found to be PC-dependent as demonstrated by treatment with PC alone or PC-BSA in place of ES-62 [183] PC-dependent anti-proliferative effects of ES-62 were also observed in cultured Jurkat T cells, rendering them anergic to TCR signalling [167], once again by interfering with the signalling cascades downstream of the Ag receptor [186]. These initial *in vitro* findings in lymphocytes provided the foundations for extensive work into the immunomodulatory and anti-inflammatory properties of ES-62.

1.5.4.3 The immunomodulatory effects of ES-62 *in vivo*

ES-62 was initially described as a TH2-promoting molecule and this was supported by several findings. Injected into BALB/c mice, ES-62 induced an IgG1, but not an IgG2a antibody response directed against the non-PC components of its structure [167]. The IgG1 antibody isotype promotes a TH2-mediated immune response and TH2 cytokine, IL-4, promotes antibody class switching to IgG1 [154]. The ES-62 specific IgG1 antibody response was dependent on IL-4, demonstrated by absence of this antibody response in IL-4KO mice [187]. IL-4 is fundamental to initiation of a TH2-mediated immune response and inhibits onset of a Th1 mediated immune response, by disruption of the IL-12 receptor β chain [188]. However, IL-4 deficient mice did not default to IgG2a antibody production in response to ES-62, indicating that suppression of the Th1 mediated immune response to ES-62 was IL-4 independent. It was uncovered that the PC component of ES-62 was responsible for preventing development of a Th1-type antibody response, and this was induced in an IL-10 dependent manner [187].

1.5.4.4 Dissection of the effects of ES-62

Further support for ES-62 as a TH2-promoting molecule was obtained in dendritic cells. Treatment of bone marrow-derived dendritic cells conferred a TH2-inducing property on the cells and enabled them to prime naïve T cells into TH2 cells, secreting high levels of IL-4 and low levels of TH1 cytokine, IFNγ [189]. This was corroborated by studies in peritoneal macrophages, where ES-62 inhibited pro-inflammatory cytokine production. In

more detail, treatment of peritoneal macrophages with ES-62 alone induced low-level activation of the cells and production of Th1-promoting cytokine production, whilst rendering them hyporesponsive to subsequent stimulation with LPS and IFNγ [190]. Moreover, the immunomodulatory effects of ES-62 on DC and macrophage function were largely mimicked by PC (conjugated to irrelevant protein, OVA or BSA) [191]. It was suggested that ES-62 diverted immune responses away from a pro-inflammatory TH1-mediated immune response in favour of a less inflammatory TH2-mediated immune response.

1.5.4.5 ES-62 is anti-inflammatory in action

Recently it was proposed that rather than promoting a TH2 immune response, ES-62 appeared to act in a more general anti-inflammatory manner. ES-62 treatment of mice *in vivo* (facilitated by subcutaneously implanted osmotic pumps continuously releasing ES-62 at concentrations similar to that secreted in filarial nematode infection) resulted in differentiation of bone marrow derived DC and macrophages that had an anti-inflammatory phenotype [191]. More direct evidence of the anti-inflammatory action of ES-62 was obtained using a murine model of TH1-mediated autoimmune inflammation, rheumatoid arthritis. ES-62 treatment of such collagen-induced arthritis delayed onset and severity of footpad inflammation and inhibited erosion of joints [192].

As mentioned previously, the immunomodulatory effects of filarial nematode infections has often been attributed to ES products generated by the parasites and secreted into the host bloodstream [161]. It has been suggested that ES products facilitate relay of inflammation-reducing signals from parasite to host immune system. Thus, it was proposed to exploit the facilitating action of a filarial nematode ES product to inhibit inflammation in allergic and autoimmune disease and ultimately, dissect the mechanisms of filarial nematode-mediated immunomodulation.

1.6 Aims of the thesis

The *in vitro* and *in vivo* immunomodulatory effects of ES-62, an ES product secreted by rodent filarial nematode *A. viteae,* have thoroughly and comprehensively been determined as a result of investigations conducted in this laboratory [193]. Therefore, the core aim of this project was to extend the previous research of this laboratory and determine the potential for exploitation of parasite product-induced immunomodulation in inflammatory disease by defining the anti-inflammatory properties of ES-62 in models of inflammatory diseases commonly exhibited in Western society. The specific aims of this thesis are described in more detail below.

It has previously been determined that ES-62 delays onset and inhibits severity of inflammation in a murine model of rheumatoid arthritis [192]. However the mechanisms of action of ES-62 in this model have yet to be determined. Thus, a primary aim of this investigation was to confirm such action of ES-62 and extend this study to dissect the immunological mechanisms of anti-inflammatory action of ES-62 in a model of Th1-mediated autoimmune disease.

Previously, it has been identified that PC, a common component of pathogen-derived substances is largely responsible for several of the immunomodulatory effects of ES-62. Thus, it was also aimed to determine the PC-dependency of the anti-inflammatory action of ES-62 in the arthritis model and furthermore, to examine the immunomodulatory properties of alternative structural components (e.g. carbohydrate) of parasite-derived substances on cells of the immune system.

It has been suggested that infection with helminth parasites inhibits murine models of allergy and TH2-mediated inflammation [110]. Following determination of ES-62-mediated inhibition of TH1-mediated inflammation in a model of arthritis [192], the second aim of this project was to determine and dissect the action of ES-62 treatment in a model of TH2-mediated allergic disease, asthma.

As mentioned previously, SLE is an inflammatory autoimmune disease of heterogeneous pathology and ambiguous immunology. By utilising the MRL/lpr mouse model, inflammation commonly exhibited in human SLE can be examined in the laboratory. Thus, in addition, it was aimed to determine the effect of ES-62 treatment of the MRL/lpr model of SLE. Furthermore, upon confirmation of any modulatory effect, it was aimed to dissect the mechanism of action of ES-62 in this model of inflammation.

Figure 1.1 Activation of dendritic cells and T cell priming

Immature dendritic cells (DC) normally reside in the peripheral tissues, where they perform a surveillance function. This function is mediated by uptake and processing of antigen which they encounter. In this maturation state DC exhibit low antigen-presentation capacity (low expression of MHCII) and express low levels of co-stimulatory molecules (such as CD40, CD80 and CD86). Following uptake and recognition of a pathogen-associated molecular pattern (PAMP; for example, lipopolysaccharide (LPS), a component Gram-negative bacterial cell wall), immature DC become activated or mature, signified by a morphological change (formation of dendrites). At this point, antigen uptake and processing functions are reduced and antigen presentation becomes the major function of the DC. Co-stimulatory molecules are upregulated on the surface of the DC and pro-inflammatory cytokines (such as TNFα and IL-12) are secreted. Following maturation, the antigen-presenting DC migrates to the secondary lymphoid organs in search of a naïve T cell, specific for the activating antigen. Priming of the naive T cell results in initiation of an antigen-specific immune response, the nature of which can be influenced by the pattern of cytokines secreted by the DC.

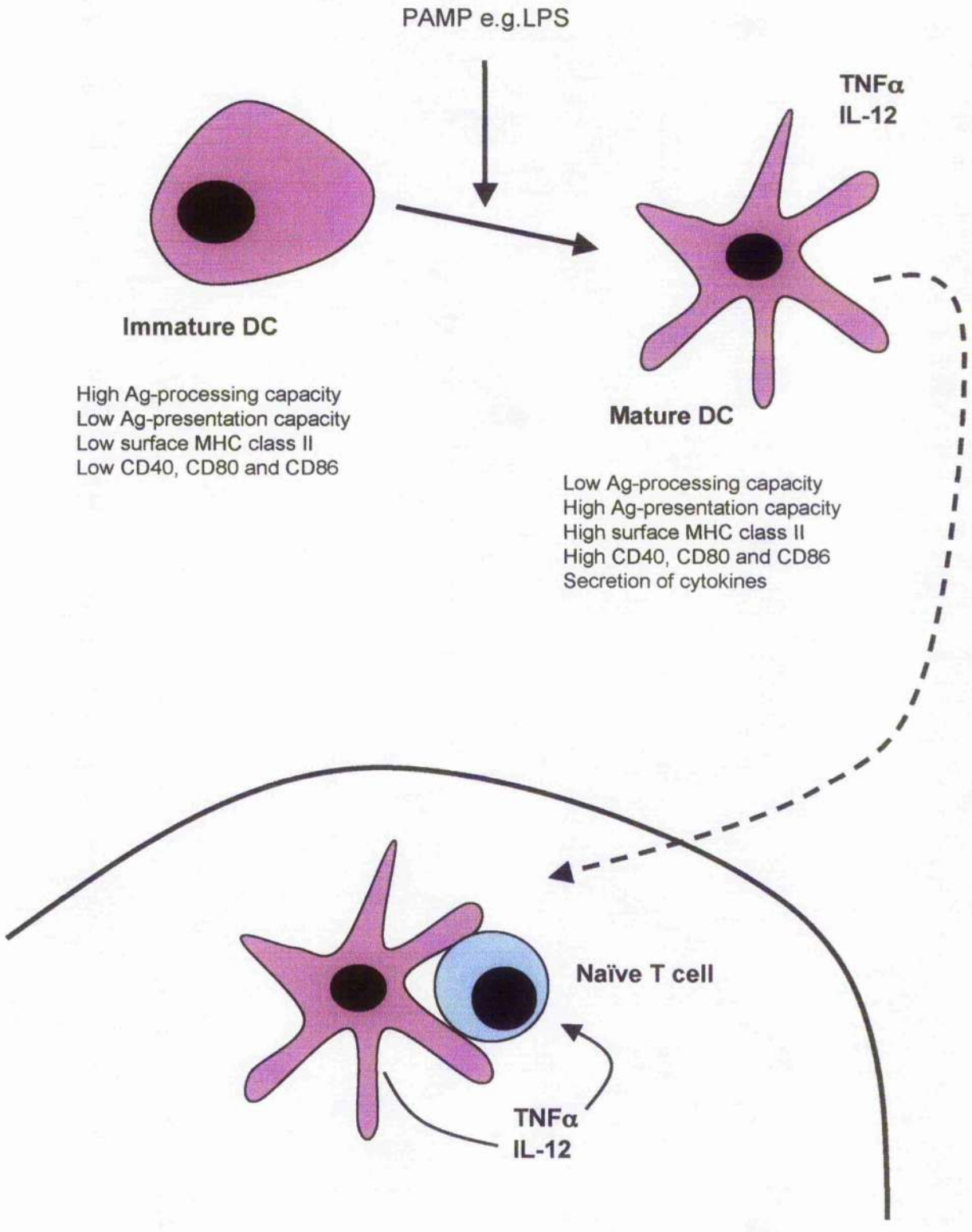
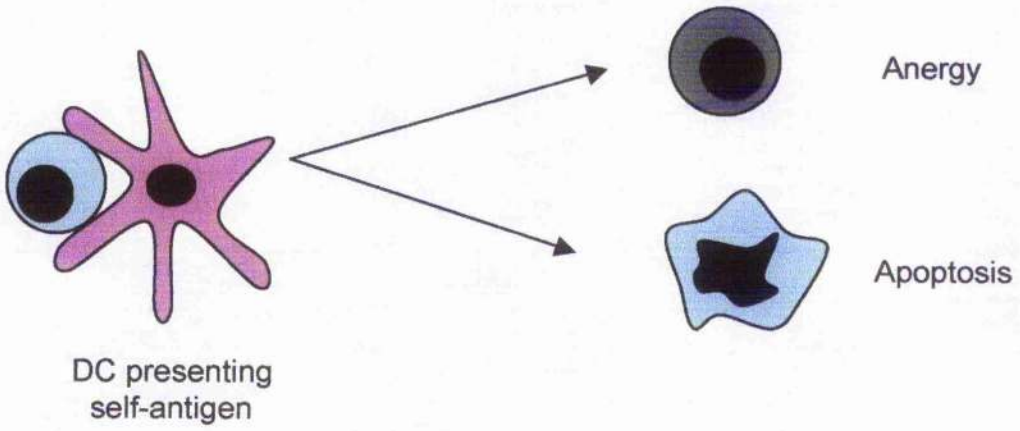

PAMP e.g.LPS
TNFα
IL-12
Immature DC
High Ag-processing capacity
Low Ag-presentation capacity
Low surface MHC class II
Low CD40, CD80 and CD86
Mature DC
Low Ag-processing capacity
High Ag-presentation capacity
High surface MHC class II
High CD40, CD80 and CD86
Secretion of cytokines
Naïve T cell
TNFα
IL-12

Figure 1.2 Induction of immunity or tolerance to antigens

(A) Normally, presentation of self-antigen by DC to naïve autoreactive T cells results in apoptosis or anergy of the T cell. Thus, autoreactive T cells are removed from the T cell repertoire and tolerance to the self-antigen is achieved.
(B) Conversely, when pathogenic antigens are presented by DC to naïve antigen-specific T cells, co-stimulatory molecules expressed on the surface of the DC are upregulated. Therefore, antigen-presentation in combination with a second co-stimulatory signal induces activation of the antigen-specific T cell, leading to proliferation and initiation of an immune response targeted at destruction and disposal of the pathogen.

A


Anergy
Apoptosis
DC presenting
self-antigen

B

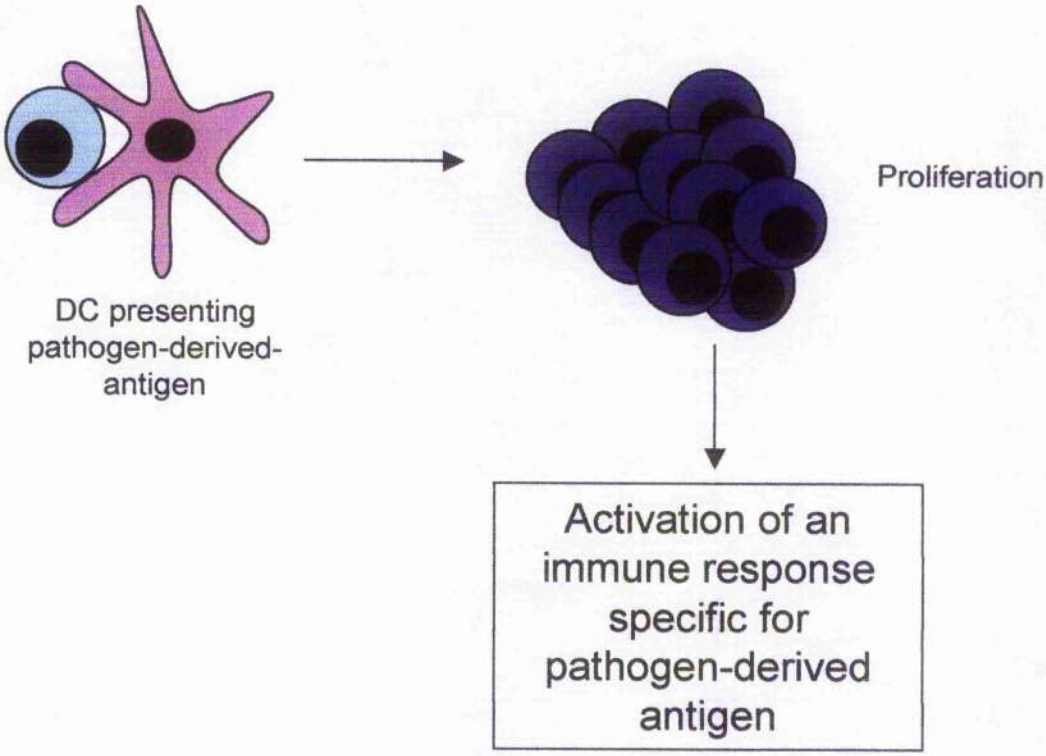
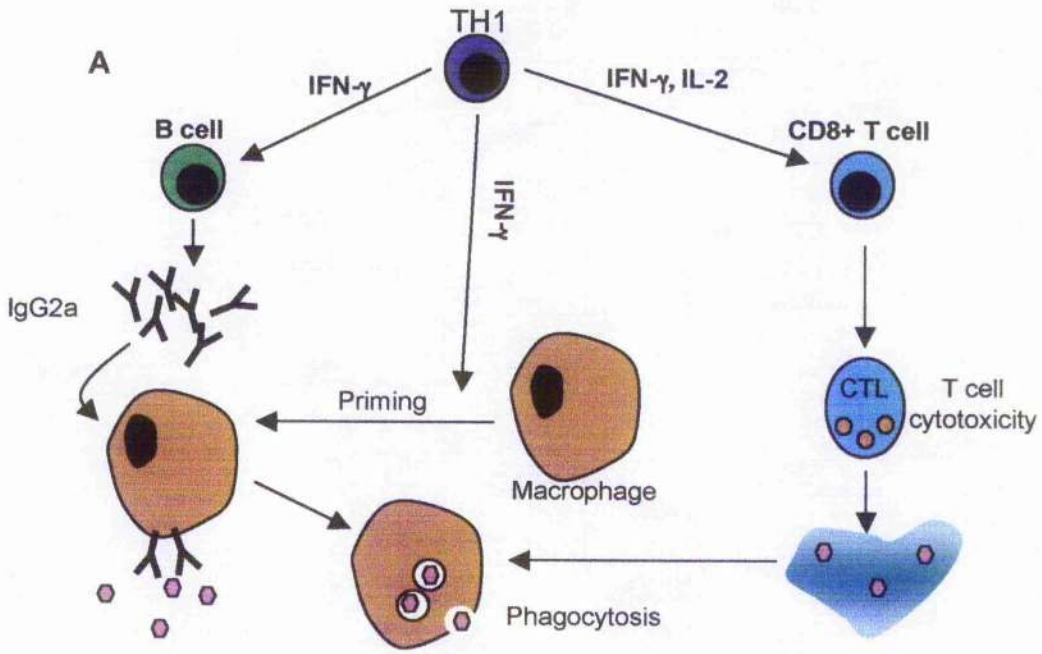

Proliferation
DC presenting
pathogen-derived-
antigen
Activation of an
immune response
specific for
pathogen-derived
antigen

Figure 1.3 Effector functions of TH1 and TH2 CD4+ T cells

(A) The production of IFN-γ by TH1 cells is important for inducing the class switching of B cells to IgG2a production. These antibodies are highly efficient at fixing complement and can bind to high affinity FcγR expressed by macrophages. This is important for the opsonisation of particulate microbes, which promotes their phagocytosis by macrophages. IFN-γ induces priming of macrophages, resulting in production of pro-inflammatory cytokines and synthesis of toxic oxygen radicals, hydrogen peroxide and nitric oxide. Finally, IFN-γ and IL-2 are important for activating $CD8^+$ CTLs to increase their cytotoxic potential by inducing the production of perforin and granzyme. Following pathogen destruction, cellular debris is cleared by macrophage-mediated phagocytosis.

(B) The production of IL-4 by TH2 cells induces class switching of B cells to produce non-complement fixing IgG1 antibodies. IgG1 acts to neutralise antigens and can promote their uptake without promoting inflammation. In addition, IL-4 induces the class switching of B cells to produce IgE antibody. IgE binds to FcεRI expressed on mast cells, which together with IL-3 and IL-9 secreted by TH2 cells, primes mast cells for degranulation and release of inflammatory mediators following recognition of specific antigen. TH2 cytokine, IL-5 is an essential activator of and growth factor for eosinophils, which leads to the production of GM-CSF, IL-4, IL-5 and IL-13, which can lead to inflammation and augment TH2 development and responses.


A
TH1
IFN-γ
IFN-γ, IL-2
B cell
CD8+ T cell
IFN-γ
IgG2a
Priming
Macrophage
CTL
T cell
cytotoxicity
Phagocytosis

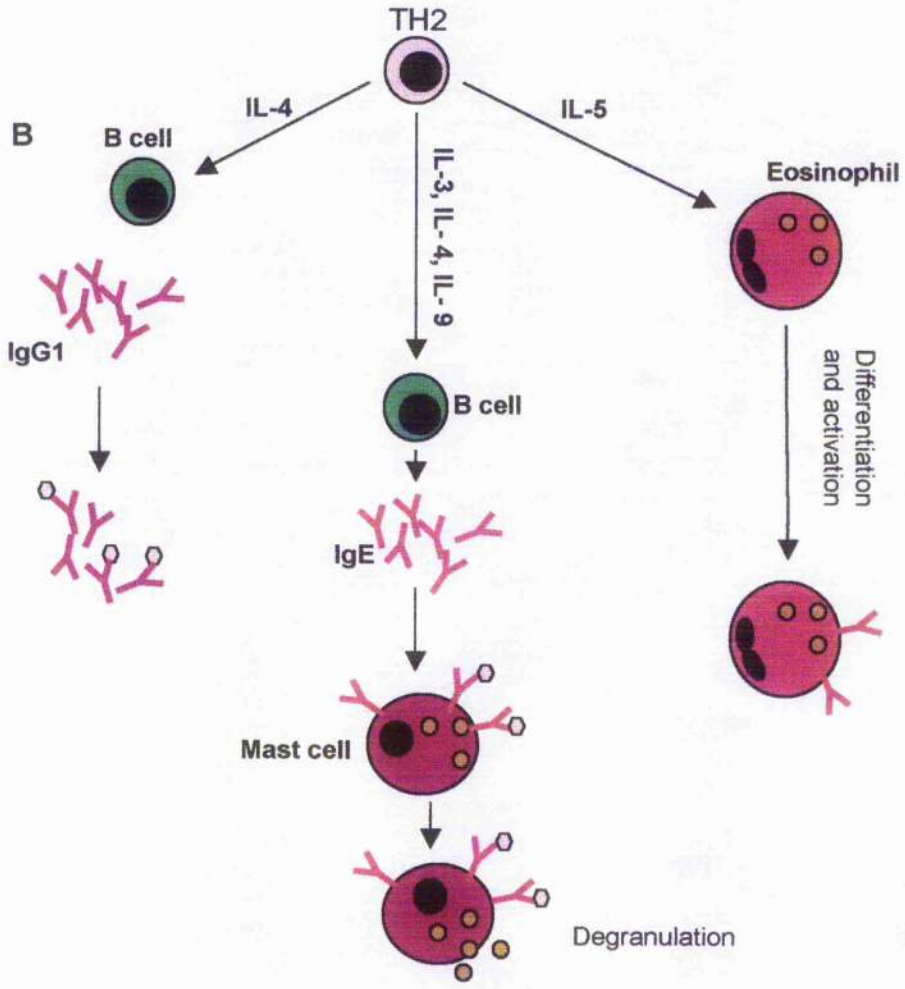

TH2
B
B cell
IL-4
IL-5
IL-3, IL-4, IL-9
Eosinophil
IgG1
B cell
Differentiation
and activation
IgE
Mast cell
Degranulation

Figure 1.4 Immune response initiation and effector mechanisms

An antigen-specific immune response is normally facilitated by initiation and effector mechanisms. Initiation of an antigen-specific immune response can be achieved by pathogen-induced DC activation and migration to the T cell area, followed by $CD4^+$ T cell priming. Following activation of antigen-specific CD4+ T cells, effector mechanisms are induced, which include CD8+ T cell mediated cytotoxicity, B cell mediated antigen-specific antibody production and macrophage activation. After destruction of pathogen (by cytotoxic T lymphocytes) and opsonisation of pathogen cells by pathogen-specific antibodies, the products of pathogen destruction are normally disposed of by phagocytosis.

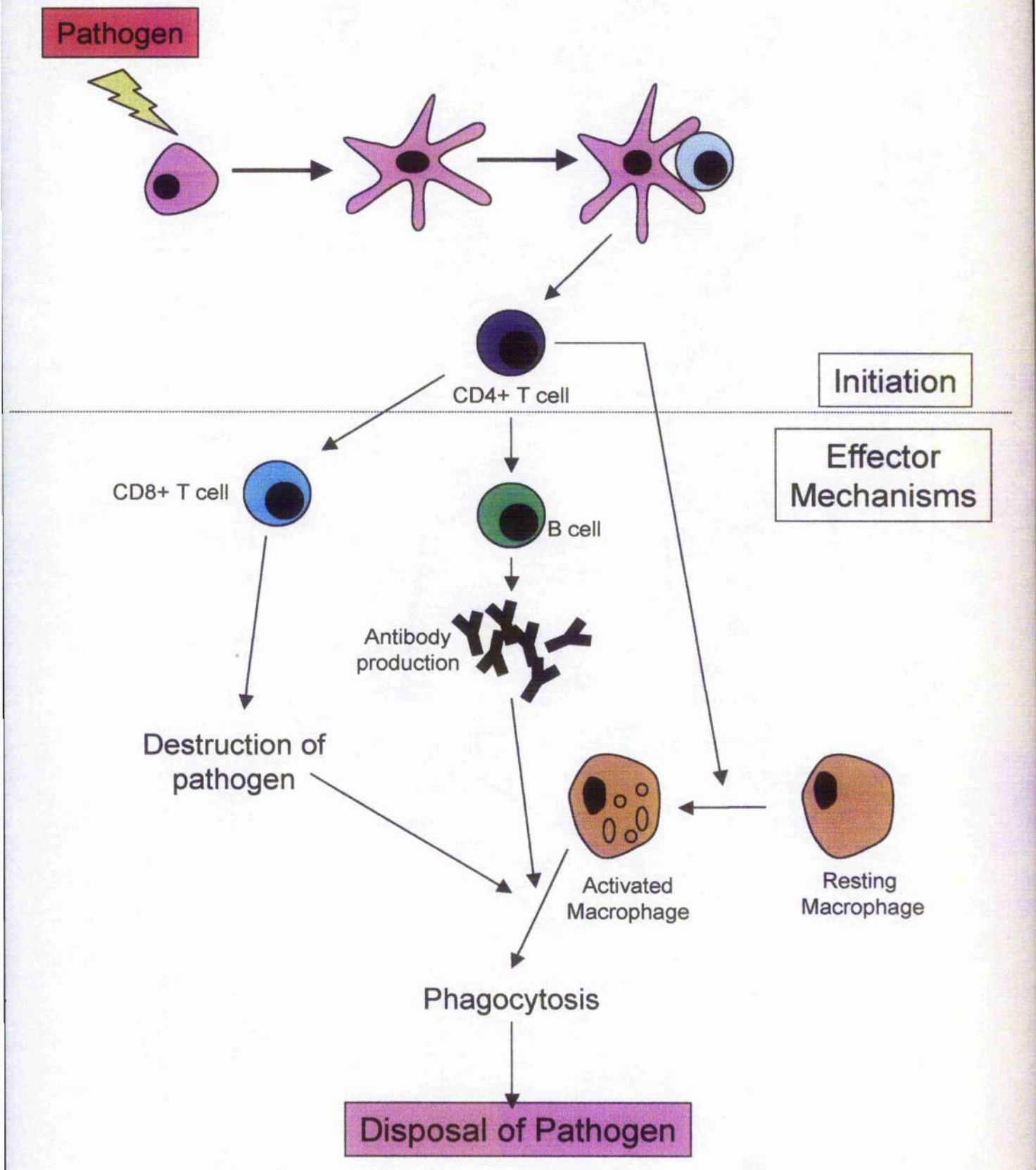

Pathogen
CD4+ T cell
Initiation
Effector Mechanisms
CD8+ T cell
B cell
Antibody production
Destruction of pathogen
Activated Macrophage
Resting Macrophage
Phagocytosis
Disposal of Pathogen

Figure 1.5 Development of an autoimmune response

An inflammatory autoimmune response can be initiated by activation of autoreactive T cells in response to recognition of self-antigen. However, the conditions required for activation of such T cells are unclear. Self-antigen might be presented by aberrant antigen-presenting cells (e.g. DC), promoted by the presence of danger signals, such as pathogen-derived antigens (e.g. LPS), products of inflammation, or pro-inflammatory cytokines. Inadequate peripheral tolerance mechanisms will ensure that activation of autoreactive T cells results in initiation of an inflammatory immune response, specific for the activating self-antigen. The inflammatory autoimmune response is mediated by the activation of normal immune effector cells (e.g. B cells and macrophages), leading to destruction of cells and tissues that express the self-antigen. Damage to tissue can lead to release of further self-antigen. Thus, in the presence of inflammatory mediators (released during mediation of the original autoimmune response), new autoreactive T cells can be activated and a positive feedback cycle of inflammation and tissue destruction may be set up.

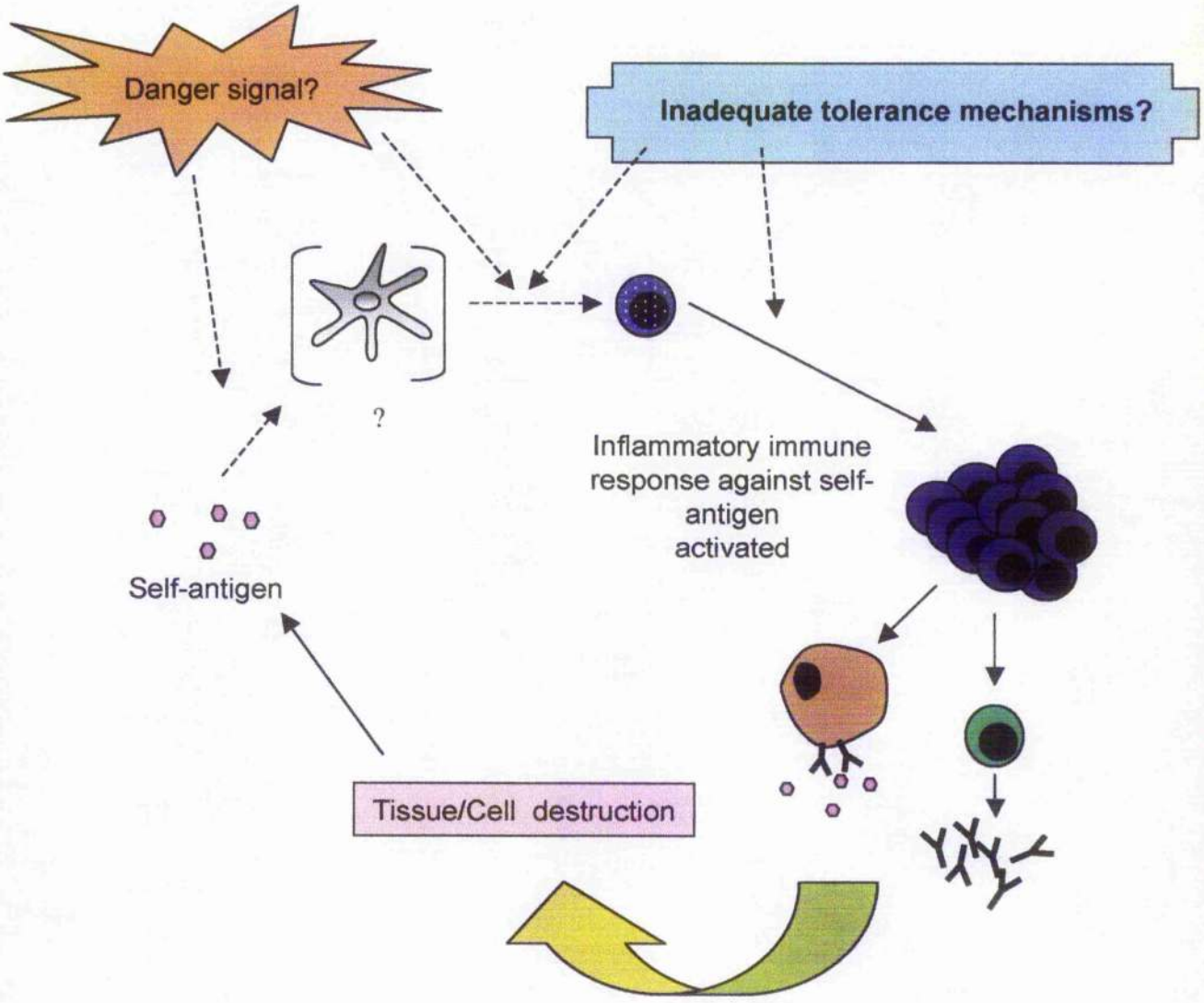

Danger signal?
Inadequate tolerance mechanisms?
?
Inflammatory immune response against self-antigen activated
Self-antigen
Tissue/Cell destruction

Figure 1.6 Development of an allergic immune response

Inhalation or ingestion of allergen (non-self, non-pathogenic antigen) is recognised immediately by allergen-specific IgE, present in the allergenic individual. Ligation of allergen and IgE bound to FcεRI on the surface of mast cells induces degranulation and release of inflammatory mediators, which recruit immune effector cells and TH2 cells to the site of allergen recognition. TH2 cells produce IL-4, which induces class switching of B cells to IgE production and mast cell activation, and IL-5, which induces differentiation and activation of eosinophils. Thus, a positive feedback cycle is set up, generating inflammation in response to recognition of allergen. Usually, this cycle can be broken by prevention of allergen intake.

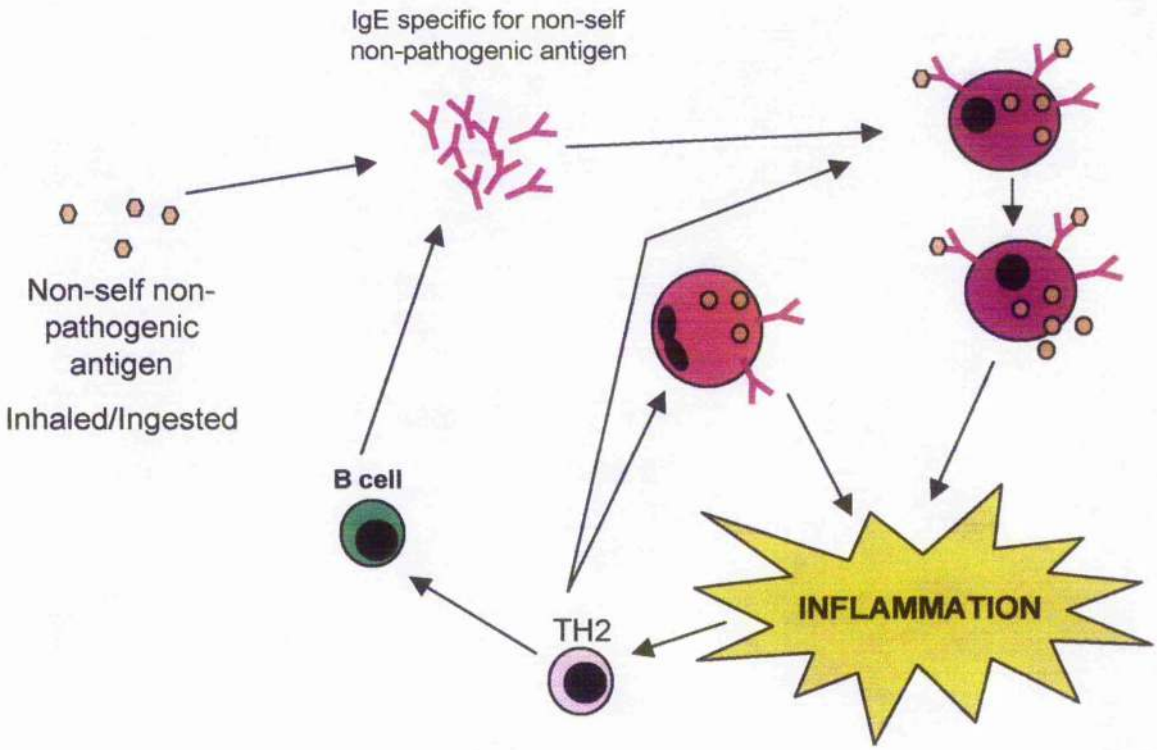
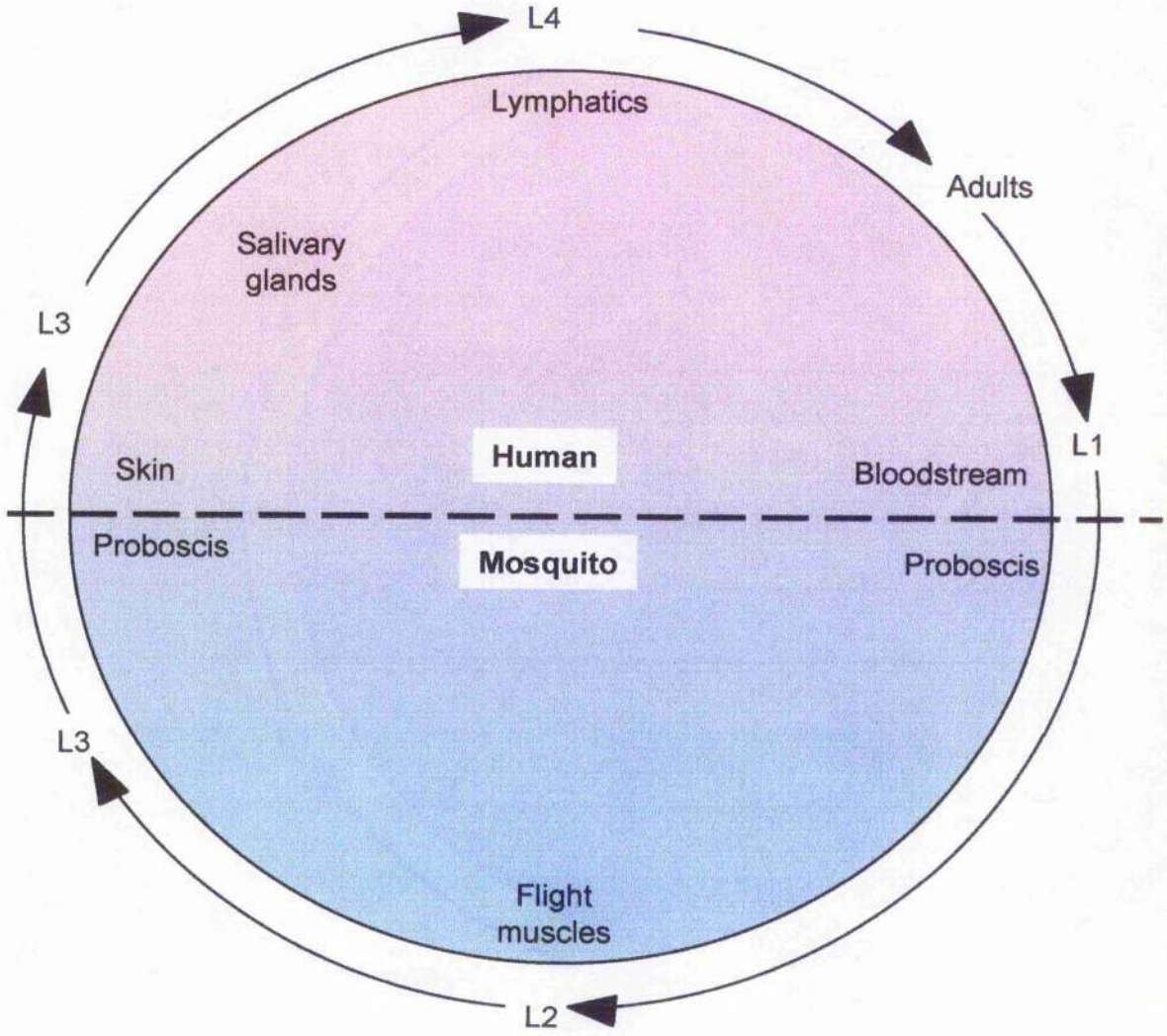

IgE specific for non-self
non-pathogenic antigen
Non-self non-
pathogenic
antigen
Inhaled/Ingested
B cell
TH2
INFLAMMATION

Figure 1.7 Typical lifecycle of *Wuchereria bancrofti* and *Brugia malayi* filarial nematode parasites

Adult worms live in the lymphatics of the human host where they reproduce sexually, releasing thousands of microfiliariae into the bloodstream. These L1 larvae are ingested by mosquitoes when they take a bloodmeal. They shed their acellular sheath, penetrate the midgut and migrate into the flight muscles where they develop into L2 larvae. About two weeks later, L3 larvae migrate through the salivary glands to the proboscis. At this stage they can infect humans by injection into the skin when the mosquito feeds. They migrate into the lymphatics where they moult and mature into L4 larvae, before further maturing and differentiating to form adult worms. The development from microfilariae to adult worms can take several months and adult worms can live for many years.


L4
Lymphatics
Adults
Salivary
glands
L3
L1
Skin
Human
Bloodstream
Proboscis
Mosquito
Proboscis
L3
Flight
muscles
L2

Appendix Figure 1 Predicted structure of ES-62 derived from *A. viteae*

ES-62 is known to be a PC-containing N-linked glycoprotein. The structure of ES-62 is yet to be conclusively determined, but predictions have been made regarding the individual components of the complete glycoprotein. These have been detailed in this figure.

A, The location of key residues within the ES-62 sequence, including N-glycosylation sites, a possible site for interaction with PC donors, leucine-rich regions (likely to be involved in protein–protein interaction), and regions containing subcellular targeting motifs.

B, Prediction of a tertiary structure for the ES-62 monomer, indicating α helices and β strands, as well as glycosylation sites, leucine-rich regions and residues involved in metal ion co-ordination (ES-62 shows homology with aminopeptidases that contain a divalent cation in their active site).

C, Proposed structure of the PC containing *N*-glycans of ES-62. There is at least one and at most three N-linked glycans within the structure of ES-62. PC is linked to the glycans by phosphodiester linkage, with at least one (and at most 4) PC residues per glycan.

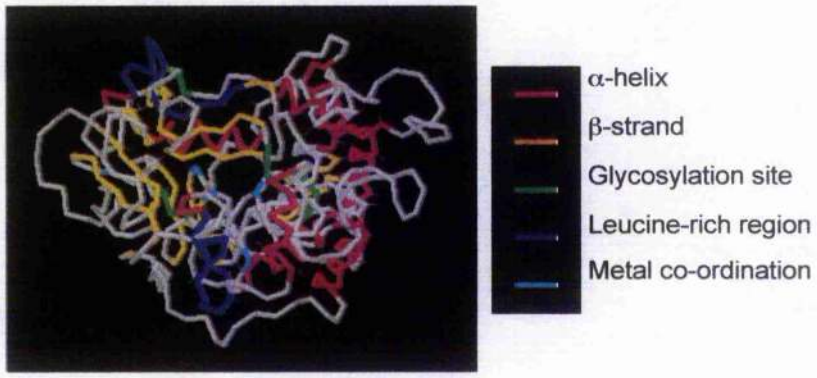
A, and B reproduced with kind permission from Goodridge et al, 2005

A

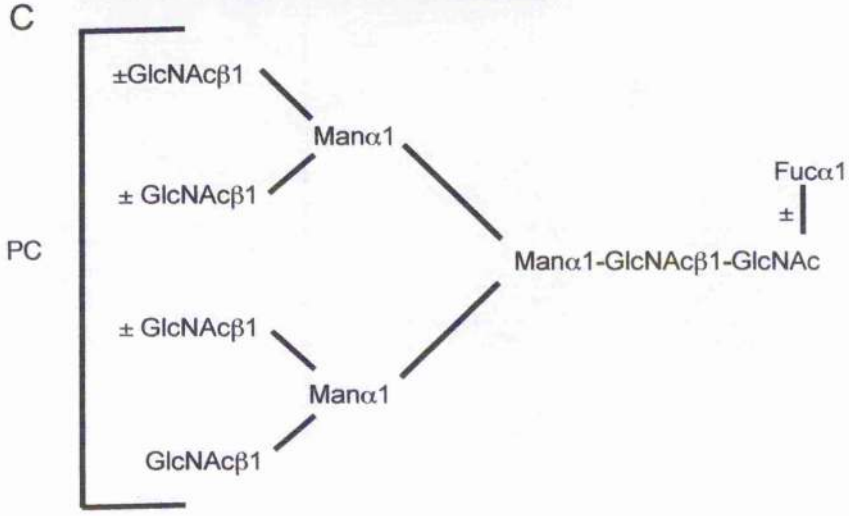
N-Glycosylation site Putative PC donor binding site Leucine-rich regions

Nuclear localisation signal Putative ER membrane retrieval signal

B



C



2 Materials and Methods

2.1 Animals

BALB/c, DBA/1, C57BL/6, ST2KO (on a BALB/c background), DO.11.10 (on a BALB/c background) and MRL/*lpr* mice were used in undertaking this study. BALB/c, DBA/1 and C57BL/6 mice were purchased from Harlan Olac (Bicester, U.K.). ST2KO mice were maintained at the University of Glasgow and were a generous gift from Prof. S. Akira, University of Osaka, Osaka, Japan. DO.11.10 mice were used to generate naïve $CD4^+$T cells for T cell-DC co-culture experiments. These mice are homozygous for the transgenic TCR, which is specific for cOVA $peptide_{323-339}$ in the context of $I\text{-}A^d$. The T cells bearing the transgenic TCR were detected by flow cytometry using monoclonal antibody KJ1.26. All animals were specified pathogen-free and were housed under standard animal house conditions with free access to water and standard rodent pellets at the University of Glasgow Central Research Facilities or Joint Animal Facilities (Glasgow, U.K.) in accordance with local Home Office regulations.

2.2 Preparation of parasite products

2.2.1 ES-62

ES-62 is a secreted product of the adult filarial nematode, *Acanthocheilonema viteae*, which infects rodents. Its structure is tetrameric; a glycoprotein composed of four 62kDa, PC-containing, monomers covalently attached to N-type glycans [194] ES-62 was prepared by Dr KM Houston and Ms C Egan at the Department of Immunology, Institute for Biomedical Sciences, University of Strathclyde, as described previously [183]. In detail, ES-62 was purified from 500 ml of spent culture medium (endotoxin-free RPMI 1640 (Invitrogen Life Technologies) with added endotoxin-free glutamine (2 mM), endotoxin-free penicillin (100 U/ml), and endotoxin-free streptomycin (100 µg/ml)) of adult *A. viteae*. To remove larval forms (microfilariae) released by the adult female worms, the medium was passed through a 0.22-µm pore size filter (Sigma-Aldrich). It was then transferred to a stirred cell ultrafiltration unit containing a YM10 membrane (Amicon). After reducing the volume of the sample to 5–10 ml and transferring the holding medium to endotoxin-free PBS, pH 7.2 (Cambrex Bioscience), it was further concentrated to 200–500 µl using Centricon microconcentrators with a 30-kDa cutoff membrane (Amicon). The sample was then applied to a 30 x 1 cm Superose 6 column (HR 10/30; BD Biosciences), fitted to an isocratic FPLC system (BD Biosciences), previously equilibrated with endotoxin-free PBS, pH 7.2, at room temperature. The column was eluted at a flow rate of 0.5 ml/min and monitored for absorbance at 280 nm. More than 95% of the protein eluted as a single peak, and this was purified ES-62. The purity and identity of each batch were confirmed by a combination of SDS-PAGE and Western blotting, the latter using a rabbit antiserum specific for ES-62. Endotoxin content of the ES-62 was determined using an Endosafe kit

(Charles River Laboratories, Kent, UK). The purified ES-62 product contained <0.003 endotoxin units/ml. In experiments where ES-62 was used *in vitro*, it was added to the culture media at 2 μg/ml.

2.2.2 GSL

Native *A. suum* glycosphingolipid was kindly prepared by Dr G Lochnit in the laboratory of Dr R Geyer, Institute of Biochemistry, University of Giessen, Giessen, Germany as described previously [195]. In detail, *Ascaris suum* worms (800g wet weight) were pulverized at 20°C in a pre-cooled Waring blender and lyophilized. Glycolipids were extracted with chloroform, methanol, water 10:10:1 (v/v/v), chloroform, methanol, 0.8 M aqueous sodium acetate 30:60:8 (v/v/v), and 2-propanol, *n*-hexane, water 55:20:25 (v/v/v) and evaporated to dryness. To remove most of the contaminating triglycerides, the residue was treated with acetone at 4 °C for 2 h. Neutral and acidic glycosphingolipids were separated by DEAE-Sephadex A-25 column chromatography (Pharmacia). The column was equilibrated with and the sample taken up in chloroform/methanol/water 30:60:8 (v/v/v). Neutral glycosphingolipids were obtained in the flow-through, and the acidic glycosphingolipids were eluted with chloroform, methanol, aqueous 0.8 M sodium acetate 30:60:8 (v/v/v). Zwitterionic glycolipid fractions were further fractionated on a silica gel_{60} column (Merck). Zwitterionic components were obtained by isocratic elution with chloroform/methanol/water 10:10:2.5 (v/v/v) from a silica gel_{60} column (1-50 cm, 70-250 mesh; Merck). After lyophilization, the glycosphingolipids were resuspended at 1 mg/ml in sterile PBS, sonicated, and stored at -20 °C until further use.

2.2.3 PC-free GSL

Removal of PC from the glycosphingolipid was also performed by Dr G Lochnit (Institute of Biochemistry, University of Giessen, Giessen, Germany). Briefly, native glycosphingolipid was dried in a stream of nitrogen and incubated for 24 h at 4 °C with 50-200 μl of hydrogen fluoride (HF, 48%; Fluka, Neu-Ulm, Germany). Excess was removed in a stream of nitrogen at room temperature.

2.3 Preparation of synthetic GSLs

The structures of three synthetic mimetics of incomplete native *A. suum* GSL are depicted in Figure 6.1. Mimetics of *A. suum* GSL were prepared as previously described [196-198]. Compound 1 resembles native glycosphingolipid but has the ceramide moiety replaced with an octyl group and lacks the two terminal sugars (galactose and *N*-acetylgalactosamine). Compound 2 differs in containing ceramide but lacks PC as well as the two terminal sugars [197, 198]. Compound 3 has features of a related PC-glycolipid from *Pheretima* species and consists of two galactose residues with PC attached to one and an octyl group to the other [196].

2.4 Preparation of recombinant ES-62

Recombinant ES-62 was prepared from *P. pastoris* by Ms C Egan (Department of Immunology, University of Strathclyde, Glasgow.) as described previously [199]. ES-62 was amplified by PCR using the primers: MJA121 5'-AAGGGGTATCTCTCGAGAAAAGAGAGGCAGCTGTCCTTCCGGACAAAACTGTCGCT and MJA122 5'-ATGGGAATTCTTATAGCTTTTTCGATCAGA TTTCTCAGTAGT at 35 cycles of 94°C for 30 secs, 54°C for 30 secs, 72°C for 90 secs, using Amplitaq (2U/100μl; Applied Biosystems, UK) according to the manufacturers instructions. pPIC9-derived plasmids containing ES-62 encoding sequences were linearised at the *Sal* I site for 4h at 37°C. The linearised plasmids were then transformed into *P.pastoris* GS115 (5μg/transformation) and the transformed strains were selected on MD plates following electroporation according to the manufacturer's instructions (Invitrogen). Selected clones were grown at high density (OD_{600nm} 3-6) in 5 x 4 L flasks containing BMG (2L) at 30°C. The culture was then centrifuged (6000 x g for 15 min), and the pellet re-suspended in BMM (10 L) and incubated with agitation (200 rpm) at 30°C for 48-72h with addition of methanol (0.5% v/v) every 12h. After induction, the supernatant was separated from cells by centrifugation (6000 x g for 15 min) and filtered through a 0.45 μm membrane. Following HPLC purification more than 95% of the protein elutes as a single peak that represents ES-62. Purity and identity of each batch was confirmed by a combination of SDS-PAGE and Western blotting, the latter employing a rabbit antiserum specific for ES-62. Finally, the level of endotoxin in the recombinant ES-62 sample was confirmed using an Endosafe kit (Charles River).

2.5 Preparation of nut derivatives

Nut derivatives were prepared by Dr MJC Alcocer (School of Life and Environmental Sciences, University of Nottingham, Nottingham, U.K.) as previously described [200]. Briefly, amplified genes for sunflower albumin 8 (SFA 8) and brazil nut albumin (Ber e 1) were ligated into pPIC9 (Invitrogen) plasmids and transformed into *Pichia pastoris* GS115 (Invitrogen). Recombinant Ber e 1 and SFA 8 proteins were secreted by the yeast and purified by FPLC using a heparin-sepharose column. Both reverse phase purified recombinant proteins were shown to be absent of endotoxin as determined by an Endosafe Kit (Charles River Laboratories, Ramsgate, UK).

2.6 Preparation of PC-OVA

OVA-PC was prepared by prepared by Ms C Egan (Department of Immunology, University of Strathclyde, Glasgow.) by combining diazoniumphenyl-PC and ovalbumin.

Diazoniumphenyl-PC was prepared by dissolving 1 mmol of aminophenyl-PC (Toronto Research Chemicals) in 3 ml of 1 N HCl and adding 1 mmol of sodium nitrite. Diazoniumphenyl-PC (125 µmol) was coupled to 1.5 µmol of OVA by incubation in 5 ml of 0.1 M borate buffer with 0.15 M sodium chloride, pH 9.0, for 12 h at 4°C, followed by dialysis against 10 mM phosphate buffer, pH 7.2. The absence of endotoxin from PC-OVA and OVA samples was confirmed using an Endosafe kit (Charles River Laboratories).

2.7 Murine Models of inflammation

2.7.1 Collagen-induced arthritis

CIA was induced and assessed by Drs A Boitelle and JA Gracie at the Centre for Rheumatic Diseases, University of Glasgow (Glasgow, U.K.) as previously described [192]. Male DBA/1 mice received 200 µg of bovine type II collagen (CII; Sigma-Aldrich) in CFA (Difco, Detroit, MI) by intradermal injection (day 0). Collagen (200 µg in PBS) was given again on day 21 by i.p. injection. Control mice received PBS alone at the same time points. Mice were monitored daily for signs of arthritis for which severity scores were derived as follows: 0, normal; 1, erythema; 2, erythema plus swelling; 3, extension/loss function, and total score, sum of four limbs.

For the prophylactic treatment model (Figure 2.1a), mice were treated with 2 µg ES-62 *s.c.* on day -2, day 0 (day of immunization with CII in CFA), and day 21 (i.p. collagen challenge). For the therapeutic studies (Figure 2.1b), mice were treated daily with 2 µg ES-62 *s.c.* for a total of 14 days commencing 1 day after CIA was clinically detectable. Control mice received PBS alone at the same time points.

2.7.2 Ovalbumin-induced asthma

The ovalbumin-induced asthma model was induced and maintained by Dr C McSharry, Department of Immunology, University of Glasgow. Following a similar previously published protocol ([201]; demonstrated in Figure 2.2), pulmonary inflammation was induced. Briefly, 6-8 week old female BALB/c mice were sensitised to whole ovalbumin (OVA) by intraperitoneal injection of 100 µg OVA in 1% alum (Alhydrogel; Brenntag Biosector, Fredriksund, Denmark) on days 0 and 14. On day 14 mice were additionally challenged intranasally with 50 µg aerosolised OVA in phosphate buffered saline (PBS) (after anaesthesia was induced with avertin (1,1,1-tribromoethanol) dissolved in amyl alcohol and diluted 1/40). On days 25, 26 and 27 mice were anaesthetised and re-challenged with 50 µg aerosolised OVA in PBS, administered intranasally. Control mice received PBS in place of OVA. Mice were euthanased on day 28 by lethal intraperitoneal injection of anaesthetic. For the prophylactic treatment model (Figure 2.2a), mice were treated with 2 µg ES-62 *s.c.* two days before and on the day of OVA administration. For

the therapeutic studies (Figure 2.2b), mice were treated daily with 2 μg ES-62 *s.c.* for three days prior to sacrifice. Lungs were carefully dissected, fixed in Formalin and stored for sectioning. Dissection and processing continued as described in section 2.8 below.

2.7.3 Ovalbumin-induced chronic airway inflammation

This chronic airway inflammation model (Figure 2.3) was designed as a preliminary model to extend the ovalbumin-induced asthma model (described above). The chronic airway inflammation model was induced and maintained by Dr C McSharry, University of Glasgow, Glasgow. 6 to 8 week-old female wild-type (BALB/c) and ST2KO mice were treated as described for the asthma model (Section 2.7.2) until day 14. After this time, mice continued to receive once-weekly challenges with aerosolised ova until day 56. ES-62 treatment of this model was prophylactic. ES-62 (2 μg) in PBS was administered subcutaneously 2 days before and on the day of ovalbumin administration. Control mice received PBS. Mice were euthanased on day 57 by lethal intraperitoneal injection of anaesthetic.

2.7.4 Systemic Lupus Erythematosus (SLE) Model

Several different inbred murine models have been investigated for different aspects of autoimmune systemic lupus erythematosus pathogenesis and pathology [202]. The MRL/Mp–*lpr/lpr* (MRL/*lpr*) mouse substrain is genetically predisposed to the development of SLE-like syndrome, including pathologies such as lupus nephritis and spontaneous lupus-like skin lesions Thus, this model has been classified as clinically similar to the human disease. It has been determined that this mouse strain carries a mutation in the *fas* gene. The *fas* defect is believed to accelerate the autoimmunity of MRL/*lpr* mice, which begins at the age of 3 months. The SLE model was maintained and continuously assessed by Drs A Boitelle, JA Gracie and Prof IB McInnes at the Centre for Rheumatic Diseases, University of Glasgow (Glasgow, U.K.). Male MRL/*lpr* mice were purchased at 5-7 weeks old. Micturation was induced twice weekly and urine monitored for proteinuria and haematuria using a commercially available kit (Multistix; Bayer, Cambridge, U.K.). Mice were also assessed for arthritis using the score system adopted in the CIA model (Section 2.7.1). ES-62 (2 μg) diluted in PBS was administered subcutaneously twice per week. Control mice received PBS in place of ES-62. Autoimmunity was allowed to develop for 11 weeks before mice were euthanased by cervical dislocation. Kidneys were carefully dissected, snap frozen in OCT embedding medium and stored in liquid nitrogen for sectioning. *Post-mortem* dissection and processing continued as described below (Section 2.8).

2.8 Disease model post-mortem procedures

2.8.1 Broncho-alveolar lavage and analysis

Broncho-alveolar lavage was performed in mice from the asthma model (Section 2.7.2) as previously described [201]. Briefly, after lethal injection and careful dissection of the thorax, a 23G needle sheathed by plastic tubing was inserted, via a small incision in the trachea, and the airways flushed with 1ml PBS. BAL was centrifuged at 400 *g* for 5 minutes and BAL fluid (supernatant) separated from BAL cells. BAL fluid was stored at –80°C until further analysis and BAL cells were resuspended in 1ml FACSflow. Cytospin preparations of 10000-100000 cells were made, fixed with methanol, dried and stained using Rapi-Diff II (rapid Romanowsky stain). Subsequently, differential cell counts were made from these cytospins by counting at least 400 cells in random high-power microscope fields. Different cell types were determined by identifying different staining and morphology. BAL fluid was analysed by ELISA or Luminex® for cytokine content.

2.8.2 Immunohistology of lung tissue

After BAL sampling, the lungs were removed and inflated with 1ml of 10% neutral-buffered formalin and then fixed in 10% neutral-buffered formalin for 72 h. The left lung was then dissected free and embedded in paraffin. Sections (6 µm) were stained with H&E and examined under x10 magnification, and peri-bronchial and peri-vascular inflammation assessed.

2.8.3 Serum preparation and analysis

Blood (1 ml) was obtained from each mouse, by cardiac puncture. This was centrifuged for 10 minutes to separate serum and blood cells. The serum was transferred to a separate tube and stored at –20°C until further analysis.

2.8.4 *In vitro* preparation of lymph node cells and splenocytes

Lymph nodes and spleen were carefully dissected from disease model mice. In the asthma and chronic airway inflammation models, lung-draining (thoracic, cervical and pulmonary) lymph nodes were kept separate from other (peripheral) lymph nodes and labelled as draining lymph nodes (DLN) and peripheral lymph nodes (PLN) respectively.

2.8.5 *In vitro* bone marrow cultures

Intact femurs were dissected from the mice and stored in RPMI on ice until further processing. Bone marrow was flushed from the bones using a 23G needle and syringe filled with RPMI 1640 (supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin). Bone marrow cells in suspension were underlaid with 1 ml FCS and incubated on ice to separate non-cell debris. After 5 min incubation time, the top layer was

removed to another tube and underlaid with 1 ml FCS. Following centrifugation 400 x *g*, 5 min, 4°C), the resulting cell pellet was re-suspended in appropriate culture media.

2.8.6 Myography of airway smooth muscle

Myography is a method commonly used to study the contraction of smooth muscle in the walls of physiological vessels. Intact vessel rings are mounted on parallel pins within the myograph apparatus (Figure 2.4) and held under transverse tension. Contraction and relaxation of the smooth muscle in the vessel wall (to decrease or enlarge the vessel lumen) is detected and, via an isometric force transducer, can be measured. Mice from the asthma model were killed by an overdose of sodium pentobarbitone (0.01 ml/g) and the heart and lungs removed and placed in cold Krebs solution (composition (mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 11.0; pH 7.4). The trachea was dissected out and cleaned of the surrounding connective tissue. The trachea from each mouse were mounted on a Multi Myograph (Model 610, 'myopin' arrangement; Danish Myotechnology, Aarhus, Denmark) in Krebs solution at 37°C and bubbled with 16% O_2, 5% CO_2, 79% N_2 throughout the entire experiment to simulate *in vivo* conditions. No attempt was made to remove the endothelial cell layer.

Preliminary experiments, using untreated BALB/c mice, were performed to assess the optimum tension at which the vessels should be held in the myograph apparatus. These experiments were conducted by increasing the tension across the vessels in a step-wise manner and measuring the maximum response to KCl (50 mM). The optimum tension was considered to be that when the maximum contractile response to KCl was greatest. These preliminary experiments indicated that 3.5 mN was the optimum tension at which the tracheal rings should be held for conducting the experimental protocols.

After experimental vessels were mounted on the myograph, they were left to equilibrate for at least 30 minutes or until they had attained a stable baseline. Tension was applied at this point and the vessels were allowed to equilibrate once more until a stable baseline had been achieved. Experiments involved: completing a maximal response to KCl (50 mM) protocol, washout and restablisation of baseline, followed by completion of a MCh cumulative concentration-response protocol (CCRP). The MCh concentration was increased in half-log increments from $1x10^{-9}$ to $3x10^{-4}$ M (Figure 2.5). Myodaq and Myodata software (Danish Myotech, Aarhus, Denmark) were used for data acquisition and data analysis respectively. Contractile responses are represented as a percentage of the vessels maximum response to KCl.

2.9 Cell culture

2.9.1 Bone marrow-derived DC

Bone marrow dendritic cells were cultured as described previously [189]. Briefly, bone marrow progenitor cells were washed with sterile culture media and cultured for 6 days in large (175 cm^2) flasks at $4x10^5$ cells/ml in DC media (RPMI 1640 with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 2-mercaptoethanol (50 μM) (all Gibco, Paisley, U.K.) and 10% culture supernatant of a GM-CSF-transfected X63 myeloma cell line at 37°C/5%CO_2/95% O_2. Cells were fed with fresh medium on day 4 of culture.

2.9.2 Bone marrow-derived macrophages

Bone marrow-derived macrophages were cultured as described previously [191]. Briefly, bone marrow progenitor cells were cultured in macrophage media, which consists of DMEM (Life Technologies, Paisley, UK) containing 10% FCS (Life Technologies), 20% L929 cell culture supernatant (containing CSF-1), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 5% horse serum. The macrophages were cultured at 37°C/5%CO_2 for at least 6 days before beginning experiments. The media was replaced on day 4 of culture.

2.9.3 Generation of T cell blasts for cell contact assay with macrophages

On day 3 before set-up of the T cell-macrophage cell contact assay, spleens and lymph nodes were dissected from BALB/c mice and a single cell suspension was obtained by pushing them through Nitex mesh. This cell suspension was cultured at $1x10^6$ cells/ml for 3 days in lymphocyte media (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.01% non-essential amino acids and 0.001% sodium pyruvate) containing PMA (10 ng/ml) and Con A (5 μg/ml) to generate T cell blasts (activated and proliferating T cells).

2.9.4 Purification of naïve $CD4^+CD62L^{high}$ T cells for co-culture with DC

On the day of co-culture set-up, lymph nodes and spleens were dissected from untreated DO.11.10 mice and a single cell suspension prepared by pressing through Nitex mesh. These cells were washed in RPMI 1640 and resuspended (10^8 cells/ml) in magnetic activated cell sorter (MACS) buffer (0.5% bovine serum albumin in PBS) and incubated with anti-CD4-MACS beads (Miltenyi Biotec), for purification using a MACS (LS) magnetic column. These purified $CD4^+$ T cells were further separated to isolate (naïve) $CD62L^+CD4^+$T cells using anti-CD62L-MACS beads and a magnetic (LS) column by following the manufacturers instructions (Miltenyi Biotec).

2.9.5 THP-1 cells

THP-1 cells are a human mononuclear cell line. These cells were cultured in RPMI (with 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin) at 37°C until required.

2.9.6 Human peripheral blood T cells

Samples were derived with approval from Glasgow Royal Infirmary Ethical Committee, Glasgow UK. Peripheral blood (PB) T cells were prepared as described previously [203]. Briefly, normal donor derived PB T cells were stimulated for 72 h with PHA (5 μg/ml) / PMA (10 nM) in RPMI, with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% FCS, then fixed in 4% paraformaldehyde. Control non-activated PB T cells were also fixed for comparative purposes. Fixed PB T cells were then co-cultured with myelomonocytic THP-1 cells that were pre-treated with ES-62 (2 μg/ml) for 18 h.

2.10 In vitro assays

2.10.1 Culture of Dendritic Cells and Macrophages with LPS

On day 6 of bone marrow cell culture, DC or macrophages were gently removed from culture flasks. DC were plated out at $2x10^6$ cells/well in a 6-well plate in a total volume of 3ml DC culture media, whilst macrophages were plated out at $2x10^5$ cells/well in triplicate in 96-well plates in a total volume of 250μl macrophage culture media. Cells were cultured unstimulated (media alone) or stimulated for 24 hours. For this stimulation DC were treated with 1 μg/ml lipopolysaccharide (LPS, from *Salmonella Minnesota*), whilst macrophages were treated with 100 ng/ml LPS plus 10^2 U/ml murine IFNγ (kindly donated by Prof. FY Liew, University of Glasgow). After this culture period, supernatants from both cell types were collected and stored at –20°C until analysis for cytokine content (section 2.11.5). DC were gently removed from the culture well and separated from the culture supernatant by centrifugation, in preparation for analysis by flow cytometry (section 2.11.3) or for co-culture experiments (section 2.10.2).

2.10.2 DC - D0.11.10 T cell co-culture

After culturing bone marrow DC for 6 days, cells were gently removed from the culture flask. DC were plated out at 1 x 10^6 cells/ml in 6-well plates and grown in DC culture medium in the presence or absence of relevant stimulus for a further 24 h at 37°C. At the end of the DC 24h culture period, DC were washed three times with PBS before co-culturing with purified DO.11.10 T cells (Figure 2.6). Briefly, $CD4^+CD62L^+$T cells were co-cultured (2.5 x 10^5 cells/ml) with DC (2.5 x 10^4 cells/ml) in a total volume of 1 ml in 24-well tissue culture plates (for cytokine analysis) or in a total volume of 200 μl in 96-well tissue culture plates, (for proliferation analysis)(Nunc, Naperville, IL). Cells were cultured with or without cOVA $peptide_{323-339}$ at various concentrations (0.03, 0.3, 3.0 and 30 nM) at 37°C. After 72h of culture, proliferation assay wells were pulsed with [^{3}H] thymidine (1 μCi/well;

Amersham, Buckinghamshire, U.K.) and cytokine assay wells were stimulated with PMA (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma). After a further 24h of culture (total co-culture time was 96h), cytokine assay culture supernantants were collected and stored at –20°C and cells were harvested using a Betaplate 96-well automated harvester (Amersham Pharmacia Biotech) onto glass fibre filter mats (Wallac, Warrington, U.K.). Incorporated thymidine was assessed by liquid scintillation counting and results are expressed as counts per minute incorporated label ± S.E.M. (of triplicate culture wells).

2.10.3 T cell - Macrophage contact assay

The T cell - macrophage contact assay was designed to investigate only contact-dependent communication between T lymphocytes and macrophages. Macrophages were cultured from bone marrow progenitor cells as described above (section 2.9.2). After 7 days of culture, macrophages were removed from their culture wells and washed three times with RPMI 1640. On the same day, T cell blasts (as described in section 2.9.3) were removed from their culture flask and fixed by incubating with 4% formaldehyde for 30 mins, followed by washing three times with RPMI 1640. Macrophages and fixed T lymphocytes were plated out in triplicate in 96-well plates. Each well contained $2x10^5$ macrophages and increasing numbers of lymphocytes (from $2x10^5$/well up to $1.6x10^6$/well) in a total volume of 200µl macrophage medium. After co-culturing at 37°C for 48h, culture supernatants were collected and stored at –20°C.

2.10.4 *In vitro* cultures of LN cells and splenocytes from disease models

Single-cell suspensions of lymphocytes were obtained by pressing lymph nodes and spleens through Nytex mesh (Cadisch Precision Meshes Ltd, London, U.K.) to obtain a single cell suspension. Splenic red blood cells were removed by hypotonic lysis. Cells were cultured in RPMI 1640 medium at $1x10^6$ cells/ml with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), 2-mercaptoethanol (50 µM), 1% non-essential amino acids and sodium pyruvate (1 mM) (all from Gibco Life Technologies, Paisley, U.K.). Cells were cultured, unstimulated (media alone) or in media containing antigen (whole OVA at 500 µg/ml (asthma model) or type II collagen at 50 µg/ml (CIA model)) or mitogen (Con A at 5 µg/ml) for a 72h culture period.

2.11 *In vitro* analysis

2.11.1 RNA Extraction

At the end of the in vitro culture period, culture supernatants were removed and cells washed twice with cold, sterile PBS (Gibco, Paisley, U.K.). RNA-Bee solution (1 ml; AMS Biotechniology, Oxon, U.K.) was added directly to the cells in the culture wells and incubated at room temperature for 1 minute, to allow time for the cells to lyse, after which the samples were stored at –20°C until further use. The RNA-Bee samples containing the

lysed cells were then transferred to 1.5 ml microcentrifuge tubes, in preparation for the RNA extraction procedure. Chloroform (200 µl) was added to the tubes and samples were incubated for 10 minutes on ice. Following centrifugation at 13,000 x *g* (15 min, 4°C), the upper phase was removed and an equal volume of isopropanol was added. RNA was precipitated by incubation on ice for 30 min and separated by centrifugation at 13,000 x *g* (20 min). The remaining RNA pellet was washed with ethanol twice and dried before dissolving in diethyl pyrocarbonate (DEPC)-treated water. The RNA sample was heated to 68°C for 2 min before further processing.

2.11.2 Taqman and RT-PCR

Taqman real-time PCR was conducted according to the manufacturer's instructions (PE Biosystems, Foster City, USA) and as described by [204]. RNA was reverse transcribed using 100 U Superscript II RT (Life Technologies, Gaithersberg, USA) at 42°C for 50 min in the presence of 50 nM Tris-Hcl (pH8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 0.5 mM dNTPs and 5 µM oligo$(dT)_{16}$ (Gibco BRL, Paisley, UK). Primers and fluorogenic probes (Table 2.1) were designed using the Primer Express™ v1.0 program and purchased from Applied Biosystems. The probes used contained a reporter dye 5'-6-carboxy-fluorescein (FAM) and a quencher dye 3'-6-carboxy-tetramethyl rhodamine (TAMRA) and were HPLC purified. Extension from the 3' end was blocked by attachment of a 3' phosphate group.

PCR reactions were performed in the ABI-prism 7700 Sequence Detector, which contains a Gene-Amp PCR system 9600 (PE Biosystems). PCR amplifications were performed in triplicate in a total volume of 25 ml 10 mM Tris-HCl buffer (pH 8.3) containing 0.5 µl cDNA sample, 75 mM KCl, 10 mM EDTA, 200 µM dATP, dCTP, dGTP and 400 µM dUTP, 5 mM $MgCl_2$, 300 nM each primer, 200 nM probe, 0.625 U AmpliTaqGold™ and 0.25 U AmpErase UracilN-Glycolase (PE Biosystems). The PCR temperature protocol was as follows: 2 mins at 50 °C, 10 mins at 94°C, followed by 45 two-temperature cycles (15 seconds at 94°C and 1 minute at 60°C). Data analysis was completed using Sequence Detection Software (PE Biosystems) and samples were normalised by the reference reporter hypoxanthine-guanine phosphoribosyltransferase (HPRT).

2.11.3 Analysis of DC surface expression

Bone marrow-derived DC were cultured and isolated from culture medium as described previously (sections 2.9.1 and 2.10.1) and surface expression was analysed by flow cytometry to determine the effect of experimental treatments on cell phenotype. DC were washed twice in 5 ml tubes (Becton Dickinson, UK) with 200 µl cold FACS buffer (PBS, 1% BSA, 0.1% sodium azide) at 400 x *g* for 5 mins, 4°C.. An aliquot of DC from each experimental group was left unstained (no stain control). Whilst the remaining DC from each treatment group were separated into 5 aliquots and stained with PE-conjugated anti-

CD11c (to determine the $CD11c^+$ dendritic cell population) and one of the following FITC-conjugated antibodies: anti-MHC class II, anti-CD40, anti-CD80 and anti-CD86, or anti-CD54-biotin followed by Streptavidin-FITC (all from BD Pharmingen) for 30 mins at 4°C. The optimum incubation concentration of these antibodies was previously determined by titration. Details of the antibodies used are presented in Table 2.2a. DC were washed with 200 µl FACS buffer and re-suspended in 300 µl. Immediately prior to data acquisition, 50 µg/ml propidium iodide (PI; Calbiochem) was added to each sample to enable identification of live cells during data analysis (live cells do not incorporate PI). Cellular fluorescence was aquired using a FACSCalibur™ Immunocytometry system (Becton Dickinson, Oxford, Oxon, UK) and CellQuest software. Data was compiled and analysed using FloJo software (Tree Star Inc., Oregon, USA) (Figure 2.7).

2.11.4 Serum antibody analysis

To detect Ig levels in serum samples from the disease model mice, Maxisorp (Nalge Nunc International, Denmark) ELISA plates were coated with 20 µg/ml OVA protein or type II collagen or 2 µg/ml purified capture antibody in PBS and incubated at 4°C overnight (details of antibodies used in this protocol are listed in Table 2.2b). Plates were washed at least 3 times with 0.05% Tween-20 in PBS before being blocked with 10% FCS for 1 hour at 37°C. Plates were washed and serum samples (diluted in 10% FCS in PBS) were titrated, added to the plate and incubated overnight at 4°C. Plates were washed again with 0.05% Tween-20 and biotin-conjugated anti-mouse detection antibody (2 µg/ml; BD Pharmingen) was added to the plate. The plate was incubated at room temperature for 1 hour and washed. The plate was finally incubated with Streptavidin-HRP (1/1000 dilution in 0,05% Tween-20; BD Pharmingen) for 1h at room temperature. Plates were washed again and developed with tetramethylbenzidine (TMB) substrate (Insight Biotechnology Limited, Middlesex, U.K.). Absorbance at 630 nm was determined using an ELISA plate reader.

2.11.5 Cytokine analysis by sandwich ELISA

Cytokine ELISA was performed according to the antibody manufacturer's recommendations. IL-4, IL-5, IL-10, IFN-γ and TNF-α were analysed using OPTEIA Mouse ELISA kits (BD Pharmingen) and IL-12p40 was analysed using antibody pairs (BD Pharmingen) Briefly, Maxisorp (Nunc, Roskilde, Denmark) ELISA plates were coated with anti-mouse capture antibody, diluted in sodium bicarbonate buffer (0.1 M, pH 7.4), or sodium phosphate buffer (0.2 M, pH 6.5) for IL-10 ELISA, overnight at 4°C. Plates were washed with PBS-Tween (PBS containing 0.05% Tween 20) and blocked with 10% FBS in PBS for 1 hour at room temperature. All subsequent steps were performed at room temperature. After washing, samples and titrated standards were added to the plate and

incubated for 2 hours. Plates were washed again and biotinylated anti-mouse detection antibody (BD Pharmingen), diluted in 10% FCS in PBS, was added to the plate. After a one-hour incubation, plates were incubated with streptavidin-HRP (BD Pharmingen) for approximately 1 hour, washed and developed using tetramethylbenzidine (TMB) substrate (Insight Biotechnology Limited, Middlesex, U.K.) and absorbance at 650 nm was determined using an ELISA plate reader (Dynex Technologies, Worthington, U.K.). Cytokine concentration was determined using the absorbance values obtained for the standards. The limit of detection was consistently 20 pg/ml.

2.11.6 Cytokine analysis by Luminex

In some experiments, the Luminex system was used to determine the concentration of several inflammatory cytokines simultaneously in culture supernatants. This was performed according to the manufacturers instructions using a Multiplex antibody bead kit (Biosource Inc., Worcester, U.S.A.) for Luminex. The Luminex method for cytokine quantitation employs spectrally encoded microbeads conjugated to antibodies specific for the range of cytokines to be measured. Each antibody is conjugated to individual beads that can be distinguished from beads conjugated to other antibodies by their individual fluorescence wavelength and size properties. Beads specific for particular cytokines can be gated to a particular region by the Luminex 100™ scanner (Bio-Rad Laboratories, Hercules, U.S.A.). Cytokine capture microbeads are incubated with samples and titrated standards at room temperature for 2 hours in a filter-bottom microplate, followed by washing and incubation with biotinylated detection antibody. This is followed by incubation with Streptavidin - R. Phycoerythrin (RPE) and a final wash step. Sample cytokine concentration is determined by comparing the fluorescence intensity of the microbeads in the sample wells with their counterparts in the known-concentration standards.

2.11.7 Tissue sectioning, staining and imaging

Intact lymph nodes, frozen in liquid nitrogen in OCT embedding medium (Miles, Elkart, USA) in cryomoulds (Miles), were cut into 6 μm thick sections using a cryostat (ThermoShandon, Cheshire, U.K.) and mounted on SuperFrost slides (ThermoShandon). Slides were fixed in formaldehyde and stored at –20°C until stained. Lymph node sections were stained using a protocol similar to that described previously [205]. Briefly, tissue sections were brought to room temperature in acetone for 10 min, air dried and rehydrated with PBS before being incubated in 0.1% azide/3% H_2O_2 for 45 min, to block endogenous peroxidase. Avidin solution (Vector Laboratories Ltd., Peterborough, UK) was added for 15 min to block unmasked endogenous biotin, then biotin solution (Vector Laboratories) was added to block excess avidin. Sections were washed in PBS after each treatment.

Prepared sections were stained for expression of IgE as follows. Sections were incubated with permeabilization buffer (2% FCS, 2 mM EDTA, pH 8.0, 0.5% saponin (Sigma-Aldrich)) for 30 min. After washing with PBS, sections were blocked with 1% blocking reagent (PerkinElmer) for 15 min. IgE was detected by incubating sections with biotinylated anti-IgE or rat anti-IgG1 isotype control (diluted in 1% blocking reagent/0.1% saponin) for 40 mins. Sections were washed with TNT wash buffer (10% Tris (pH 7.5), 10% NaCl and 0.05% Tween-20 (all Sigma) in distilled water) between incubations with Streptavidin-HRP (diluted 1/100 with 1% blocking reagent/0.1% saponin; Perkin Elmer) for 30 min and biotinylated tyramide (diluted 1/50 with amplification diluent; Perkin Elmer) for 10 min. Fluorescence was attached to the IgE signal by incubating the sections with Streptavidin – Alexa Fluor 647 (diluted 1/500 in TNB (10% Tris (pH 7.5), 10% NaCl and 0.5% blocking reagent); Molecular Probes Inc., Eugene, U.S.A.) for 30 mins. At this stage, sections were also stained with FITC-anti-B220 (diluted 1/100 in TNB; BD Pharmingen) to detect B cell follicles. Sections were washed with TNT and slides were mounted using Vectashield mounting medium (Vector). Photographs of sections were taken at x20 magnification using a Hammamatsu camera and analysed using Openlab imaging software (Improvision, Coventry, U.K.). IgE positive cells were depicted in red.

2.12 Statistics

Where presented, statistical significance was determined by student's t-test or ANOVA followed by Tukey's multiple comparison, as appropriate. Statistical significance was assumed when $p<0.05$.

Table 2.1 Primers and probes for Taqman Real-Time PCR

All probes and primers were purchased from Applied Biosystems

Gene	Forward Primer	Reverse primer	Probe 5'-3'
c-MAF	AAGAGGCGGACCCTGAAAA	TCCGACTCCAGGACGTGTCT	TGCCCAGTCCTGCCGCTTCAAG
Foxp3	CCCAGGAAAGACAGCAACCTT	TTCTCACAACCAGGCCACTTG	ATCCTACCCACTGCTGGCAAATGGAGT
GATA-3	TCCTCCTCTACGCTCCTTGCTA	ACACTGATTCCTTGGCGCTC	TCGTGATCGGAAGAGCAACCGTCTC
T-bet	GCCAGGGAACCGCTTATATG	AACTTCCTGGCGCATCCA	CCCAGACTCCCCCAACACCGGA

Table 2.2 Antibodies used for flow cytometry and serum immunoglobulin

Table 2.2a Antibodies used for flow cytometry

All antibodies were purchased from BD Pharmingen except KJ1.26, which was prepared in house from the B cell KJ1.26 hybridoma.

Table 2.2b Antibodies used for serum immunoglobulin analysis

All antibodies were purchased from BD Pharmingen. OVA and Type II Collagen were purchased from Sigma.

Table 2.2a

Specificity	Conjugate	Isotype
CD4	PE, APC, PerCP	Rat IgG2aκ
CD11c	PE	Hamster IgG1λ
CD40	FITC	Rat IgMκ
CD54	Biotinylated	Hamster IgG1λ
CD62L	PE	Rat IgG2aκ
CD80	FITC	Hamster IgG2κ
CD86	FITC	Rat IgG2aκ
MHC class II	FITC	Mouse IgG2bκ
TCR KJ1.26	Biotinylated	Mouse IgG2a

Table 2.2b

Measurement	Capture Antibody or Protein	Detection Antibody
Total IgE	Anti-mouse IgE	Biotinylated anti-mouse IgE
OVA-specific IgE	OVA protein	Biotinylated anti-mouse IgE
OVA-specific IgG1	OVA protein	Biotinylated anti-mouse IgG1
OVA-specific IgG2a	OVA protein	Biotinylated anti-mouse IgG2a
Collagen-specific IgG1	Bovine type II collagen	Biotinylated anti-mouse IgG1
Collagen-specific IgG2a	Bovine type II collagen	Biotinylated anti-mouse IgG2a
Collagen-specific IgG3	Bovine type II collagen	Biotinylated anti-mouse IgG3
Collagen-specific IgG3	Bovine type II collagen	Biotinylated anti-mouse IgM
Single stranded DNA-specific IgG2a	heat-denatured single-stranded DNA	Biotinylated anti-mouse IgG2a
Double stranded DNA-specific IgG2a	heat-denatured double-stranded DNA	Biotinylated anti-mouse IgG2a
Total IgG1	Anti-mouse IgG1	Biotinylated anti-mouse IgG1
Total IgG2a	Anti-mouse IgG2a	Biotinylated anti-mouse IgG2a
Total IgG3	Anti-mouse IgG3	Biotinylated anti-mouse IgG3
Total IgM	Anti-mouse IgM	Biotinylated anti-mouse IgM

Figure 2.1 Collagen-induced arthritis induction protocol

Male DBA/1 mice received 200 µg of bovine type II collagen in Complete Freund's Adjuvant (CFA) by intradermal (*i.d.*) injection (day 0). Collagen (200 µg in PBS) was given again on day 21 by *i.p.* injection.

Prophylactic treatment protocol: Mice were treated with 2 µg ES-62 (*s.c.*) on day -2, day 0, and day 21.

Therapeutic treatment protocol: Mice were treated daily with 2 µg ES-62 (*s.c.*) for a total of 14 days commenced 1 day after CIA was clinically detectable.

Control mice received PBS in place of ES-62 at the same time points.

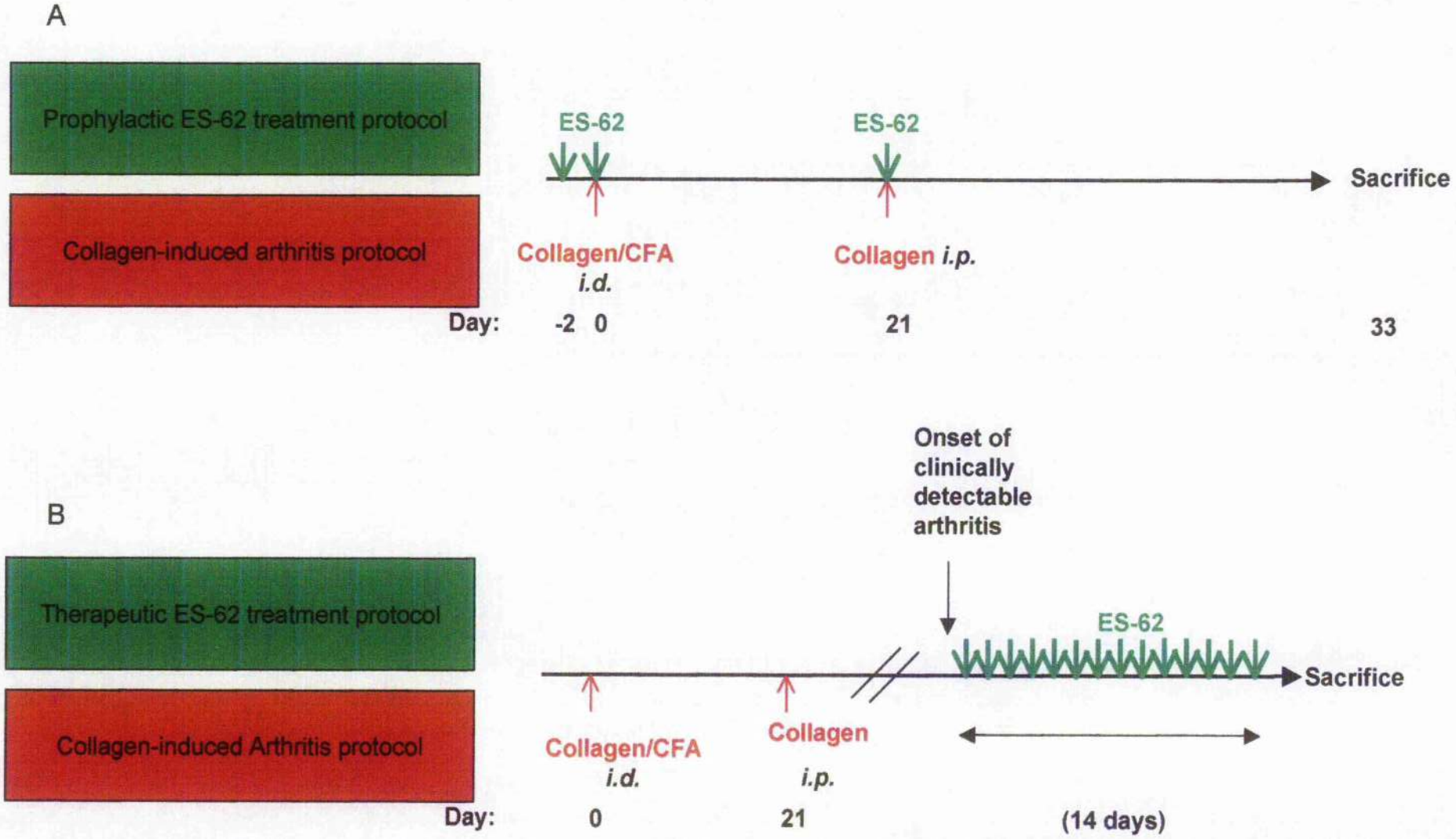

A
Prophylactic ES-62 treatment protocol
Collagen-induced arthritis protocol
ES-62
ES-62
Sacrifice
Collagen/CFA
i.d.
Collagen i.p.
Day:
-2 0
21
33
B
Therapeutic ES-62 treatment protocol
Collagen-induced Arthritis protocol
Onset of clinically detectable arthritis
ES-62
Sacrifice
Collagen/CFA
i.d.
Collagen
i.p.
Day:
0
21
(14 days)

Figure 2.2 Ovalbumin-induced airway inflammation protocol: prophylactic treatment with ES-62

BALB/c mice were sensitised with an ovalbumin (100 μg)/aluminium hydroxide (2 mg) emulsion intraperitoneally (OVA/ALUM *i.p.*) on days 0 and 14 and challenged with OVA (diluted in PBS) intranasally (OVA *i.n.*) on days 14 (50 μg), 25 (2 μg), 26 (2 μg) and 27 (2 μg) to stimulate onset of airway inflammation. There were four experimental groups denoted: *control, ES-62, asthma and asthma+ES-62*. The term 'Asthma' was used because the allergic pulmonary inflammation exhibited in these mice was similar to that observed in asthma.

Prophylactic treatment protocol: ES-62 treatment (2 μg) was administered subcutaneously two days before and on the day of OVA administration, i.e. on days –2, 0, 12, 14, 25 and 27.

Therapeutic treatment protocol: ES-62 treatment (0.4 μg, 2 μg or 10 μg) was administered subcutaneously on days 25, 26 and 27.

Mice in control treatment groups were administered with PBS in place of OVA/ALUM, OVA or ES-62. On day 28 of the protocol mice were sacrificed by lethal injection of anaesthetic.

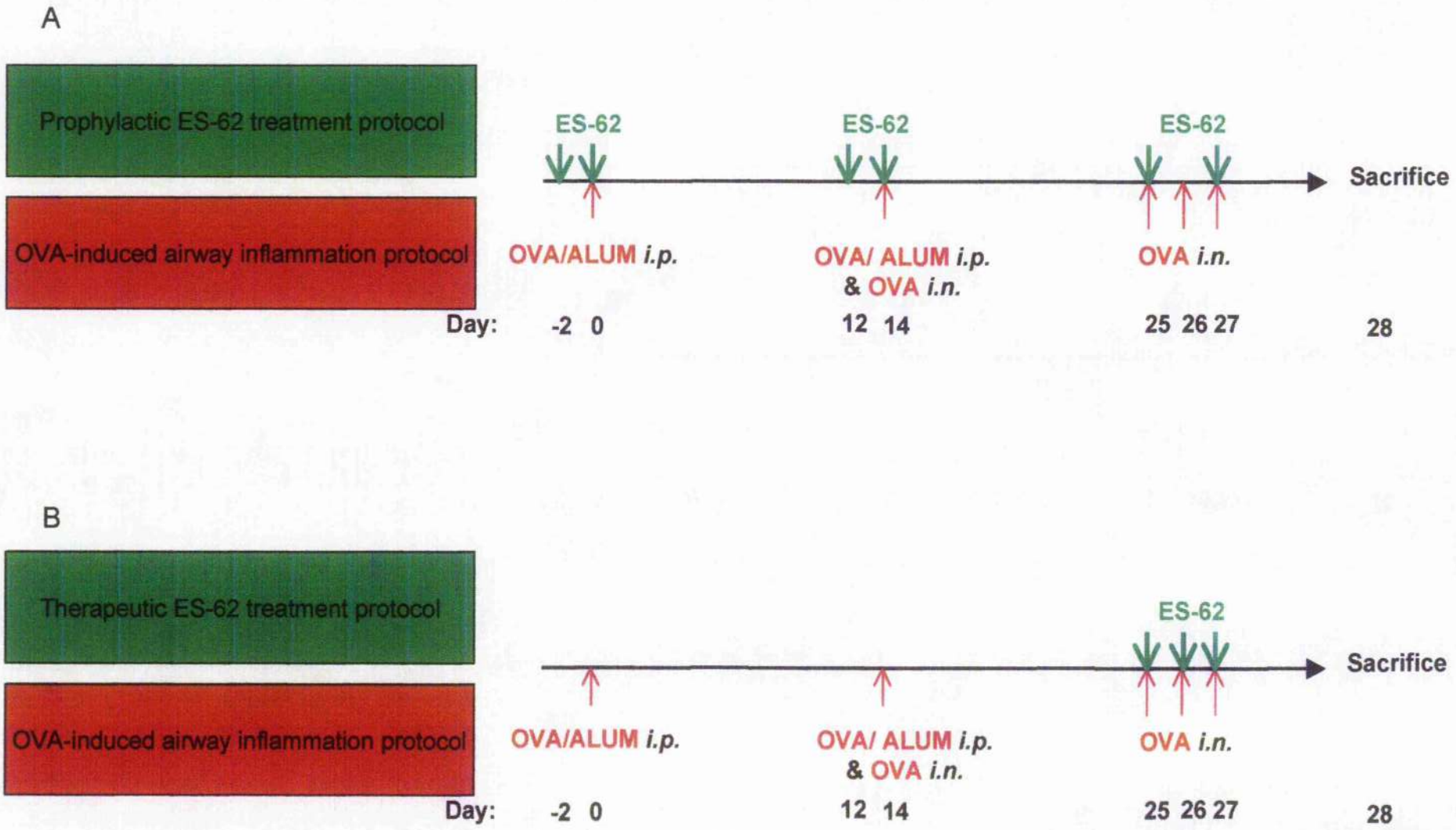

A
Prophylactic ES-62 treatment protocol
OVA-induced airway inflammation protocol
ES-62
ES-62
ES-62
Sacrifice
OVA/ALUM i.p.
OVA/ ALUM i.p.
& OVA i.n.
OVA i.n.
Day:
-2 0
12 14
25 26 27
28
B
Therapeutic ES-62 treatment protocol
OVA-induced airway inflammation protocol
ES-62
Sacrifice
OVA/ALUM i.p.
OVA/ ALUM i.p.
& OVA i.n.
OVA i.n.
Day:
-2 0
12 14
25 26 27
28

Figure 2.3 Ovalbumin-induced chronic airway inflammation protocol: prophylactic treatment with ES-62

BALB/c mice were sensitised with an ovalbumin (100 μg)/aluminium hydroxide (2 mg) emulsion intraperitoneally (OVA/ALUM *i.p.*) on days 0 and 14 and challenged with OVA (diluted in PBS) intranasally (OVA *i.n.*) on day 14 (50 μg) and once every seven days until day 56 (2 μg), to induce chronic airway inflammation and airway remodelling. ES-62 treatment (2 μg) was administered subcutaneously two days before and on the day of OVA administration, i.e., on days –2, 0, 12, 14, 19, 21, 26, 28, 33, 35, 49, 42, 47, 49, 54 and 56. Mice in the control treatment groups were administered with PBS in place of OVA/ALUM, OVA or ES-62. On day 57 of the protocol mice were sacrificed by lethal injection of anaesthetic.

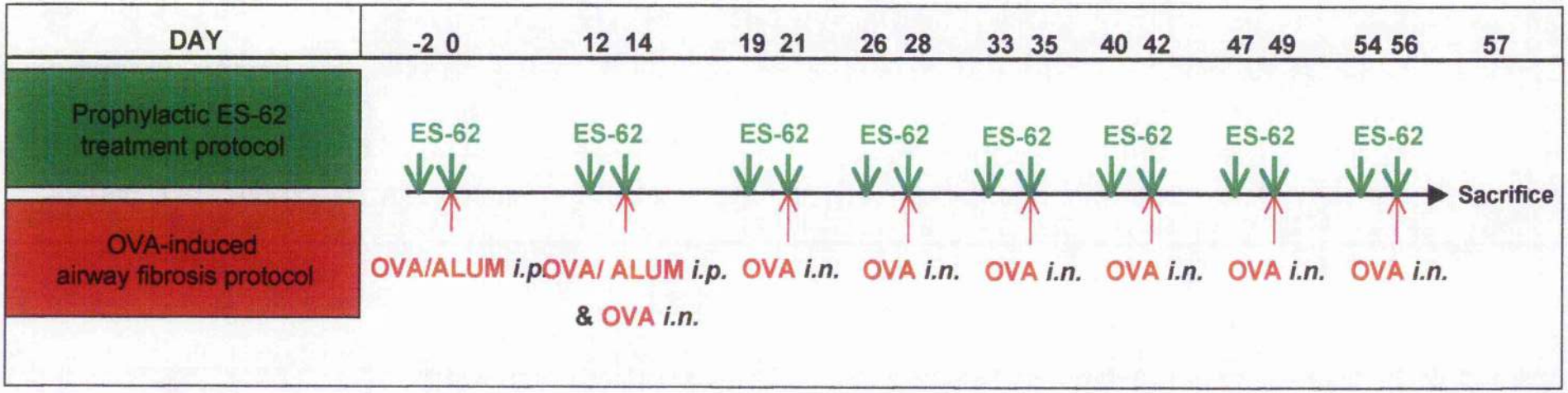

DAY
-2 0
12 14
19 21
26 28
33 35
40 42
47 49
54 56
57
Prophylactic ES-62 treatment protocol
OVA-induced airway fibrosis protocol
ES-62
Sacrifice
OVA/ALUM i.p
OVA/ ALUM i.p.
& OVA i.n.
OVA i.n.

Figure 2.4 Myograph apparatus

Myography is an experimental method used to determine the contractile properties of small vessels (of internal diameter 100 μm-3 mm) in terms of responsiveness to hormones, agonists or contractile agents. Using the Multi Myograph apparatus (Danish Myotechnology) in the 'myopin' arrangement, four individual vessels can be analysed in identical *in vitro* conditions. The (5 ml) organ baths are continually perfused with Krebs solution (bubbled with 16% O_2, 5% CO_2, 79% N_2) and maintained at a concentration of at 37°C throughout the entire experiment, to simulate *in vivo* conditions. Micro-dissected tracheal rings are fed onto the myopins taking care not to damage the tissue and the pre-determined optimum tension is applied using the micrometer screw gauge. Treatments are applied directly to the organ bath therefore the treatment concentrations are calculated based on a final volume of 5 ml. The distal myopin (A) is fixed, whilst the proximal myopin (B) moves in response to contraction of the tissue or alteration of the micrometer screw gauge. Changes in tension of the tissue are detected by the transducer, which is electronically linked to the Myodaq (Danish Myotechnology) acquisition software.

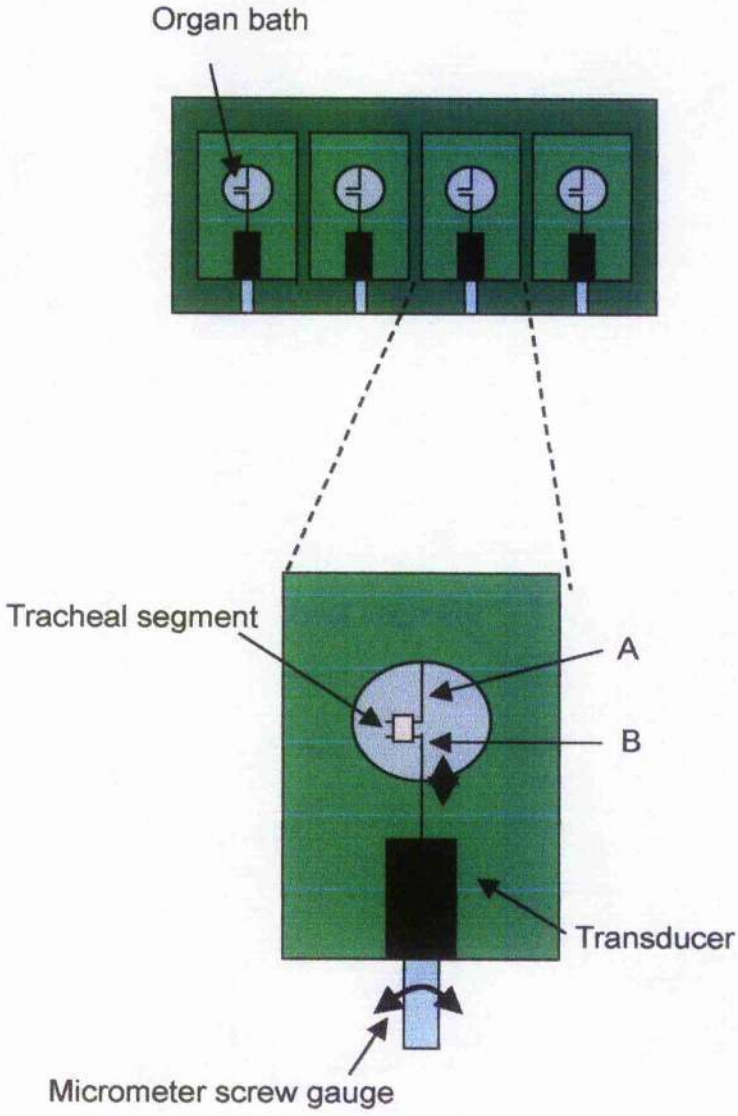

Organ bath
Tracheal segment
A
B
Transducer
Micrometer screw gauge

Figure 2.5 A typical cumulative concentration reponse protocol using the myography technique

This figure represents a typical tension plot obtained during induction of a cumulative concentration-response protocol, using the Multi Myograph apparatus (Figure 2.4) and Myodaq acquisition software (Danish Myotechnology). After the vessel is mounted on the myograph apparatus, it is left to equilibrate for at least 30 minutes or until it attains a stable baseline. The pre-determined optimum tension (3.5 mN) is then applied and the vessel is allowed to equilibrate once more until a stable baseline tension had been achieved. At this point KCl is added to the organ bath (at a final concentration of 50 mM). Once the contractile response to KCl has plateaued (KCl_{max}) the organ bath solution is washed and the vessel is allowed to attain a stable baseline once more. The cumulative concentration – response protocol (CCRP) to a contractile agent (e.g. methacholine) is started by adding the lowest concentration (e.g. $1x10^{-9}$ M) of the agent to the organ bath and allowing the tension response of the vessel to plateau. Without washing, the concentration of the agent in the bath is increased to the next determined concentration and this pattern is repeated in (half log) increments until the vessel has been exposed to the highest concentration of the agent (e.g. $3x10^{-4}$ M) and the tension response allowed to plateau. At this point the bathing solution in the organ bath is refreshed several times to wash the vessel. The maximum tension response of the vessel at each concentration of contractile agent can then be expressed as a percentage of KCl_{max}.

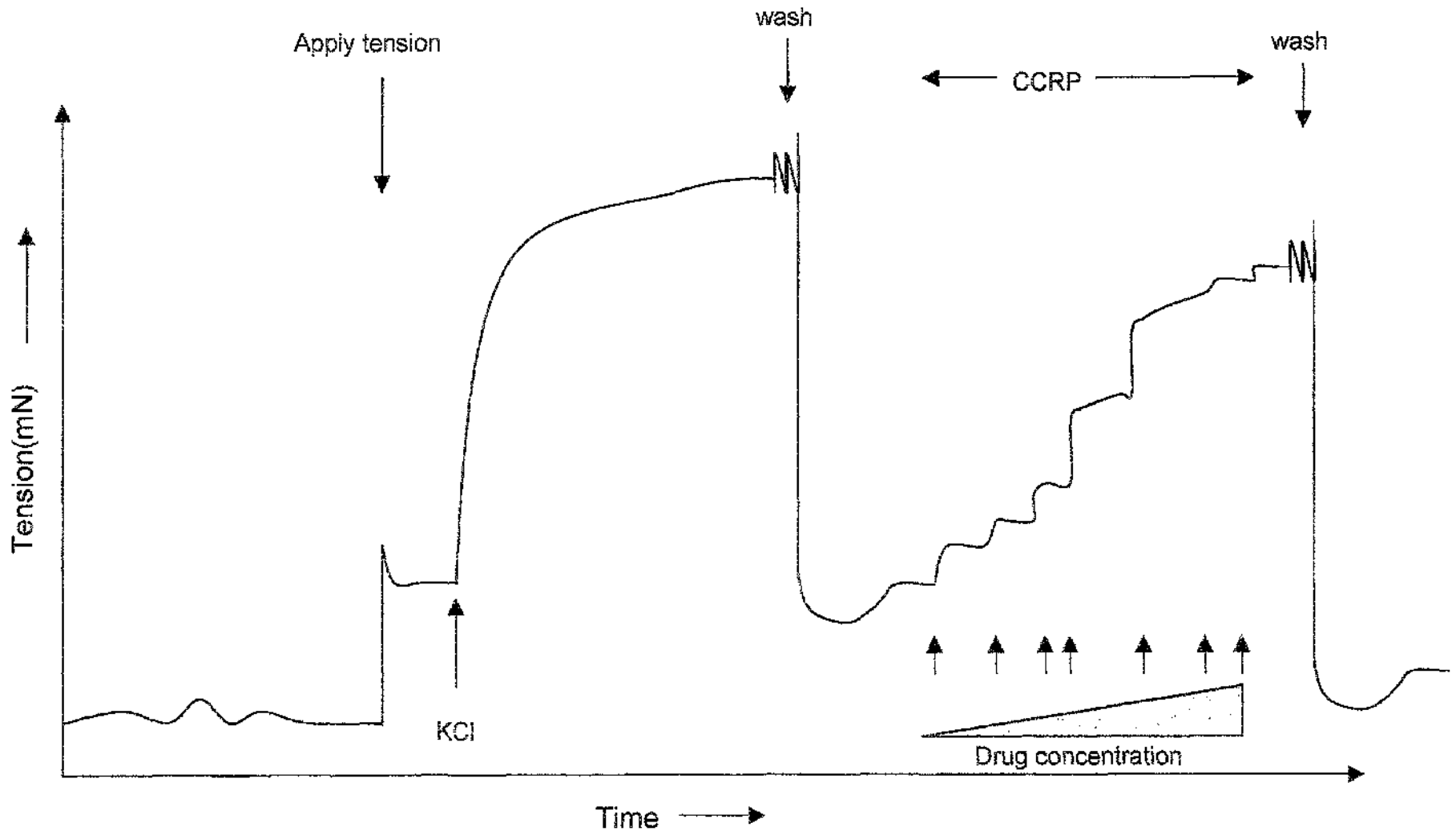

Appiy tension
wash
CCRP
wash
Tension(mN)
KCl
Drug concentration
Time

Figure 2.6 Determination of the percentage of $CD4^+$ transgenic OVA-specific TCR T cells in lymph node cell and splenocyte cultures from DO.11.10 BALB/c mice

A Dot plot of forward scatter versus side scatter, showing the population of cells deemed healthy by their position on this plot. Forward scatter gives a measure of the size of cells, whilst side scatter gives a measure of their granularity. The gate indicates cells of the correct size and granularity associated with lymphocytes.

B-D Dot plot showing unstained lymphocytes (**B**). Dot plot of lymphocytes single stained with anti-CD4-PerCP, thus allowing identification of $CD4^+$ T lymphocytes (**C**) Dot plot of lymphocytes single stained with the anti-clonotypic KJ1.26-biotin-streptavidin-PerCP. This antibody binds the TCR expressed by $CD4^+$ T cells from DO.11.10 BALB/c mice, which is specific for the chicken ovalbumin peptide 323-339 bound to I-Ad MHC class II (**D**).

E Dot plot showing the population of transgenic $CD4^+KJ1.26^+$ T cells from a naïve mouse with gate for double positive $CD4^+KJ1.26^+$ T cells.

F Dot plot showing the population of transgenic $CD4^+KJ1.26^+$ T cells from an immunised mouse. From this plot the population of $CD4^+KJ1.26^+$ T cells can be clearly identified and gated, thus allowing the percentage of $CD4^+KJ1.26^+$ T cells from the total lymphocyte population to be calculated.

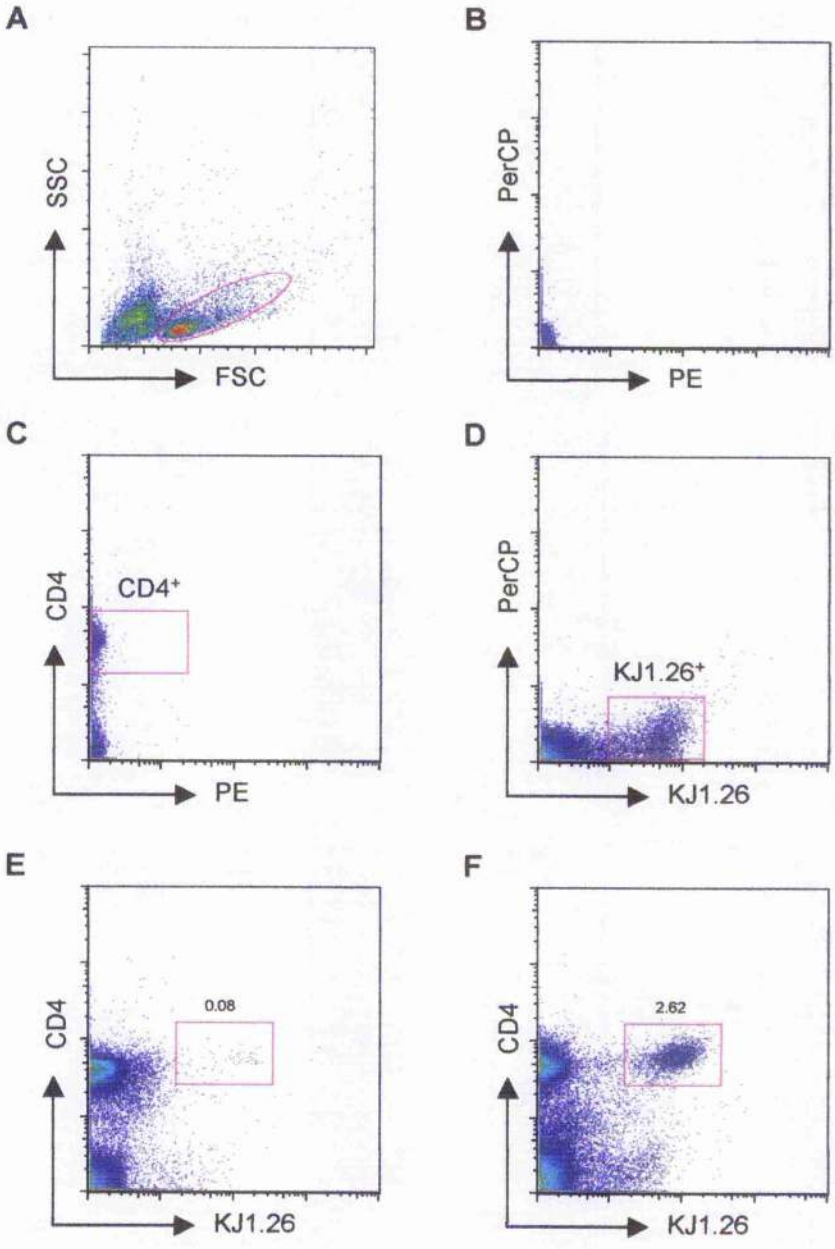
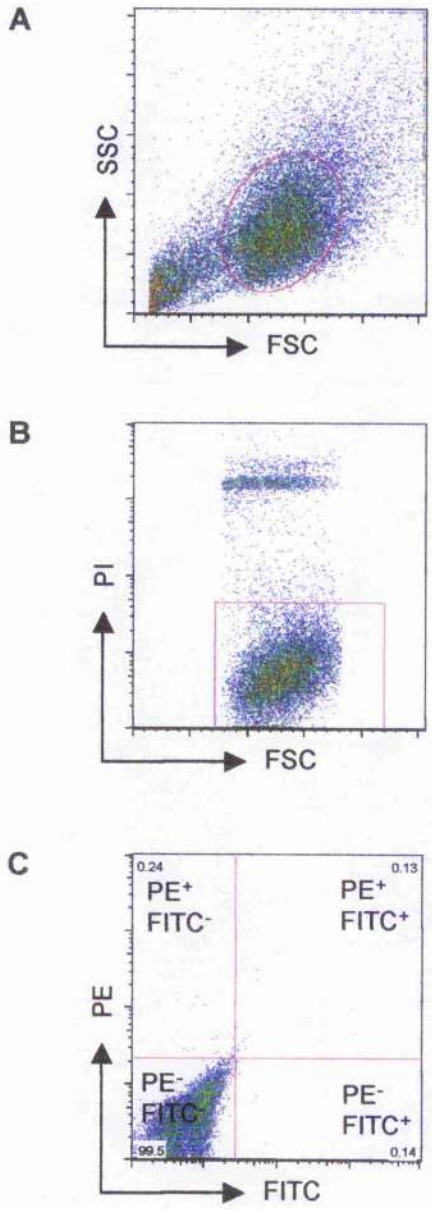

A
SSC
FSC
B
PerCP
PE
C
CD4
CD4+
PE
D
PerCP
KJ1.26+
KJ1.26
E
CD4
0.08
KJ1.26
F
CD4
2.62
KJ1.26

Figure 2.7 FACS analysis of costimulatory molecule expression by dendritic cells

A Dot plot of forward scatter versus side scatter, showing the population of cells deemed healthy by their position on this plot. Forward scatter gives a measure of the size of cells, whilst side scatter gives a measure of their granularity. The gate indicates cells of the correct size and granularity associated with dendritic cells

B Dot plot of forward scatter versus propidium iodide (PI) staining, showing the population of dendritic cells deemed viable by their lack of PI fluorescence. PI is excluded from viable cells, but can enter cells with porous membranes, such as dead cells, whereupon it binds the cellular DNA in a stoichiometric manner.

C Dot plot of cells from a murine bone marrow-derived dendritic cell culture (bmDC). Dead cells were excluded from analysis by adding propidium iodide (50 μg/ml) immediately prior to data collection, as healthy cells should not accumulate PI. Positive and negative PE and FITC quadrants were then established using unstained dendritic cells to take into account the auto-fluorescent nature of dendritic cells. Alternatively, positive and negative quadrants were similarly established using dendritic cells stained with the relevant isotype control antibodies for the costimulatory molecule being analysed.


A
SSC
FSC
B
PI
FSC
C
0.24
0.13
PE+
FITC-
PE+
FITC+
PE-
FITC-
PE-
FITC+
99.5
0.14
PE
FITC

3 Investigation of the mechanisms of ES-62 mediated anti-inflammatory action in Collagen-Induced Arthritis

3.1 Introduction

Rheumatoid arthritis (RA) is a TH1-mediated autoimmune disease characterised by chronic articular inflammation, particularly of the small joints of the hands and feet. With a prevalence of 0.8%, RA is the most common and the most disabling chronic inflammatory joint disease in the UK [206]. In extreme cases the inflammation can also affect the major organs of the body. Progression of the disease leads to erosion of cartilage and, eventually, periarticular bone, which has crippling effects for the patient [207]. The aetiology of RA is currently unclear and it has recently been concluded that RA susceptibility and outcome are influenced by a combination of several genetic and environmental factors [208]. Evidence indicates that many RA patients have similar genetic backgrounds. A significant number of patients (more than 80%) express similar mutations in the HLA-DR locus [209], which encodes proteins that form part of the major histocompatibility complex (MHC) class II binding site. MHC expressed on the cell surface facilitates antigen presentation, thus, auto-antigen presentation has been suggested in theories of RA pathogenesis [210]. However, it must be noted that RA is not classified as a genetically-linked disease because not all RA patients exhibit HLA-DR anomalies and not all individuals exhibiting this genetic abnormality develop RA. As observed in several autoimmune disorders, the majority of RA sufferers are female, implicating gender and, therefore, sex hormones in susceptibility to RA.

3.1.1 Animal models of rheumatoid arthritis

Several laboratory models of RA have been developed to help dissect the mechanisms of pathogenesis and chronic pathology exhibited in the human disease. Adjuvant arthritis was the first described model of induced RA and is brought about by administration of Complete Freunds Adjuvant (CFA) [211]. However, it remains unclear how (CFA) is able to induce joint-specific arthritis-like inflammation. Due to a lack of correlation of effectiveness of treatment in this model and human RA, models of antigen-induced arthritis (AgIA) superceded adjuvant arthritis [212]. AgIA can be induced by immunization, involving administration of irrelevant antigen (usually BSA) in CFA, followed by challenge (administration of antigen without CFA) [213]. This administration of antigen is usually directly to the site of inflammation, i.e. the joint, and induces inflammation similar to that observed in human RA joints. Progress in the design of arthritis models and increasing knowledge about RA pathology led to development of the first collagen-induced arthritis model in rats [214]. This model of arthritis reflects several features of human RA, including histopathological features, such as joint erosion and several immunological aspects, such as the inflammatory cell infiltrate and cytokines involved. When compared with previous

models, it is believed that the CIA model exhibits the greatest similarity to the human disease [215, 216]. Unresponsive to NSAIDs or methotrexate (early RA treatments), CIA has been viewed as an ideal model for investigation of new RA therapies designed to treat patients not responding to conventional RA treatment [217, 218]. In laboratory research, CIA remains a versatile and economically viable method of investigating arthritis pathology and therapy. The most renowned and widely used CIA model is set up in mice by immunising (intradermally) with type II collagen and CFA and challenging (intraperitoneally) with type II collagen to create the pathology of arthritis in the joints of the feet [214, 219]. Thus, CIA has provided the field of arthritis research with insight into the immunopathology of human RA, highlighted several potential molecular targets for immunotherapy of RA patients and provided a system with which these targets can be investigated [220].

3.1.2 The immunopathology of rheumatoid arthritis

Samples obtained from animal models and patient biopsies have enabled the immunology of RA to be analysed and the cell types involved to be uncovered. In inflamed RA synovial joints there is mass infiltration of blood-derived cells. Synovial fluid is enriched with neutrophils, whilst T cells, macrophages and DC are also detected [207], suggesting that these cell types play an important role in the pathogenesis of RA. The synovial membrane encasing the joint becomes infiltrated by macrophages and T cells and, as a result, is increased in diameter. All the cell types present in this tissue are in an activated state, expressing surface molecules necessary for antigen-presentation and secreting pro-inflammatory mediators, which sustain the chronicity of the inflammatory environment.

Dendritic cells (DC) facilitate communication between cells of the innate and adaptive immune responses. They are also pivotal in initiation and direction of adaptive immune responses. Following activation in the periphery and migration to the secondary lymphoid organs, DC communication with T cells, via antigen-presentation, co-stimulation and cytokine secretion, helps to stimulate and co-ordinate inflammatory immune responses throughout the body tissues [3]. Mature antigen-presenting DC have been identified in RA synovial fluid and are thought to play an important role in amplification of the inflammation exhibited in RA [26, 221-224]. It has been identified that activated myeloid DC present within synovial fluid and synovial tissue may promote the inflammatory environment of the RA synovial joint [225, 226]. Hence, it has been suggested that DC may be an important therapeutic target for treatment of RA [227]. Interestingly, it has been shown that anti-TNF therapy (Infliximab) strongly inhibited the maturation and activation of circulating DC, demonstrated by reduced expression of the DC activation marker, CD83 in RA patients [228] and indicated that activation of DC is induced by TNFα.

Activation of T cells marks the beginning of the antigen-specific phase of an immune response. It has been suggested that RA is a T cell-driven disease and that T cells direct the destructive action of the other cell types involved [229]. Moreover, it has been suggested that antigen-induced pathogenesis of RA could be brought about by autoreactive T cells. Indeed, T cells continuously infiltrate the RA synovium and comprise 20-30% of the mononuclear cells present [207]. In support of the proposed TH1-phenotype of RA inflammation, the majority of T cells present are TH1-type, identified by their secretion of IFNγ and many arthritis models rely on T cells to bring about the full extent of the pathology. For example, a direct role for antigen-specific T cells has been identified in the collagen-induced arthritis model [230]. Further support for T cell-mediated inflammation in CIA has been provided by blockade of CIA with cyclosporin, an antagonist of T cell signalling [231]. Moreover it is widely established that T cells have a role in helping B cell antibody secretion [232] and activation of other immune cell types present in the inflamed joint [233, 234]. Whether this is representative of the immunology in RA patients is yet to be determined.

Whilst it has yet to be confirmed, it has been recognised that B lymphocytes, by secretion of antibodies, play a significant role in the pathogenesis of many autoimmune diseases [235]. It has thus been postulated that RA is, at least in part, mediated by auto-antibody production. Auto-reactive B cells, present in the synovium, may be responsible for the autoimmune response observed in RA, via production of joint protein-specific immunoglobulin. Indeed, B cell depletion therapy is currently under trial as a new method of treating RA inflammation [236-238] as collagen specific antibodies are detected in circulation of RA patients. Consistent with this, mice deficient in B cells are resistant to CIA [239], however, in contrast, it has been demonstrated that injection of Rag-/- mice (that do not contain T cells or B cells) with type II collagen (without adjuvant) induced onset of arthritis. This indicated that collagen was capable of inducing chronic inflammation without activating cells of the adaptive immune response [240]. Therefore the relative importance of T and B cells in CIA inflammation and joint erosion remains unclear.

Monocytes infiltrate the RA synovial membrane and differentiate into mature macrophages [241], hence, the synovial lining in RA patients comprises activated macrophages alongside fibroblast-like synoviocytes [242]. Macrophages also become enriched in the invading pannus, the area where the synovial lining layer meets cartilage and tissue damage begins. It has been demonstrated that the number of macrophages present in the joint correlates with the severity of damage exhibited in RA patients [243]. Therefore, it is believed that macrophages play an important role in chronicity of RA. By secreting pro-inflammatory cytokines, such as TNFα and IL-1, and possibly participating in

auto-antigen presentation, macrophages could recruit and activate other leukocytes and induce maintenance of the inflammatory environment in RA synovium. Nevertheless, it has been demonstrated that macrophages are not contributory to initiation of RA pathogenesis [244], suggesting that their primary function is facilitation of inflammatory effector mechanisms. However, the therapeutic action of traditional RA treatment, methotrexate, in some patients has been attributed to the anti-inflammatory effects of this anti-metabolite drug on effector synovial macrophages [244].

Clearly the inflamed synovium contains several inflammatory cell types, all with potentially complex roles in RA pathogenesis and maintenance of ongoing pathology. It has been suggested that arthritis pathology and pathogenesis is the result of interactions between these cell types. Dissection of these interdependent relationships may provide further insight into pathology and potential targets for therapy. For example, macrophages differentiate from myeloid precursor cells and have important functions in antigen presentation and maintenance of the inflammatory environment via cytokine production and cell surface co-stimulatory molecule expression. Within the synovial joint, macrophages, T cells and fibroblast-like synoviocytes (FLS) lie in close proximity [245]. Evidence has suggested that the pathogenesis of RA is facilitated by communication between such cells in the synovium, particularly in a cell-contact dependent manner. McInnes et al [245] formulated a hypothesis dissecting the communication between these three cell types into cell-contact-dependent and cytokine-secretion-dependent categories, which work together to bring about the production of matrix metalloproteinases and prostaglandins that facilitate the inflammation and erosion pathology of RA (Figure 3.1; [245]). In this model, T cells reciprocally activate macrophages and FLS. Facilitated by cell-cell contact, via membrane-bound cytokines and/or co-stimulatory molecules, T cells stimulate macrophages to produce RA-promoting, pro-inflammatory cytokines, such as TNFα, IL-1, IL-15 and IL-18. In turn, these cytokines re-stimulate the T cell to further activate the macrophage by cell contact, or perhaps via the production of IFNγ, a T cell-specific, pro-inflammatory cytokine. In this manner a positive feedback communication system is set up. Synovial T cells interact in a similar manner with FLS and an inflammatory environment is created, initiating the production of matrix metalloproteinases and prostaglandins, which help to facilitate the cartilage destruction and inflammation exhibited in this disease respectively

3.1.3 Immunology of rheumatoid arthritis

RA has generally been associated with an imbalance of cytokines and the repertoire of cytokines in the inflamed synovium has been intensely investigated. Despite the majority of T cells found in the RA synovium being of the TH1 phenotype [246], the presence of T cell-produced cytokines, such as IL-2 and IFNγ, is relatively low. In fact, cells of the innate

immune system produce most of the synovial cytokines detected. TH1-promoting, pro-inflammatory cytokines, TNFα, IL-1, IL-6, IL-15, IL-18 and GM-CSF are most prevalent [203, 247] and likely secreted by infiltrating macrophages (and T cells) and resident synovial fibroblasts. TNFα, a pro-inflammatory cytokine predominantly secreted by macrophages and DC, is thought to have a paramount role in RA pathogenesis and IL-1, a cytokine whose production is induced by TNFα [248], are both chronically elevated in synovial fluid and serum of RA patients. Furthermore, a transgenic mouse that overexpresses TNFα exhibited spontaneous onset of arthritis [249]. Based on these combined findings it was concluded that TNFα was seminal in pathogenesis of arthritis and therefore an appropriate target for reduction of arthritic inflammation. It is believed that the major role of TNFα is in initiation and maintenance of the inflammatory environment in the synovium, whilst IL-1 appears to have a predominant role in joint destruction [250].

Identification of the complex role of cytokine networks within RA has led to development of anti-cytokine therapies for the disease [251]. The CIA model has been used extensively in pre-clinical trials for development of drugs now regularly used to treat RA patients [220, 252], including inhibitors of TNFα [253] and IL-1 [254]. Anti-TNFα therapy has been most widely used [255], in combination with methotrexate, an anti-metabolite drug. As mentioned above, TNFα is upstream of IL-1 therefore, blockade of TNFα inhibits IL-1 production. Furthermore, it was identified that TNFα antagonists induced inhibition of other pro-inflammatory cytokines found in RA synovium, promoting the theory that complex cytokine networks are involved [207]. Blockade of TNFα is mediated in RA patients by treatment with recombinant TNF receptor, or anti-TNF antibody, prescribed as Etanercept and Infliximab, respectively. Treatment with these drugs is successful in many patients, underlining the integral role of TNFα in inflammation progression. However, 30 to 40% of RA patients do not experience anti-inflammatory effects from this type of therapy [256], highlighting the heterogeneous nature of this disease in different cases. Anti-IL-1 treatment has been therapeutic in some patients but clearly, anti-cytokine therapy is not universally effective and new methods of therapy are currently being investigated. For example, blockade of B-cell activity with anti-CD20 monoclonal antibody (Rituximab) and the inhibition of T-cell activation with fusion protein CTLA4Ig are currently undergoing trials as new treatments for RA [257].

3.1.4 Aims

As previously discussed in Chapter 1, in countries endemic for parasite infections, autoimmune disease is relatively rare [258], suggesting parasite infection may protect us from development of autoimmunity . In support of this theory, it has previously been

demonstrated that infection with the parasite *Schistosoma mansoni* prevents induction of a model of autoimmune diabetes [140]. Could this inhibition of autoimmunity by parasite infection be mirrored in a model of RA? It has been suggested that parasite excretory-secretory (ES) products possess immunomodulatory properties and that diversion of inflammatory immune responses in parasitized individuals, to favour the survival of the parasite, is due to the action of these substances [161]. ES-62 is a 62kDa phosphorylcholine (PC)-containing glycoprotein that is secreted by the rodent filarial nematode, *Acanthocheilonema viteae* [170]. Research conducted previously in this laboratory [184-187, 189-191] has demonstrated that ES-62 has immunomodulatory actions on cells of the immune response. Indeed, it has been determined that the immunomodulatory effects of ES-62 extend to anti-inflammatory action in the murine CIA model. More specifically, prophylactic administration of ES-62 delayed the onset and reduced the severity of inflammation exhibited in CIA. In addition, in established disease, ES-62 suppressed mean articular index and articular swelling, two measures of the severity of the arthritis. These actions of ES-62 were manifested by a reduction in antigen-specific TH1-type cytokine production and serum TH1-type antigen-specific immunoglobulin levels. Interestingly, no compensatory increase in collagen-specific TH2-type immunoglobulin was induced by ES-62, indicating that ES-62 did not induce these changes by counter-regulatory mechanisms. In summary, the *in vivo* mechanisms underlying the anti-inflammatory actions of ES-62 in CIA are yet to be established. The therapeutic potential of ES-62 in arthritic inflammation has also been determined using human RA samples. Co-culture of peripheral blood T cells from RA patients with macrophages *in vitro* induced contact-dependent TH1 cytokine production by the macrophages. This cytokine production was inhibited by pre-treatment of the macrophages with ES-62. These results indicated that ES-62-mediated down-modulation of the inflammatory immune response exhibited in RA might be advantageous, as a method of treatment.

Following demonstration of the ES-62 mediated inhibition of inflammation exhibited in murine CIA [192], the core aim of this investigation was to extend analysis of this model and to more thoroughly characterise the anti-inflammatory effects of ES-62. In addition, it was proposed to identify the structural components of ES-62 involved, the cell types employed to facilitate ES-62 action and the key signalling mechanisms used. The specific aims are outlined below:

1. It has previously been identified, *in vitro*, that several of the immunomodulatory effects of ES-62 on single cell types were facilitated by parasite-specific phosphorylcholine (PC). PC is a post-translational modification of the ES-62 structure. Therefore, a primary aim of this investigation was to dissect the role of PC in the observed anti-inflammatory effects of ES-62 in the CIA model.

2. The varied roles of different immune cell types involved in pathogenesis and chronic pathology of RA have previously been mentioned. It was a necessary aim of this investigation to identify the immune cell types that facilitated the actions of ES-62 in the CIA model of RA.
3. The marked immunomodulatory actions of ES-62 treatment on DC and macrophages have previously been characterised *in vivo* and *in vitro*. These two cell types are also known to have important roles in the CIA inflammatory processes. Therefore, specific dissection of the action of ES-62 on DC and macrophages in this model of arthritis was an integral aim of this investigation.
4. Modulation of the macrophage-T cell interaction hypothesised to be integral to arthritis pathogenesis [245] may be a viable method for inhibition of disease onset and/or progression. It was also planned therefore to develop an *in vitro* system with which to determine whether this was a method by which ES-62 might inhibit onset and progression of CIA mediated inflammation.

3.2 Results

3.2.1 ES-62 inhibits onset of inflammation and reduces established inflammatory pathology in the collagen-induced arthritis model.

It has previously been demonstrated in this laboratory that ES-62, both prophylactically and therapeutically, mediates inhibition of inflammation in the CIA model of arthritis [192] by inhibiting antigen-specific TH1-type inflammation in this TH1-mediated autoimmune disease model. To review, and as confirmation of these studies, when ES-62 is administered in a prophylactic manner, it significantly reduces arthritic swelling and delays the onset of arthritis induced by the collagen administration protocol. This is demonstrated by inhibition of arthritic score (a measure of severity of inflammation) and reduction in the incidence of footpad inflammation (Figure 3.2). As shown previously [192], ES-62-mediated inhibition of such inflammation in this arthritis model is associated with significantly reduced collagen-specific proliferation and TH1-promoting cytokine production by draining lymph node cells (Figure 3.2). Furthermore, ES-62-mediated inhibition of TH1-promoting cytokine production does not stimulate a compensatory increase in TH2 (IL-4 and IL-5) cytokine production (results not shown), but enhances production of antigen-specific regulatory cytokine, IL-10. Additionally, ES-62 treatment of CIA significantly diminishes the collagen-specific serum IgG2a levels (Figure 3.3) in this model. Furthermore, inhibition of antigen-specific serum IgG2a by ES-62 is not associated with any compensatory modulation of collagen-specific IgG1, IgG3 or IgM (Figure 3.3).

When ES-62 is administered therapeutically (after the onset of inflammation), it significantly inhibits progression of inflammation and joint erosion (Figure 3.4). The inhibitory action of ES-62 in this therapeutic treatment model is also facilitated by modulation of antigen-specific responses in draining lymph node cells. More specifically, therapeutic ES-62 treatment reduces collagen-induced lymph node cell proliferation and abrogates IFNγ, TNFα and IL-6 (Figure 3.5), but not IL-10 (results not shown) release ex vivo. Although serum levels of collagen-specific IgG2a and IgG1 were also previously reported to be slightly inhibited by ES-62 in this therapeutic treatment model [192], no statistical reduction could be detected in these studies (Figure 3.6). In summary, ES-62 treatment prevents both onset of inflammation and reduction of existing inflammation exhibited in the collagen-induced arthritis model.

3.2.2 The effects of treating CIA model inflammation with a PC-free recombinant homologue of parasite-derived ES-62

Phosphorylcholine (PC) has been characterised as an immunomodulatory moiety utilised by several pathogens and it has been identified as a component of ES-62 [177], with PC being covalently attached to the glycoprotein backbone of ES-62. Interestingly, *in vitro*

treatment of immune system cells with PC conjugated to an irrelevant protein, BSA (bovine serum albumin), mimicked many of the effects of ES-62 [167], demonstrating that PC may be the active component of ES-62. To test this theory a PC-free recombinant homologue of ES-62 (rES-62) has been synthesised utilising the protein synthesis machinery of the yeast *P. pastoris*. As the yeast expression system does not possess the necessary machinery to attach PC to the glycoprotein backbone, rES-62 does not contain PC. Despite exhibiting increased mannose content, and differences in secondary and tertiary structure compared with parasite-derived ES-62 [199], rES-62 has been recognised by rabbit antisera against native ES-62 and therefore represents a potential PC-free version of the *A. viteae*-derived ES product. Therefore, to address whether PC was necessary for ES-62-mediated inhibition of CIA model inflammation, rES-62 was used to treat CIA model mice in place of native ES-62 in the prophylactic and therapeutic treatment models.

In contrast to the action of native ES-62, prophylactic treatment of the CIA model with rES-62 did not significantly inhibit the severity of CIA (Figure 3.7). In more detail, the arthritic score of mice treated with rES-62 was not significantly different to those treated with PBS from day 23 onwards. However, interestingly, rES-62 treated CIA model mice exhibited earlier onset of prominent arthritic inflammation (days 16-17) when compared with PBS treated mice (days 19-20). Perhaps surprisingly, given the observed differences in pathology, the average levels of collagen-specific serum immunoglobulin (Ig) were similar in ES-62 and rES-62 treated mice. Thus, as with the serum from ES-62 treated mice, serum obtained at the end of the protocol from rES-62 treated mice exhibited significantly reduced levels of collagen-specific IgG2a, when compared with serum from PBS treated mice (Figure 3.7). Similarly, a compensatory modulation of collagen-specific IgG1, IgG3 or IgM by rES-62 was not observed.

Having determined that rES-62 treatment did not inihibit onset of CIA inflammation in the prophylactic treatment model, we continued our analysis of rES-62 action in the therapeutic treatment model. Treatment of CIA model mice with rES-62 after the onset of inflammation did not significantly modulate the incidence or severity of arthritis exhibited. On average, the mean arthritic score of CIA model mice treated therapeutically with rES-62 was maintained at a high level, not significantly different to PBS treated mice, until sacrifice (Figure 3.8). Furthermore, the average arthritic score of mice treated with rES-62 was significantly greater than that of mice treated with native ES-62 in this treatment protocol. It appeared that, in a similar manner to the results of prophylactic rES-62 treatment, therapeutic treatment with rES-62 did not exhibit a significant inhibitory effect on collagen-induced inflammation. Consistent with this, serum samples obtained from rES-62, ES-62 and PBS treated mice, at the end of the therapeutic treatment protocol, did

not reveal significantly different profiles of collagen-specific IgG1, IgG2a, IgG3 or IgM (Figure 3.8).

In summary, the results were consistent with previously determined findings of this laboratory, which concluded that PC may be required for the anti-inflammatory actions of ES-62 in vitro [167]. Following this investigation it was concluded that the PC component of ES-62 might be necessary for adequate inhibition of inflammation in the CIA model. This was illustrated by the inability of PC-deficient rES-62 to inhibit CIA-mediated inflammation. This finding was consistent and independent of the pattern of rES-62 administration. In other words, rES-62 did not inhibit CIA mediated inflammation when administered during initiation of inflammation or after the onset of clinically detectable inflammation. However, it was of interest that prophylactic rES-62 treatment appeared to promote premature inflammation during days 16-21 of the collagen administration protocol. This indicated that this recombinant glycoprotein may have immunostimulatory potential. Due to lack of immune-dampening PC [177] or increased immunostimulatory mannose content [199] within the structure of rES-62, compared with parasite-derived ES-62. The similarity of the collagen-specific Ig profile of serum from rES-62 treated mice and serum from ES-62 treated mice (in the prophylactic treatment model) indicated that ES-62 mediated inhibition of IgG2a is PC-independent. Furthermore, it therefore appeared that ES-62-mediated modulation of serum IgG2a was not directly related to modulation of pathology.

3.2.3 The effects of treating CIA model inflammation with phosphorylcholine

It is clear from the results described in section 3.2.2 that PC may be a required component of ES-62, in mediation of the anti-inflammatory effects observed in the CIA model. However, as mentioned previously, rES-62 is not simply a PC-free copy of native ES-62; it also contains high levels of mannose. An experiment was therefore designed to determine whether PC, independently of the rest of the ES-62 structure, was capable of inducing inhibition of the TH1-type inflammation exhibited in this model. To facilitate this investigation CIA model mice were treated (in place of ES-62) with PC conjugated to an irrelevant protein, ovalbumin (OVA-PC). It has been suggested that PC conjugated to a protein (instead of PC alone) is more likely to resemble ES-62 with respect to circulation in the bloodstream [199]. Furthermore, it has previously been shown that PC conjugated to BSA or OVA can mimic several of the immunomodulatory effects of ES-62, in vitro and in vivo [167, 183, 185, 191]. As a negative control for OVA-PC treatment, a group of CIA model mice were additionally treated with sham-conjugated ovalbumin (OVA). Prophylactic treatment of mice with OVA-PC significantly reduced the onset and severity of CIA inflammation, when compared with the OVA treatment group (Figure 3.9). This

result provided support for the findings described above, that the PC component of ES-62 may be responsible for ES-62-mediated inhibition of CIA model inflammation. In addition, these findings suggested that PC was independently capable of anti-inflammatory action in this arthritis model and that the ES-62 glycoprotein structure is dispensable. However, serum samples from OVA and OVA-PC-treated CIA model mice exhibited very similar average levels of collagen-specific IgG1, IgG2a and IgG3, indicating that, unlike ES-62, PC did not specifically modulate antigen-specific IgG production or isotype (Figure 3.9). Interestingly, collagen-specific serum IgM levels were reduced in OVA-PC treated mice compared to OVA treated mice. The reduction of antigen-specific IgM by PC was similar to the previously observed (non-significant) action of ES-62. Nevertheless, at this stage, it was only possible to conclude that prophylactic treatment with OVA-PC mediated inhibition of coliagen-induced inflammation, but not modulation of serum antigen-specific IgG.

Treatment of CIA model mice with OVA-PC after the onset of clinically detectable arthritis also prevented progression of the inflammation severity (Figure 3.10). Thus, whilst OVA-treated mice exhibited an increase in this measure of inflammation severity, the arthritic score of OVA-PC treated mice did not progress throughout the treatment period, although the incidence of arthritis was not modulated in either treatment group. These combined observations suggested that treatment with OVA-PC offered some protection from progression of arthritic inflammation in this model. In particular, PC was responsible for this action because inhibition of disease progression was not observed in OVA-treated mice. As with the prophylactic model, analysis of serum immunoglobulin revealed no significant differences in the average levels of collagen-specific IgG1, IgG2a, IgG3 and IgM subclasses between OVA and OVA-PC treated mice (Figure 3.10). This indicated that PC, administered therapeutically, did not modulate antigen-specific antibody production in this model.

3.2.4 Analysis of the serum cytokine profile of CIA model mice in the prophylactic treatment model

It has been well documented that serum levels of certain circulating cytokines and chemokines represent the severity of inflammatory pathology exhibited in RA [259, 260]. For example, serum samples from RA patients exhibit high concentrations of pro-inflammatory cytokines, TNFα and IL-1 and these cytokines have been identified as integral to arthritis pathogenesis [207]. It was therefore proposed that examination of the cytokine and chemokine content of serum samples from mice in the CIA model would provide useful information about the inflammatory and immunological phenotype of mice in each treatment group and how the structural components of ES-62 were acting.

Serum samples were obtained from CIA model mice in each treatment group and tested for several cytokines commonly identified in inflammation. Samples from the PBS treatment group were found to contain measurable quantities of pro-inflammatory cytokines, IL-1α, IL-12, and IL-2 (Figure 3.11). IL-1α has been implicated in arthritis pathogenesis [207], IL-12 is required for induction of TH1-type inflammation [261] and IL-2 is a potent activator and mitogenic stimulator of T cells [262]. Therefore, the presence of these cytokines implied that inflammatory processes were ongoing in the CIA model mice. TH2-type cytokine, IL-5 [263] and anti-inflammatory cytokine, IL-10 [264], were also identified in these serum samples (Figure 3.11), however they were detected in relatively low concentrations, which indicated that TH2 or anti-inflammatory signals were minimal in this model. Other pro-inflammatory cytokines (IL-1β, IL-6, IL-17, IFNγ and TNFα) and TH2 cytokines (IL-4 and IL-13) analysed were not detected in the serum samples from PBS treated CIA model mice. In summary, the overall cytokine profile of serum samples from PBS-treated CIA model mice confirmed a predominantly inflammatory TH1 phenotype of the inflammation exhibited in this disease model. However, the pro-inflammatory cytokine, TNFα, was not detected in any of the serum samples analysed. This was surprising, considering TNFα has been identified as integral to RA pathogenesis [265].

Treatment of CIA model mice with ES-62, in a prophylactic manner, did not significantly modulate the mean concentrations of the serum cytokines detected compared with those of the PBS treated mice (Figure 3.11). It was observed however, that the range of concentrations of some serum cytokines displayed by mice in the PBS treatment group had been modulated by ES-62 treatment. More specifically, serum samples from 4 out of 9 mice in the ES-62 treatment group exhibited increased serum IL-1α concentration relative to the top concentration detected in the PBS treatment group. This was surprising, given the observed reduction in inflammatory pathology observed in the ES-62 treatment group mice. Furthermore, it appeared that serum IL-1α concentration exhibited by individual mice might be inversely related to inflammation status, because the mice in the ES-62 treatment group that exhibited the highest IL-1α concentrations also displayed minimal arthritic scores.

The mean serum concentration of anti-inflammatory/TH2 cytokine, IL-10, was subtly elevated in serum samples from ES-62-treated mice (as a result of an outlier), compared with the mean level detected in the PBS treatment group. This difference was not significant and no correlation was observed between inflammation scores and serum IL-10 levels, however, the promotion of IL-10 by ES-62 treatment suggested potential enhancement of anti-inflammatory signals by ES-62.

Serum samples from the rES-62 treatment group mice also contained detectable levels of all the cytokines observed in serum from PBS and ES-62 treatment group mice (described above) (Figure 3.11). As described in section 3.2.2, prophylactic treatment of CIA model mice with rES-62 did not prevent onset of inflammation, unlike prophylactic treatment with native ES-62, suggesting that PC was the major component of ES-62 responsible for inhibition of CIA-mediated inflammation. It was therefore of interest to examine the cytokine profile of serum samples from rES-62 treatment group mice to enable direct comparison with the ES-62 treatment group mice. Interestingly, serum samples from rES-62 treated mice contained significantly higher levels of IL-12 than serum from PBS and ES-62 treatment groups. Therefore, rES-62 administration appeared to induce IL-12 secretion, *in vivo*, by CIA model mice. It has previously been demonstrated that administration of IL-12, in combination with type II collagen, induced severe arthritis in DBA/1 mice [266]. Therefore, the increased production of IL-12 induced by rES-62 may underlie the enhanced inflammation observed in rES-62-treated mice compared with PBS-treated mice. Also of interest, although not significantly different, the mean serum concentration of IL-1α in rES-62 treated mice was reduced compared to the mean level detected in the ES-62 treatment group and similar to the level detected in the PBS treatment group. Furthermore, the inverse correlation between serum IL-1α concentration in individual mice and arthritic score observed in the ES-62 treatment group was no longer apparent in the rES-62 treatment group. Based on these comparisons, it appeared that whilst, IL-1α concentration might be related to the *in vivo* effects of ES-62, it was not directly related to inflammatory pathology in this model. Anti-IL-1 treatment has been shown to ameliorate murine CIA [267] therefore, the inverse relationship observed in this model was surprising. Interestingly, the mean concentration of IL-10 detected in serum samples from rES-62 treated mice was less than detected in serum samples from native ES-62 treated mice, however, this difference was not significant and most likely due to the outlier from the ES-62 treatment group. In summary, a modest enhancement of the serum TH1 cytokine balance of CIA model mice was induced by rES-62 treatment and this change appeared to be due to lack of PC and/or presence of additional immunogenic components within the structure of rES-62.

As previously described (section 3.2.3), treatment of CIA model mice with OVA-PC inhibited onset of inflammation in a similar manner to that induced by native ES-62 treatment, implicating PC in mediation of ES-62-induced anti-inflammatory effects. It was proposed that comparison of serum cytokine changes in ES-62- and OVA-PC-treated CIA model mice (in terms of their control treatments, PBS and OVA, respectively) would help to clarify the relationship between serum profile and inflammatory pathology. Serum samples obtained from OVA and OVA-PC treated mice contained measurable levels of all the cytokines detected in serum from PBS and ES-62 treated CIA model mice (described

above) (Figure 3.11). Careful scrutiny of the pattern in cytokine levels between serum from PBS and ES-62 treated mice and serum from OVA and OVA-PC treated mice, respectively, revealed similarities between ES-62 and OVA-PC. Thus, the mean value of serum IL-1α concentration was significantly elevated in OVA-PC treated mice compared with OVA treated mice. Although not significant, increased serum IL-1α concentration had also been detected in response to ES-62 treatment, compared with PBS. As mentioned above, promotion of IL-1α was not induced by rES-62. Therefore, serum IL-1α concentration appeared to be inversely related to inflammation severity in these PC-treated models. It was interesting to observe that serum IL-10 levels were particularly low in OVA and OVA-PC treated mice compared with PBS and ES-62 treated mice. This appears to negate any proposal that promotion of serum IL-10 (observed in ES-62 treated mice) was PC-induced.

3.2.5 Analysis of the serum chemokines and growth factors in CIA model mice in the prophylactic treatment model

Chemokines and growth factors secreted by several different cell types in chronic inflammation are important mediators of the inflammatory immune response, which act to recruit cells and, in conjunction with cytokines, promote maintenance of the inflammatory environment. KC (keratinocyte-derived chemokine) and MIP-1α (macrophage inflammatory protein-1a), powerful chemokines that function in recruitment of neutrophils [268, 269], were detected in serum samples from PBS treated CIA model mice (Figure 3.12). KC is induced by TNFα [270], which indicates that TNFα must have been produced, perhaps at an earlier time-point, in this model. Also detected in PBS-treated mice were interferon inducible protein-10 (IP-10) and MIG (monokine induced by gamma interferon), which are important markers of IFNγ-mediated inflammation [271, 272], and fibroblast growth factor-basic (FGF-2), a potent stimulator of cellular proliferation and angiogenesis [273]. These findings indicated that, in addition to TNFα, IFNγ is likely to also have been involved in development of the inflammation exhibited in these mice. FGF is an important growth factor implicated in stimulation of several cell types and promotes development of new blood vessels, necessary for chronicity of inflammation. To obtain a comprehensive overview of the chemokines and growth factors produced in the CIA model mice, serum samples were also analysed for, but found not to contain, detectable levels of GM-CSF, MCP-1 and VEGF. In summary, several inflammatory chemokines, important for promotion of inflammation, were detected in PBS-treated CIA model mice and this corresponded with the predominantly pro-inflammatory repertoire of serum cytokines also present (section 3.2.4).

Treatment of CIA model mice with ES-62, in a prophylactic manner, induced some changes to the repertoire of serum chemokines exhibited in CIA model mice (Figure 3.12).

It was observed that the inflammatory neutrophilic chemokine, KC, was only detected in 2 out of 9 mice in the ES-62 treatment group (compared with 4 out of 9 in the PBS treatment group), which distinctly, but not significantly, reduced the mean serum concentration of KC in response to ES-62 treatment. However, no correlation was observed between the concentration of serum KC and arthritic score in individual mice in these treatment groups. As mentioned previously, it has been shown that KC is induced by TNFα and, hence, may represent TNFα levels in this model [270]. Furthermore KC has been found in the blood and joints of arthritic rats in an adjuvant-induced arthritis model [274] and has been shown to correlate with the development of arthritis in Lyme disease [275]. Therefore, whilst it is not clear why TNFα was not detected in the serum of CIA model mice, it appeared that reduced levels of serum KC, in the ES-62 treated mice, were consistent with the observed reduction in inflammatory pathology in this treatment group.

Serum samples from the rES-62 treatment group CIA model mice contained measurable levels of all the chemokines detected in PBS and ES-62 treatment group mice, except KC (Figure 3.12). The absence of KC was surprising, as this chemokine had been observed in serum samples from PBS and ES-62 treatment groups. It was therefore proposed that the reduction in KC levels observed in the ES-62 treatment group may have been induced by the non-PC components of ES-62 and hence, was more potently observed in CIA model mice treated with PC-deficient rES-62. However, this theory contradicted the previous proposal that serum KC levels reflected the severity CIA pathology.

In an effort to further clarify the role of PC in the observed actions of ES-62 on serum chemokines. Serum samples obtained from OVA and OVA-PC treated CIA model mice were analysed and found to contain measurable levels of all the chemokines detected in serum from PBS and ES-62 treated CIA model mice (described above) (Figure 3.12). A pattern was observed in the subtle changes to mean serum levels of MIG, MIP-1α and KC induced by ES-62 and OVA-PC treatment. More specifically, a modest promotion of MIG and MIP-1α was observed upon treatment with ES-62 and treatment with OVA-PC relative to PBS and OVA treatment, respectively. Furthermore, non-significant reduction in the mean serum KC levels observed in ES-62 treated mice (compared with PBS treated mice) was also observed in OVA-PC treated mice (compared with the OVA treatment group). It was interesting that the pattern of serum MIG and MIP-1α levels inversely reflected the severity of inflammatory pathology in the PBS, ES-62, OVA and OVA-PC treatment groups. The reduction in serum KC induced by OVA-PC questioned the theory put forward above, that KC was inhibited by a non-PC component of ES-62. Furthermore, the suggested pattern of reduced serum KC concentration in ES-62 and OVA-PC treated mice directly reflected the pattern of inflammatory pathology in these treatment groups, implying that, in this model, KC might be an indicator of inflammation severity. In

conclusion, the similarities in the action of ES-62 and OVA-PC on inflammatory serum chemokines suggested that PC was responsible for these changes, however, it must be remembered that the changes in serum chemokines were non-significant, therefore, conclusive statements cannot be made at this time.

3.2.6 Analysis of the serum cytokine profile of CIA model mice in the therapeutic treatment model

After observation of minimally modulated patterns in serum cytokine profiles of CIA model mice treated prophylactically with ES-62 (and derivatives), analysis of serum from therapeutic model mice was conducted to determine whether treatment of CIA after the onset of inflammation led to more obvious modulation of serum factors. However, few of the serum cytokines detected at the end of the prophylactic protocol could be detected in the serum of CIA model mice culled from the therapeutic treatment protocol (Figure 3.13). More specifically, IL-2 and IL-12, pro-inflammatory cytokines identified in the prophylactic treatment model mice, were identified in serum samples from PBS-treated CIA mice, however, IL-1α, IL-10 and IL-5, were not. As conducted in the prophylactic treatment model, serum samples from therapeutically treated CIA mice were also analysed for IL-1β, IL-4, IL-6, IL-13, IL-17, IFNγ and TNFα content, but these cytokines were also not detected.

Although there was a spread of concentrations within the PBS and ES-62 treatment groups, treatment of CIA model mice with ES-62 after the onset of inflammation did not significantly modulate the mean serum levels of IL-2 or IL-12 (Figure 3.13). Therefore, unlike the findings from the serum analysis in the prophylactic treatment model, there were no indications of ES-62-mediated immunomodulation, at least in terms of serum cytokine levels. Nevertheless, there did appear to be positive correlation between serum IL-12 concentration and inflammation score in individual PBS- and ES-62-treated mice, because mice exhibiting the highest concentrations of serum IL-12 also exhibited the highest inflammation scores. However, this pattern was not reflected in serum IL-2 concentrations.

Serum samples obtained from rES-62 treated mice also contained IL-2 and IL-12, as had been identified in PBS and ES-62 treated mice (Figure 3.13). In a similar manner to ES-62, therapeutic treatment with rES-62 did not significantly modulate the mean levels of the serum cytokines detected. Therefore, it appeared that therapeutic treatment of CIA mice with ES-62, independent of the PC-content, did not modulate the concentration or type of serum cytokines.

Interestingly, IL-2 was detected in serum samples from OVA-, but not OVA-PC-treated CIA model mice (Figure 3.13). As mentioned above, the inhibitory effect of OVA-PC on IL-2 was not observed in ES-62 treated mice, however it must be noted that relatively low concentrations of IL-2 were detected overall. The analogous patterns between serum cytokine profiles in OVA/OVA-PC and PBS/ES-62 treated mice described in the prophylactic treatment model clearly was not observed upon analysis of serum samples from these treatment groups in the therapeutic treatment model. Furthermore, although not significant, the mean serum concentration of pro-inflammatory cytokine, IL-12, was also reduced in OVA-PC treated mice, when compared with OVA-treated mice in this model. This reduction had not been observed in mice treated therapeutically with ES-62. Furthermore, the positive relationship between serum IL-12 and arthritic score displayed by PBS- and ES-62-treated mice was not observed in the OVA or OVA-PC treatment groups. Reduction of serum IL-2 and IL-12 might suggest a reduced pro-inflammatory phenotype in keeping with the observed reduction of inflammation in the OVA-PC treatment group. However, based on the results of the cytokine analysis, it was unclear why therapeutic treatment with OVA-PC, but not ES-62, had modulated the levels of serum pro-inflammatory cytokines.

3.2.7 Analysis of the serum chemokines from CIA model mice in the therapeutic treatment model

Many of the inflammatory chemokines that were detected in serum samples from the prophylactic treatment model, were also detected in serum samples from therapeutically treated CIA model mice (Figure 3.14). More specifically IP-10, MIG and MIP-1α, but not KC, were detected in serum from PBS-treated mice in this model, indicating that an inflammatory immune response was ongoing in these mice. Treatment of CIA model mice with ES-62 after the onset of inflammation did not significantly modulate the average levels of the three serum chemokines detected, however, some non-significant trends were observed (Figure 3.14). The range of serum concentration of inflammatory chemokine, MIP-1α was noticeably reduced in the serum of ES-62 treated mice. This drop was consistent with the observed reduction in inflammation in the ES-62 treatment group. Furthermore, MIP-1α is a neutrophilic chemokine induced by inflammation-promoting TNFα [276] and therefore may represent a measure of inflammation severity within these mice.

To determine whether MIP-1α reduction was a PC-dependent effect of ES-62 treatment, serum samples from rES-62 treated mice were also analysed for chemokine content. Similarly to PBS and ES-62 treated mice, IP-10, MIP-1α and MIG were detected in serum samples from rES-62-treated mice (Figure 3.14). Although not significant, rES-62 treatment induced mild enhancement of the mean serum concentrations of IP-10 and MIG

compared with PBS and ES-62 treatment. Furthermore, it appeared that the serum concentrations of MIG (but not IP-10) in individual PBS- and ES-62-treated mice correlated positively with inflammation status, determined by arthritic score, because mice that exhibited the highest MIG concentrations in the treatment groups also exhibited the highest arthritic scores. As previously mentioned, IP-10 and MIG are inflammatory chemokines and their secretion is induced by the TH1 cytokine, IFNγ. The promotion of IP-10 and MIG by rES-62 was, therefore, consistent with enhanced TH1-mediated inflammation in this treatment group. Interestingly, rES-62 treatment appeared to maintain the mean concentration of serum MIP-1α, which had been inhibited in ES-62 treated mice. More specifically, treatment with rES-62 resulted in mean MIP-1α levels similar to that exhibited in the PBS treated mice, potentially supporting a role for PC in inhibition of this chemokine.

It was more difficult to elucidate the profile of the pro-inflammatory chemokines, MIP-1α, IP-10 and MIG, in serum samples from OVA-PC treated mice (Figure 3.14) due to outlier responses. Indeed, the mean serum concentrations of MIP-1α, IP-10 and MIG appeared to be elevated in response to OVA-PC, compared with samples from OVA, PBS and ES-62 treated mice. This was surprising as an increased serum concentration of pro-inflammatory chemokines was not consistent with OVA-PC mediated inhibition of inflammatory pathology in this model, however, modest promotion of MIG and MIP-1α in response to OVA-PC treatment had been observed in the prophylactic treatment model (Figure 3.12). However, these changes to mean chemokine levels were non-significant and probably due to outlying readings, therefore, most likely they do not represent modulation of the overall inflammatory status of these mice. Furthermore, the correlation between arthritic score and MIG concentration observed in individual mice in the PBS, ES-62 and rES-62 treatment groups was not displayed by the OVA or OVA-PC treatment groups.

3.2.8 Analysis of the serum growth factors in CIA model mice in the therapeutic treatment model

The growth factor, FGF-2, that had been detected in serum samples from CIA mice in the prophylactic treatment model, was also found to be circulating in the serum of mice from the therapeutic treatment CIA model (Figure 3.15). In addition, VEGF, an important inducer of angiogenesis and a common feature of chronic inflammatory pathology [277], was also detected in serum from these mice. Identification of two important mediators of cellular growth, proliferation and angiogenesis indicated that inflammatory mechanisms associated with induction of chronicity and tissue remodelling had been activated in these mice.

Serum FGF concentration in CIA model mice was not significantly modulated by therapeutic ES-62 treatment (Figure 3.15). However, surprisingly, the mean serum concentration of VEGF was elevated in ES-62 treated mice, compared with PBS treated mice, indicating that ES-62 treatment might induce promotion of VEGF secretion. This was unexpected, because circulating levels of VEGF have previously been associated with inflammation severity in RA [278]. As it had previously been identified that therapeutic ES-62 treatment inhibited arthritic inflammation in the CIA model, promotion of VEGF by ES-62 appeared counter-intuitive. Nevertheless, the overall detected concentrations of VEGF in PBS and ES-62 treated mice were very low.

Therapeutic treatment of CIA with rES-62 significantly increased the mean serum FGF concentration and markedly increased the mean serum VEGF concentration exhibited, compared with the PBS treatment group (Figure 3.15). Although not significantly different to the concentrations exhibited by ES-62 treated mice, the enhancement of VEGF and FGF concentration was consistent with the increased severity of inflammation exhibited in the rES-62 treated mice. The concentration of serum VEGF was promoted to a greater degree by rES-62 treatment of CIA model mice than that exhibited in response to ES-62 treatment (described above), hence this comparison indicated that, given the range of concentrations across which VEGF might act, the magnitude of the modulation induced by ES-62 may not be important.

Interestingly, the mean serum VEGF concentration was abrogated in OVA-PC treated CIA model mice when compared with OVA treated mice (Figure 3.15). Considering the previously determined anti-inflammatory action of OVA-PC in CIA, reduction of VEGF was consistent with a role for VEGF in mediation of inflammation in this model. Furthermore, it was observed that the mean VEGF levels were higher in OVA treated mice than PBS treated mice, however this difference was not significant. It was interesting to observe that despite the previously described analogous effects of ES-62 and OVA-PC treatment on serum chemokine levels, OVA-PC mediated inhibition of VEGF was not mirrored by ES-62. This apparently inconsistent pattern had previously been observed during analysis of serum cytokines in this CIA model (described above, Figure 3.13).

In summary, whilst a predominantly TH1-like phenotype cytokine and chemokine profile was observed in both treatment models, a reduced number of cytokines were detected in serum samples from therapeutically treated, compared with prophylactically treated CIA model mice. The reduction in cytokine content in the latter model might be explained by the fact that serum samples obtained from mice in the prophylactic and therapeutic treatment models were taken at different time-points (day 33 and days 29-35, respectively). Apparent inconsistencies in the effects of ES-62 and OVA-PC treatments on

serum cytokine and growth factor content were also observed in the therapeutic, but not the prophylactic treatment model. These apparently contradictory effects may represent differences in the immunomodulatory potencies of ES-62 and OVA-PC, however, as mentioned earlier, it must be considered that serum cytokine analysis reflects a 'snapshot' of cytokines circulating at the time of sacrifice (which was different for each treatment model) and may not directly represent the overall immunology of the model.

Clearly PC is an important component of the ES-62 molecule, capable of independent anti-inflammatory action, which mimics the inhibitory actions of ES-62 observed in TH1-type CIA model inflammation. Furthermore, the PC-dependent anti-inflammatory actions of ES-62 were apparent when CIA model mice were treated before or after the onset of inflammation. More specifically, prophylactic treatment of CIA model mice with ES-62 partially prevented the development of a potent inflammatory TH1-type immune response, which was facilitated by antigen-specific IgG2a and TH1 cytokine production in control (PBS-treated) mice. Alternatively, therapeutic treatment of CIA model mice with ES-62 reduced the inflammatory pathology exhibited in the joint and therefore prevented exacerbation of disease, but did not modulate the established antigen-specific antibody response mediated by collagen-specific IgG production. Thus, it appeared that the anti-inflammatory effects of ES-62 in each type of treatment model might be the result of ES-62 action on different cell types. For example, in the prophylactic treatment model, ES-62 action may be most prevalent on cells involved in the development of the TH1-type immune response. Alternatively, in the therapeutic treatment model, ES-62 may mediate its anti-inflammatory action via modulation of the function of immune effector cells, responsible for maintenance and progression of the immune response.

3.2.9 Investigating the effect of ES-62 on the *in vitro* function of bone marrow DC derived from naïve mice

Dendritic cells (DC) comprise an integral cellular component of immune response development and it is well-established that the precise DC phenotype directs the resultant immune response. Therefore, dissection of ES-62 action in the CIA model began by investigating the effect of *in vivo* exposure to ES-62 on DC phenotype and function. The first step was to confirm the *in vitro* actions of ES-62 on DC phenotype and function described by this lab previously. Thus, DC were cultured from bone marrow of BALB/c mice, pre-treated with ES-62 for 24h and then stimulated with media or LPS for a further 24h. Treatment with ES-62 alone induced low-level IL-12p40 and TNFα production by DC (Figure 3.16), suggesting that the cells were modestly activated by ES-62. However, the ES-62-induced DC cytokine production was significantly less than that induced by LPS treatment. Furthermore, pre-treatment of DC with ES-62 before subsequent stimulation with LPS resulted in significant inhibition of LPS-induced IL-12 and TNFα production.

Thus, in terms of DC cytokine production, ES-62 exhibited dual activatory and inhibitory actions on DC *in vitro*.

To determine whether the dual actions of ES-62 were also exhibited in terms of cell surface marker expression, DC from each in vitro treatment group were stained and analysed by flow cytometry for expression of co-stimulatory (CD40, CD80, CD86) and adhesion (CD54) molecules required for DC-mediated activation of T cells and the adaptive immune response. Interestingly, low-level activation action of ES-62 treatment alone, observed in the cytokine secretion patterns, was not mirrored in terms of cell surface expression levels (Figure 3.17). Occasionally CD40 expression was upregulated in response to ES-62, but usually, ES-62-treated DC appeared immature in phenotype. LPS treatment of DC induced marked upregulation of all cell surface molecules analysed. However, in contrast to the cytokine analysis results, ES-62 pre-treatment of DC did not notably modulate subsequent LPS-mediated upregulation of cell surface expression. In summary, the *in vitro* actions of ES-62 on DC appeared to target cytokine secretion, but not expression of surface markers.

3.2.10 Bone marrow DC derived from CIA model mice exhibit a modulated phenotype

Taking together how ES-62 can modulate bone marrow-derived DC immunology *in vitro* with previous work suggesting that similar effects could be obtained following *in vivo* exposure [191], it was decided to investigate whether ES-62 treatment induced modulation of bone marrow-derived DC phenotype in CIA model mice. Thus, femurs were dissected from CIA model mice at the end of the prophylactic treatment protocol and bone marrow from each treatment group was pooled. DC were derived from such pooled bone marrow and cultured *in vitro* for 7 days before being stimulated for a further 24h with media or LPS before phenotype analysis.

Firstly, it was investigated whether bone marrow-derived DC from CIA model mice exhibited a different cell surface phenotype to naïve (non-diseased) MHC-matched (DBA/1) mice. In addition, it was also planned to investigate whether exposure to ES-62 *in vivo* modulated the phenotype of bone marrow-derived DC obtained from the CIA model mice.

When compared with DC cultured from bone marrow of naïve DBA/1 non-model mice, DC derived from CIA model mice exhibited upregulated expression levels of antigen-presentation, co-stimulatory and adhesion molecules MHC II, CD40, CD80, CD86 and CD54 (Figure 3.18). This indicated that bone marrow-derived DC from CIA model mice exhibit a more activated or mature phenotype, compared with DC cultured from naïve syngeneic mice.

LPS treatment of naïve and CIA model DC *in vitro* uniformly upregulated the expression levels of the cell surface markers analysed on DC from non-model and CIA model mice. As a result, under LPS conditions, differences in surface expression of DC from the model and naïve mice were not as obvious. Culture supernatants of the CIA model and non-model DC were also analysed for cytokine content, firstly under control conditions and then following LPS treatment. Detectable concentrations of IL-10, IL-12 and TNFα (Figure 3.19) were spontaneously secreted by DC from CIA model and naïve mice. Consistent with their mature surface expression profile, DC cultured from CIA model mice secreted enhanced levels of TH1-type/pro-inflammatory cytokines, TNFα and IL-12, but not regulatory cytokine, IL-10, when compared with DC cultured from naïve mice. This difference supported the previous conclusion, that DC cultured from CIA model mice exhibited a matured, somewhat activated phenotype compared with DC from naïve mice. LPS treatment *in vitro* markedly enhanced the overall levels of IL-12, TNFα and IL-10 cytokine production by both naïve and CIA model DC. Interestingly, the TNFα response to LPS was less and the IL-10 response to LPS was greater in DC from CIA model mice compared with DC from non-diseased mice. However, despite statistical significance these differences in cytokine production levels were relatively small and did not appear to represent any real trend of distinction between the two types of DC. In summary, LPS treatment of CIA model and naïve derived DC revealed that cells from both backgrounds exhibited normal upregulatory and cytokine production responses to inflammatory stimuli. Nonetheless, the inflammatory response to LPS masked real differences in DC phenotype that were induced by *in vivo* treatments).

3.2.11 The effect of ES-62 treatment *in vivo* on the phenotype of bone marrow DC derived from CIA model mice

To determine whether ES-62 treatment of CIA model mice *in vivo* induced yet further modulation of bone marrow-derived DC phenotype, DC were cultured from pooled bone marrow samples of ES-62-treated CIA model mice. Following analysis of these DC for surface marker expression it was concluded that prior ES-62 treatment *in vivo* did not induce discernible modulation of the surface expression profile of DC derived from CIA model mice. In more detail, ES-62 treatment of CIA model mice resulted in differentiation of DC that exhibited similar expression profiles of MHC II, CD80, CD86 and CD54 to DC from CIA model mice (Figure 3.18). Occasionally, modest increases in CD40 expression by DC from ES-62-treated CIA model mice were observed. To summarise, the activated, matured phenotype of DC derived from CIA model mice was maintained despite ES-62 treatment *in vivo*. Interestingly, DC from ES-62 treated CIA model mice exhibited a somewhat reduced CD40, CD54 and CD86 upregulation response to LPS, compared with DC from control (PBS-treated) CIA model mice. These subtle differences in the

upregulation response to LPS suggested that DC from ES-62 treated CIA model mice might exhibit a restricted response to inflammatory stimuli *in vivo*.

However, ES-62 treatment of CIA model mice resulted in differentiation of DC that secreted significantly increased levels of IL-12, but not TNFα or IL-10, when compared with DC from control CIA model mice (Figure 3.19). The enhanced IL-12 production was, therefore also significantly increased from the levels secreted by DC from naïve mice, which indicated that, in terms of IL-12 production, the activated phenotype of DC from CIA model mice was maintained despite *in vivo* ES-62 treatment. Interestingly, the IL-12 and TNFα response to LPS was significantly greater by DC from ES-62 treated CIA mice compared with DC from control CIA mice. In summary, analysis of DC from CIA model mice revealed an activated matured cell phenotype, compared with DC derived from naïve mice and this was not prevented by ES-62 treatment of CIA model mice *in vivo*. Nevertheless, some differences in expression of cell surface markers and production of cytokines were apparent between DC from ES-62 treated and control CIA model mice.

3.2.12 Investigation of the role of PC in ES-62-mediated action on bone marrow DC derived from CIA model mice

It was decided to investigate the nature of the subtle effects of ES-62 treatment of the CIA model mice on subsequently differentiated bone marrow DC. To determine whether the PC component of ES-62 was involved in these effects, DC from CIA model mice that had been treated with rES-62 or OVA-PC were cultured and compared with DC cultured from CIA model mice that had been treated with PBS, ES-62 or OVA respectively.

3.2.12.1 The effect of PC on DC surface expression profile

DC cultured from native ES-62 and rES-62 treated CIA model mice exhibited similar expression profiles of MHCII antigen-presenting molecules and co-stimulatory molecules, CD40, CD80 and CD86. Interestingly, CD54 expression was marginally increased on DC from rES-62 treated mice, compared with DC from ES-62 treated mice (Figure 3.18). Nevertheless, the overall surface expression phenotype of DC cultured from CIA model mice treated with rES-62 or native ES-62 was largely similar. In response to LPS stimulation *in vitro*, DC cultured from rES-62 treated mice exhibited somewhat enhanced LPS-induced upregulation of MHC II and CD86, compared with DC from ES-62 treated mice. This difference suggested that DC from rES-62 treated CIA model mice exhibited an augmented response to LPS treatment. Nevertheless, this effect was modest.

As previously observed between DC from ES-62 and rES-62 treated mice, expression of the surface markers analysed was highly similar on DC cultured from OVA and OVA-PC treated mice (Figure 3.21). LPS treatment *in vitro* induced marked uniform upregulation of

MHCII, CD40, CD54, CD80 and CD86 expression on DC from OVA and OVA-PC treatment groups. However, although DC from both OVA-PC and OVA treated mice expressed high levels of MHCII in response to LPS treatment, DC from the latter mice expressed the greatest levels of MHCII. The pattern of inhibited LPS-induced upregulation of MHCII expression had previously been exhibited by DC from ES-62, but not rES-62 treated mice. Thus, it was suggested that *in vivo* treatment of CIA model mice with PC-containing compounds might result in development of DC that exhibit an inhibited response to LPS, in terms of MHCII expression. However, on the whole, the surface expression profiles of DC cultured from bone marrow samples of CIA model mice were not extensively modified by prior *in vivo* treatment with ES-62, rES-62 or OVA-PC.

3.2.12.2 The effect of PC on DC cytokine production

To complete the analysis of the role of PC in ES-62-mediated actions on DC derived from CIA model mice, cytokine production by DC from rES-62 and OVA-PC-treated CIA model mice was compared with DC from ES-62 and OVA-treated CIA model mice respectively. DC from rES-62-treated mice spontaneously secreted less IL-12, but not TNFα or IL-10 than DC from ES-62 treated mice. Nevertheless, the difference was minimal and was maintained at a significantly higher level than was secreted by DC from CIA model mice (Figure 3.20). Although DC cytokine production levels were uniformly elevated by LPS stimulation *in vitro*, LPS-induced TNFα and IL-10 production by DC cultured from the rES-62 treated mice was less than that secreted by DC from ES-62 treated mice under the same conditions. However, similar to the responses of DC from ES-62 treated mice, the level of LPS-induced IL-12 and TNFα production by DC from rES-62 treated mice remained greater than that produced by DC from PBS-treated CIA mice. This indicated that DC from rES-62 treated mice generally exhibited similar cytokine production profile to DC from ES-62 treated mice, under control and LPS conditions.

DC cultured from bone marrow of OVA and OVA-PC treated CIA model mice also consistently secreted increased levels of IL-12 and TNFα when compared with DC cultured from naïve mice. Interestingly, DC cultured from bone marrow samples of OVA-PC treated mice spontaneously secreted significantly less TNFα, but not IL-12 or IL-10, than DC cultured from OVA treated mice (Figure 3.22). This trend of reduced TNFα production had previously been exhibited by DC cultured from ES-62 treated mice (Figure 3.19), but the difference was not significant.

Treatment of these DC *in vitro* with LPS induced marked increases in the production of IL-12, IL-10 and TNFα by DC from OVA and OVA-PC treated CIA model mice. However, LPS-induced IL-12 and TNFα production by CIA model-derived DC *in vitro* was enhanced by prior *in vivo* treatment of CIA model mice with OVA-PC compared with OVA. This

enhanced LPS response by DC from OVA-PC treated CIA mice mirrored the response of DC from ES-62 treated CIA mice, as described above, indicating that this action may be PC-induced.

In summary, DC cultured from CIA mice treated with OVA-PC exhibited similar surface expression and cytokine production profiles to DC cultured from CIA mice treated with ES-62. This correlation indicated that the changes to DC phenotype induced by prior *in vivo* exposure to ES-62 were PC-dependent. However, in comparison to the previously published effects of ES-62 treatment *in vivo* [191], the differences in phenotype of bone marrow-derived DC from the CIA model as a result of ES-62 treatment *in vivo* were minimal. Therefore, the major alterations in DC phenotype were reflected by comparison of DC from naïve and CIA model mice, with only minor changes to DC phenotype induced by further *in vivo* treatment of CIA model mice with ES-62, rES-62 or OVA-PC. Thus, despite treatment of CIA model mice with PC (in the form of ES-62 or OVA-PC) promoting differentiation of DC that secrete reduced levels of arthritis-promoting TNFα, an effect which might be anti-inflammatory in this model, it was concluded that the majority of changes to bone marrow-derived DC phenotype by ES-62 treatment of CIA were most likely not associated with inhibition of inflammation. Nevertheless, it was interesting to observe that many of the weak modulatory effects of ES-62 observed were PC-dependent.

3.2.13 The effect of therapeutic ES-62 treatment on the phenotype and function of DC derived from CIA model mice

In addition to their functions in initiation of immune response development, DC also form an important part of the effector immune response. Through communication with other innate immune cells, DC mediate maintenance and progression of inflammation at the inflammatory site [225, 279]. As previously described, ES-62 treatment of CIA model mice after the onset of clinically detectable arthritis inhibited progression of inflammation and joint erosion ([192] and section 3.2). Following confirmation that the *in vivo* collagen administration protocol responsible for inducing CIA inflammation brings about differentiation of bone marrow-derived DC with an activated, matured phenotype, it was decided to determine whether the therapeutic ES-62 treatment protocol was associated with modulation of bone marrow-derived DC phenotype. Therefore, DC were cultured from bone marrow of CIA model mice in the therapeutic treatment model and surface expression levels of MHCII, CD40, CD54, CD80 and CD86 were analysed *ex vivo*.

3.2.13.1 The effect of therapeutic ES-62 exposure on CIA model-derived DC surface expression

DC cultured from mice treated therapeutically with ES-62 exhibited reduced expression of antigen-presentation molecule, MHCII and co-stimulatory molecule, CD86 when compared with DC cultured from control (PBS-treated) CIA mice (Figure 3.23). This suggested that exposure of bone marrow cells to ES-62 *in vivo* resulted in differentiation of DC with a reduced antigen-presentation and co-stimulatory capacity, in other words, a less mature phenotype. Interestingly, moderately reduced expression levels of MHCII had previously been exhibited by DC cultured from ES-62 treated mice in the prophylactic treatment model. This consistency indicated that inhibiting the expression of this antigen-presentation molecule was an effect induced irrespective of the pattern of ES-62 administration *in vivo*.

LPS stimulation of DC cultured from CIA model mice *in vitro* induced upregulated expression of the co-stimulatory molecules CD40, CD80 and CD86 and the adhesion molecule CD54. Interestingly DC cultured from ES-62 treated CIA mice displayed somewhat inhibited LPS-induced upregulation of CD54 and CD80. Similarly, the reduced level of expression of MHCII on DC cultured from ES-62 treated CIA mice was maintained following LPS stimulation. Therefore, therapeutic ES-62 treatment of CIA model mice *in vivo* (after the onset of inflammation) resulted in consequent differentiation of DC that displayed an inhibited response to LPS stimulation *in vitro*. Slight hints of this effect of ES-62 treatment had previously been observed during analysis of DC cultured from CIA model mice treated prophylactically with ES-62, however the effect appeared more obvious in DC from CIA mice that had undergone therapeutic treatment with ES-62. The enhanced effect of ES-62 treatment in the therapeutic treatment protocol, suggested that the term and pattern of ES-62 treatment *in vivo* influenced the effect of ES-62 on resultant bone marrow derived DC phenotype.

3.2.13.2 The effect of therapeutic ES-62 on CIA model-derived DC cytokine production

Consistent with the response observed in DC from the prophylactic treatment model, the level of spontaneous IL-12 production by DC cultured from therapeutically ES-62 treated CIA model mice was greater than that produced by DC from PBS treated mice, although this difference was not significant (Figure 3.24). In contrast, when compared with DC cultured from bone marrow of control CIA model mice, DC cultured from ES-62 treated CIA model mice secreted significantly reduced levels of TNFα. It was of particular interest that ES-62 treatment of CIA model mice *in vivo* reduced TNFα secretion by DC derived from these mice *in vitro*, because this pro-inflammatory cytokine has been identified as important for pathogenesis of arthritis inflammation. This trend of reduced TNFα, but not

IL-12 production by bone marrow-derived DC was also exhibited by DC cultured from ES-62 treated CIA model mice in the prophylactic treatment model. Therefore, it appeared that this inhibitory action of ES-62 was specific for TNFα production by DC, which might suggest that modulation of TNFα, not IL-12, was most important for amelioration of inflammation in this model. Interestingly, DC cultured from CIA model mice that had been treated therapeutically, rather than prophylactically, with ES-62 exhibited the TNFα-reducing effect of ES-62 treatment *in vivo* much more prominently. Therefore, this action of ES-62 was consistent but influenced by the term and timing of ES-62 treatment *in vivo*. LPS stimulation *in vitro* of DC cultured from the CIA model mice induced increased production of cytokines overall, however DC cultured from CIA model mice treated therapeutically with PBS or ES-62 did not exhibit significantly different patterns of cytokine production in response to LPS (Figure 3.24).

In summary, the therapeutic ES-62 treatment protocol, administered to CIA model mice *in vivo*, appeared to induce a more pronounced modulation of the phenotype of DC subsequently cultured from the bone marrow of the ES-62 treated mice than that observed for DC derived from the prophylactic treatment model. The amplification effect (from prophylactic to therapeutic ES-62 treatment) probably reflected the influence of the term and timing of ES-62 treatment *in vivo* on bone marrow derived cell phenotype.

3.2.14 Comparison of the effects of recombinant ES-62 and OVA-PC treatment on DC derived from CIA model mice

Unlike treatment of the CIA model mice with native ES-62, treatment with rES-62 after the onset of established inflammation did not inhibit progression of inflammation. However, therapeutic treatment with OVA-PC, in place of ES-62 did mimic the anti-inflammatory effects of the latter in this model of inflammation. It was therefore important to determine the phenotype of DC derived from bone marrow of rES-62 or OVA-PC treated mice in the therapeutic treatment model. DC derived from CIA mice treated therapeutically with rES-62 or PBS were cultured *in vitro* and analysed by flow cytometry to determine the cell surface expression profile of antigen-presentation, co-stimulatory and adhesion molecules (Figure 3.23). Interestingly, DC cultured from rES-62 treated CIA model mice exhibited elevated expression of MHCII, CD80 and CD86, when compared with DC cultured from PBS treated CIA model mice. As described above, DC cultured from ES-62 treated CIA mice displayed reduced MHCII and CD86 expression compared with DC from PBS treated CIA mice. Thus, the action of rES-62 treatment on the phenotype of subsequently cultured DC was opposite to the action of parasite-derived ES-62 treatment.

DC cultured from bone marrow of selected OVA and OVA-PC treated mice were similarly analysed by flow cytometry to determine their cell surface expression profile (Figure 3.23).

DC cultured from OVA-PC treated mice exhibited reduced MHCII, CD40, CD80 and CD86 expression and increased CD54 expression, when compared with DC from OVA treated mice. This observed reduction in the expression levels of MHCII and CD86 on the surface of DC had previously been exhibited by DC cultured from ES-62 treated mice, but not rES-62 treated mice, in this therapeutic treatment model. Thus, these combined findings supported a role for PC in this inhibitory action of ES-62. Furthermore, it appeared that the inhibitory effect of OVA-PC was more potent than that of ES-62, resulting in differentiation of DC with reduced expression of additional co-stimulatory molecules, CD40 and CD80. It was unusual, however, that CD54 expression was elevated on DC from OVA-PC treated CIA mice.

LPS stimulation *in vitro* of DC from CIA model mice treated with PBS, rES62, OVA or OVA-PC induced upregulation of CD40 and CD86 expression. Additionally, LPS stimulation of DC from mice treated with OVA induced upregulation of CD54, and CD80. The respective expression profiles of DC from rES-62 treated mice compared with that of DC from PBS treated mice were maintained under LPS conditions. That is, DC cultured from rES-62 treated CIA mice exhibited increased expression of MHCII and CD86 and similar expression of CD54, CD40 and CD80 when compared with DC from PBS treated mice, following LPS stimulation. As described above, native ES-62 treatment of CIA model mice resulted in development of DC with an inhibited upregulation response to LPS *in vitro*. The absence of this modulated phenotype in DC from rES-62 treated mice, suggested that this action of native ES-62 might be due to PC.

Treatment with LPS induced upregulation of CD40, CD54, CD80 and CD86 expression on DC cultured from OVA and OVA-PC treated CIA model mice. However, DC cultured from OVA-PC treated CIA mice displayed an inhibited CD80 upregulation response to LPS, compared with DC cultured from OVA-treated CIA model mice. The inhibited upregulation response to LPS observed in DC from OVA-PC treated mice had been a feature of ES-62, but not rES-62, action on DC cultured from CIA mice in this treatment model. Thus, this effect of ES-62 treatment was most likely PC-dependent.

3.2.15 Comparison of the effects of therapeutic OVA-PC or rES-62 treatment on CIA model-derived DC

DC cultured from CIA mice treated therapeutically with rES-62 did not produce significantly different levels of TNFα, IL-12 or IL-10, when compared with the cytokine production by DC from such PBS treated CIA mice (Figure 3.25). By contrast, it had previously been shown (Figure 3.24) that DC, cultured from therapeutically ES-62 treated mice, secreted significantly less TNFα than DC cultured from control CIA mode mice. Therefore this inhibitory action of prior ES-62 treatment on DC phenotype was lost when

CIA model mice were treated with rES-62 *in vivo*. This difference indicated that PC might be responsible for the reduced spontaneous TNFα production observed in DC from mice treated therapeutically with native ES-62.

Likewise, there were no significant differences in IL-12 or IL-10 production by DC cultured from OVA treated or OVA-PC treated CIA model mice (Figure 3.26). However, surprisingly, DC cultured from OVA-PC treated CIA model mice spontaneously secreted increased levels of TNFα compared with DC cultured from OVA-treated CIA model mice. This was opposite to the effect induced on DC cytokine production by ES-62 treatment of CIA in this model (Figure 3.24). Therefore it was unclear what the role of PC was in ES-62 mediated inhibition of TNFα production by DC cultured from CIA model mice.

LPS stimulation *in vitro* of DC cultured from CIA model mice that had been treated therapeutically with PBS, rES-62, OVA or OVA-PC induced elevated production of IL-12, IL-10 and TNFα by all types of DC. There were no significant differences in the LPS-induced cytokine production between DC from PBS treated mice and DC from rES-62 treated mice. Interestingly, the IL-12 response to LPS was significantly inhibited in DC cultured from OVA-PC treated CIA mice compared with DC cultured from OVA-treated mice. This inhibited cytokine production response to LPS stimulation had not previously been displayed by DC cultured from CIA model mice in the ES-62 therapeutic treatment model, indicating that this was a novel effect of OVA-PC treatment in this model of inflammation.

In summary, the effects of ES-62 treatment of CIA model mice on the surface expression levels, but not cytokine production profile, of DC subsequently derived from bone marrow of those mice appeared to be PC-dependent. In conclusion, it was identified that the patterns observed in the phenotype of DC cultured from the CIA model mice that had been treated prophylactically with ES-62 were observed more potently in phenotype of DC cultured from CIA model mice that had been treated therapeutically with ES-62.

3.2.16 The effects of *in vitro* treatment with ES-62 on bone marrow macrophages derived from naïve mice

During an established inflammatory immune response, macrophages play a key role in maintenance or progression of inflammation and recruitment of cells to the inflammatory site, via production of cytokines [280]. Thus, it was postulated that examination of macrophages was required as part of the process to dissect the mechanisms comprising the observed anti-inflammatory actions of ES-62 in CIA. Initially it was important to clarify the direct action of ES-62 treatment on macrophages *in vitro*.

It has previously been identified that ES-62 treatment of bone marrow-derived macrophages *in vitro* induces modulation of macrophage cytokine production [190, 191]. To confirm, bone marrow-derived macrophages were pre-treated with ES-62 for 18h and then stimulated with media or LPS plus IFNγ for a further 24h. After the culture period, supernatants were analysed for cytokine production. This combination stimulus is used as macrophages, unlike DC, need be to primed with IFNγ prior to stimulation with LPS, to enable a complete response *in vitro*. Such IFNγ treatment simulates the inflammatory environment in which macrophages normally function, thus inducing preparation for an inflammatory immune response. Treatment of macrophages with ES-62 alone induces low level IL-12 and TNFα production (Figure 3.27). This cytokine production is at a much lower level than that induced by LPS/IFNγ treatment. Moreover, pre-treatment of macrophages with ES-62 before subsequent stimulation with LPS/IFNγ results in significant reduction in LPS-induced IL-12 and TNFα production. This analysis clearly demonstrates that ES-62 can directly modulate macrophage cytokine production in response to an inflammatory stimulus.

3.2.17 Investigating inflammation induced by cell-cell contact in arthritis

It has been well documented that the arthritic inflammatory joint is enriched with antigen-presenting cells and T lymphocytes [265]. The communication between these two cell types is central to progression and maintenance of the inflammatory environment and erosion of the joint. In particular, it has been suggested that cell-cell contact dependent communication between T cells and macrophages within the synovial joint is important for maintenance of inflammation in the progression of arthritis. This theory was proposed by McInnes' et al [245] as a potential target for therapy of arthritis. More specifically, contact with activated T cells induces activation of macrophages and production of pro-inflammatory cytokines, such as TNFα and inflammatory mediators, such as matrix metalloproteinases and prostaglandins. As previously discussed, the macrophage-derived cytokine, TNFα, has been identified as integral to the onset and progression of inflammatory arthritis. Furthermore, TNFα induces activation of macrophages. Thus, contact-dependent communication between T cells and macrophages enables amplification of cytokine production, which is observed in the inflammatory RA joint. Based on the observed anti-inflammatory effects of ES-62 treatment in the CIA model of arthritis, it was proposed that ES-62 might disrupt this particular communication pathway between T cells and macrophages to facilitate (at least in part) inhibitory action, particularly in established CIA (therapeutic treatment model).

Support for McInnes' theory [245] was provided by co-culturing paraformaldehyde-fixed, PHA-activated peripheral blood T cells from healthy humans with macrophage-like cells from a human cell line (THP-1 cells; [192]). It was observed that the resulting T cell

contact induced marked TNFα production by the THP-1 cells (Figure 3.28). Interestingly, pre-treatment of THP-1 cells with ES-62 *in vitro* significantly inhibited the observed contact-induced TNFα production. Thus, it was suggested that the inhibitory action of ES-62 demonstrated *in vitro* might be a method by which ES-62 mediates inhibition of inflammation in the CIA joint. However, it was decided that further investigation into this artificial human cell system would not be as profitable as developing an *in vitro* murine assay, which would be directly applicable to the murine CIA model system that had been investigated up to this point. Therefore it was proposed to develop an *in vitro* murine cell system for analysis of macrophage cytokine production in response to T cell contact.

3.2.18 Development of an murine cell system for investigation of contact-dependent communication between T cells and macrophages *in vitro*

The McInnes et al ([245]) theory of RA pathogenesis indicated that antigen-independent, T cell-contact dependent macrophage pro-inflammatory cytokine production is a core component in maintenance of autoimmune disease pathology. In an analogous manner to the human T cell-THP-1 cell system, cell contact assays were designed (using murine bone marrow-derived macrophages and PMA/Con A-activated lymph node T cells) to develop a working cellular model, which could be manipulated in the laboratory for investigation of the precise mechanisms underlying contact-induced cytokine production by macrophages. Pro-inflammatory TNFαand IL-12 production were measured as an indication of macrophage activation. Preliminary experiments established a positive relationship between T cell to macrophage ratio and macrophage cytokine production (Figure 3.29). In more detail, production of TNFα and IL-12 by macrophages was positively associated with the number of fixed T cells present in the co-culture, indicating that T cell contact induced macrophage cytokine production.

3.2.19 The effect of ES-62 exposure on contact-dependent communication between T cells and macrophages.

To determine the effect of direct ES-62 treatment on contact-dependent communication between T cells and macrophages, bone marrow-derived macrophages, PMA/ConA-activated T cells or both cell types were pre-treated with ES-62 before co-culture. When untreated macrophages were co-cultured with T cells that had been pre-treated with ES-62 prior to activation with PMA/ConA and fixation, T cell contact induced macrophage TNFα production was slightly decreased relative to that observed with such macrophages alone (Figure 3.30). By contrast, pre-treatment of macrophages with ES-62 significantly reduced their production of IL-12, but not TNFα, in response to T cell contact. However, pre-treatment of both, macrophages and T cells with ES-62 significantly inhibited T cell contact-induced TNFα and IL-12 production by macrophages. It was concluded that, in this system, ES-62 could act to inhibit T cell mediated induction of macrophage TNFα and

IL-12 production. Furthermore, the observed TNFα-reducing effect of ES-62 treatment on T cells and macrophages may be of therapeutic value *in vivo*, given that TNFα has been shown to be integral to arthritis pathogenesis.

Thus, it was proposed that this inhibitory effect of ES-62 *in vitro* might be a method employed by ES-62 *in vivo*, for mediation of anti-inflammatory action in the CIA model. To determine whether the inhibitory action of ES-62 on T cell contact-mediated cytokine production by macrophages might be induced *in vivo*, a further investigation was designed. Mice were treated twice *in vivo* with ES-62 and following sacrifice, bone marrow-derived macrophages were cultured and T cells were obtained from lymph nodes. Bone marrow derived macrophages and T cells were also cultured and obtained from untreated mice for comparison. Firstly, T cells from ES-62 treated mice were co-cultured with bm-macrophages from untreated mice. Overall, IL-12 and TNFα production by bone marrow-derived macrophages from untreated mice was low in both the presence and absence of co-culture with activated T cells. (Figure 3.31) Nevertheless it appeared that there was some T cell contact-induced IL-12, but not TNFα production by macrophages, identified because IL-12 production was positively associated with increasing ratios of T cells to macrophages. This effect was maximal at the ratio 8:1; T cells to macrophages. The observed lack of T cell contact-induced macrophage TNFα production (when T cells are from mice treated with ES-62) indicated that T cell contact-induced TNFα and IL-12 production by macrophages might be differentially regulated, that is, the *in vivo* effects of ES-62 treatment on T cells disrupts contact-dependent induction of TNFα, but not IL-12 in this system.

Secondly, bone marrow macrophages derived from mice that had received ES-62 treatment *in vivo* were co-cultured with fixed, activated T cells from untreated mice (Figure 3.32). Interestingly, although macrophages cultured alone spontaneously produced elevated levels of TNFα and IL-12, co-culture with increasing numbers of T cells did not significantly modulate macrophage cytokine production, indicating that T cell contact-dependent macrophage production of IL-12 *and* TNFα does not occur when macrophage pre-cursors have been pre-treated with ES-62 *in vivo*.

In summary, it appeared that treatment of mice *in vivo* with ES-62 disrupted T cell contact-induced cytokine production by macrophages. Furthermore, it was concluded that prior treatment of either cell type with ES-62 *in vivo* induced disruption of this method of macrophage activation and inflammatory cytokine production, indicating that this was a potential mechanism by which ES-62 might mediate anti-inflammatory action *in vivo* in the CIA model.

3.3 Discussion

It has previously been established that ES-62 can induce inhibition of TH1-type immune responses *in vitro* [189]. In this chapter, evidence has been provided to confirm, the previously identified, ES-62-mediated inhibition of TH1-type inflammation *in vivo*, in a model of inflammatory autoimmune rheumatoid arthritis [192]. Furthermore, progress has been made towards dissection of the mechanisms of ES-62 action in this model, by analysing the roles of sub-structural components of ES-62 and investigating the immune cells involved in mediation of ES-62 action.

3.3.1 The role of PC in the anti-inflammatory action of ES-62 on CIA model inflammation

As stated above, ES-62 significantly inhibited the severity of joint-specific inflammation in a laboratory model of arthritis. Moreover, inhibition of CIA inflammation was observed when ES-62 was administered either prophylactically (during set-up of the inflammatory condition) or therapeutically (after the onset of inflammation). The inflammation exhibited in the collagen-induced model of rheumatoid arthritis was confirmed as TH1-type, demonstrated by antigen-specific lymphocyte production of TH1-promoting cytokines (IFNγ and TNFα), detection of an antigen-specific TH1-type antibody response (IgG2a) and a pro-inflammatory cytokine milieu in the serum. Prophylactic ES-62 treatment of the CIA model mice reduced the development of such hallmark features of TH1-type inflammation in this model. Following confirmation of the previously published [192] anti-inflammatory actions of ES-62 in this model, the investigation progressed to analyse the role of PC in the effects of ES-62. The anti-inflammatory action of ES-62 was found to be lost when a PC-deficient recombinant homologue of ES-62 (rES-62) was employed. This highlighted the potentially important role of PC in ES-62 mediated inhibition of CIA inflammation. Furthermore, PC conjugated to albumin protein (OVA-PC), was capable of mimicking the anti-inflammatory action of ES-62 treatment in the CIA model. PC is a common component of pathogen-derived substances, with important dual functions in immune system activation and immune response evasion [177]. Previously, it has been demonstrated that PC, alone or conjugated to an irrelevant albumin protein, can largely mimic the *in vivo* and *in vitro* immunomodulatory actions of ES-62 on individual cell types [167, 185, 190, 191, 281]. Furthermore, it has also been previously identified that continuous exposure of mice to PC *in vivo* (via the use of osmotic pumps) induced modulation of peritoneal macrophage cytokine production *ex vivo* and development of bone marrow-derived DC with a modulated phenotype [191]. This was concluded to be similar to the effects of ES-62 exposure on these cell types. The results presented in this chapter represent new information demonstrating that PC (conjugated to OVA) can largely mimic the anti-inflammatory actions of ES-62 in a model of autoimmunity.

Given that substances such as ES-62 are secreted continually into the host bloodstream during infection with filarial nematodes it might be proposed that parasite infection may prevent development of inflammatory autoimmune disease, such as arthritis, in the host. Indeed, it has been demonstrated that infection with protozoan parasite, Trypanosoma brucei brucei (Tbb) ameliorates CIA inflammation in Dark Agouti (DA) rats [282]. In more detail, DA rats were infected with Tbb prior to collagen immunisation, employing a similar collagen administration protocol to that used in the murine CIA model. Similar to the effects of prophylactic ES-62 treatment in murine CIA, Tbb infection inhibited development of collagen-specific IgG and decreased expression of TH1 cytokine, IFNγ in draining lymph nodes of the CIA model rats. This provides evidence to support the statement made above, that active parasite infection might prevent the development of inflammatory autoimmune disease *in vivo*. Interestingly, Tbb-mediated amelioration of CIA inflammation in the DA rat was also associated with and induction of anti-inflammatory cytokine, TGFβ. Thus, it would be interesting to determine if TGFβ was also induced by ES-62 in the murine CIA model. Furthermore, in the rat CIA model, it was observed that the anti-inflammatory action of Tbb was only mediated by infection with live and not inoculation with dead parasites, suggesting that the anti-inflammatory effects were due to a product of Tbb and not the parasite itself. Thus, it would also be prudent to determine whether the anti-inflammatory effects of Tbb infection in the DA rat model of CIA are due to the immunomodulatory actions of a parasite-derived product, analogous to ES-62.

3.3.2 ES-62-mediated inhibition of antigen-specific IgG2a is PC-independent and not anti-inflammatory

Ultimately, inflammation and pathology in CIA occurs as a result of developing a chronic collagen-specific TH1-type immune response. Analysis of serum antibody levels is a method often used to determine the nature of the immune response underlying inflammatory pathology. Previously, it was demonstrated that ES-62-mediated inhibition of inflammation in the CIA model was associated with reduced antigen-specific IgG2a, a TH1-promoting antibody subclass. Furthermore, such action was observed when CIA model mice were treated with ES-62 prophylactically or therapeutically. Serum samples from CIA model mice in both treatment models were also analysed for IgG1, IgG3 and IgM and revealed no modulation, indicating that ES-62 action on the antibody response was specific for TH1-promoting IgG subclass, 2a.

Interestingly, the anti-inflammatory action of OVA-PC on CIA model mice was not associated with significant modulation of the level or nature of the serum antibody response, indicating that the inhibitory action of ES-62 on antigen-specific IgG2a was PC-independent. Furthermore, despite lacking anti-inflammatory action in the CIA model, treatment of CIA model mice with PC-deficient rES-62 induced inhibition of antigen-

specific IgG2a production, similar to that induced by treatment with parasite-derived ES-62. Thus, inhibition of collagen-specific IgG2a by native and rES-62 indicates that this effect was mediated by component(s) common to both substances. The apparent inconsistency in anti-inflammatory action and IgG2a inhibition confirmed that the IgG2a-reducing action of ES-62 in the prophylactic treatment model was PC-independent, furthermore, it indicated that serum antibody profile did not reflect the inflammatory status, in terms of cytokine production and arthritic score, of the mice in the CIA model.

These findings are particularly interesting as it was previously shown that PC (as a component of ES-62) prevents development of an IgG2a antibody response specific for non-PC epitopes of ES-62 [187], whilst not modulating the IgG1 antibody response. However it must be highlighted that such IgG2a-inhibiting action of PC was targeted to the antibody response to ES-62. In the CIA model the antibody response measured was collagen specific, thus it appears that PC functions to prevent development of a TH1-mediated antibody response to non-PC parts of ES-62 (or possibly other substances to which it is conjugated), but not other distinct antigens (such as collagen). Indeed, this action of PC may have a role in maintaining the circulatory concentration of ES-62 during infection with *A. viteae*, as antibody ligation would result in opsonisation of ES-62, preventing its action.

Nevertheless, the action of OVA-PC in CIA was anti-inflammatory, therefore it appears that modulation of the collagen-specific IgG2a antibody response is not necessary for inhibition of inflammation in this model. The anti-inflammatory action of OVA-PC in the CIA model, without corresponding modulation of serum IgG2a may appear inconsistent as IgG2a levels are generally considered to correspond with inflammation severity in CIA. However, previous studies have argued against a correlation in IgG2a levels and CIA incidence in mice and demonstrated maintenance of a Th1 immune response despite absence of an IgG2a antibody response [283, 284]. It was particularly interesting that treatment with OVA-PC slightly reduced collagen-specific IgM levels, an effect not observed in ES-62-treated mice. Therefore, this might be considered an action of PC that is masked when PC is incorporated within the structure of parasite-derived ES-62.

In this series of investigations, prophylactic but not therapeutic treatment of CIA model mice with ES-62 inhibited antigen-specific IgG2a levels, however, inhibition of collagen-specific IgG2a had previously been documented as an effect of both prophylactic *and* therapeutic ES-62 treatment in this model [192]. Coinciding with this slight change in the actions of ES-62, a shift in the severity of induced inflammation in the CIA model has been observed. Indeed, several models of CIA have been investigated in this laboratory since publication of the initial data. During this time, the supply of mice and collagen has

changed and challenges concerning induction of inflammation have been encountered and resolved. As a result, recent models of CIA have exhibited lower levels of overall induced inflammation, compared with the preliminary models used to determine the anti-inflammatory action of ES-62 in CIA. Thus, whilst the anti-inflammatory action of ES-62 remains, loss of the IgG2a-reducing capacity of ES-62 in the recent therapeutic treatment models may be a consequence of lower overall inflammation. Indeed, inhibition of antigen-specific IgG2a was also not observed when CIA was treated therapeutically with rES-62, therefore, like ES-62, the inhibitory action of rES-62 induced when administered prophylactically was also lost, when this treatment was administered after the onset of inflammation. In summary, it can be concluded that ES-62 (and rES-62) treatment of the recent models of CIA (presented in this thesis) prevents development of the collagen-specific IgG2a response, but does not induce reduction of existing antigen-specific IgG2a levels.

3.3.3 The serum profiles of cytokines, chemokines and growth factors in CIA model mice

Serum samples from the CIA model mice exhibited expression of a range of cytokines, chemokines and growth factors. However, the range of factors detected in serum from CIA mice in the therapeutic model compared with the prophylactic model was reduced. As these mice have received identical collagen treatment *in vivo*, this difference must be due to the timing of sacrifice and serum collection in each model. In more detail, CIA model mice in the prophylactic treatment model were sacrificed on day 33 and serum obtained, whilst CIA model mice in the therapeutic treatment model were sacrificed between days 29 and 35 of the experiment (depending on the time of inflammation onset and hence, the end of the treatment period, 14 days later). Thus, it is clear that the levels of serum cytokines, chemokines and growth factors exhibited by the CIA model mice change over a period of days. Nevertheless, certain cytokines (IL-2, IL-12), chemokines (IP-10, MIG, MIP-1α) and growth factors (FGF) were consistently detected, indicating that these soluble mediators might play important roles in the pathogenesis of this model. Cytokines help to form and define the nature of the developing immune response in arthritis, whilst the directed movement of immune cells during an immune response is highly dependent on the chemokine network [207, 285]. More specifically, chemokines orchestrate the trafficking of dendritic cells, T cells and B cells needed to generate an immune response. IL-12 is an important cytokine necessary for development of a TH1-mediated immune response [261], whilst IL-2 is important for T cell survival and function [262]. Furthermore, IL-10, a cytokine often identified in serum samples of CIA model mice in this investigation, has been identified as upregulated in peripheral blood of RA patients [286] and has been demonstrated as acting in an anti-inflammatory, regulatory manner on synovial cells [207]. Furthermore, IL-10 has been identified as regulatory to TNFα in RA [287].

It has been well established that the chemokines MIG, MIP-1α and IP-10 are released by innate immune cells, such as macrophages, in response to activation by inflammatory mediators such as IFNγ. Upon recognition of such chemokines, immature dendritic cells are recruited to the site of inflammation [285]. At the site of inflammation, immature DC are activated by the inflammatory milieu and are enabled to present antigen to and activate naïve T cells. Chemokines are also involved in recruiting lymphocytes from the blood across the endothelium of venules and to the inflammatory site. As ligands for CXC-chemokine receptor-3 (CXCR3) receptors on activated T cells, IP-10 and MIG are believed to be important for development of immune responses mediated by TH1 cells [285]. MIG and IP-10 also bind to and activate CXCR3 expressed on NK cells and is important for the recruitment of these cells into tissues. All of these cellular recruitment factors are required and appropriate for development of inflammation such as that exhibited in CIA.

Along with PDGF and TGFβ, fibroblast growth factor (FGF) is a major contributor to synovial cell hyperplasia in RA [207]. Furthermore, VEGF, which was detected in serum samples from the therapeutic treatment model, is important for neovascularisation in RA [288]. Thus, the serum factors detected have appropriate inflammation-promoting roles in CIA.

3.3.4 The effect of ES-62 treatment of CIA on the serum cytokine, chemokine and growth factor profile

Prophylactic ES-62 treatment of the CIA model induced modestly increased serum levels of pro-inflammatory cytokine, IL-1α, in some mice. Indeed, this IL-1α-enhancing effect was potently mimicked by treatment with OVA-PC, but was not induced by treatment with rES-62, indicating that this was likely to be a PC-dependent action of ES-62. IL-1 has been identified as contributory in the pathogenesis of RA [207, 267], thus the increased serum IL-1α concentration would appear inconsistent with the anti-inflammatory effects of treatment with ES-62 or OVA-PC in these mice. However, it has recently been demonstrated that whilst over-expression of IL-1α in a transgenic mouse led to development of severe arthritis, the severity of arthritis was related to activity of *membrane associated* IL-1α and not to serum concentration or activity of *soluble* IL-1α [289]. This may offer some explanation for the apparent discrepancy between the serum IL-1α levels and inflammation exhibited in this model. Furthermore, in accordance with the findings of the aforementioned study, it might be proposed that ES-62 acts to reduce membrane-bound IL-1α, as a potential mechanism of anti-inflammatory action in CIA model mice. Interestingly, serum IL-1α concentration appeared to correlate inversely with the inflammatory score of the mice in the ES-62 treatment group, suggesting that an

increase in IL-1α was somehow related to the anti-inflammatory effect of ES-62 in these mice. However, this pattern was not maintained throughout the treatment groups, indicating that this association may not be real.

In addition to IL-1, TNFα has also been identified as integral to development of arthritis inflammation, indeed blockade of TNFα is routinely used to reduce RA inflammation in humans [255]. Thus, it was surprising to note that TNFα was not present in serum samples from the CIA model mice. It has been suggested that TNFα is expressed in synovial membrane at onset of arthritis in the CIA model, [207], whilst IL-1 is detected later (1-2 days after the development of clinical inflammation). Therefore, it is possible that TNFα would be detected in serum samples obtained from CIA model mice at an earlier time-point (e.g. day 18-20). Nevertheless, KC and MIP-1α (that are induced by TNFα) were consistently detected in serum samples from the prophylactic treatment model, implicating upstream TNFα action. Interestingly, the expression of GRO-2 (the human counterpart of murine KC) and MIP-1α have been shown to be upregulated in synovial fibroblasts in RA patients [290]. GRO-2 functions chiefly in recruitment of neutrophils to the inflammatory site whilst MIP-1α recruits mainly monocytes and T cells, cell types known to be involved in synovial inflammation in RA.

Thus, although TNFα was not detected in serum samples from CIA model mice, it is possible that TNFα was still acting locally, at the inflammatory site for example, inducing production of KC and MIP-1α at this location, but not circulating in the bloodstream. Furthermore, serum KC concentration was modestly reduced by treatment with ES-62 and OVA-PC, indicating that earlier production of TNFα may have been reduced by both of these treatments. Interestingly, serum IL-10 levels were not modulated by ES-62 treatment indicating that promotion of this anti-inflammatory cytokine [291] was not a method employed by ES-62 for inhibition of inflammation, at least at this stage of the protocol.

Clearly, analysis of serum cytokines, chemokines and growth factors provided informative data about the soluble mediators circulating at the time of sacrifice, however it was not clear whether the factors detected were representative of the overall immune status of the CIA model mice (such as the serum antibody profile) or whether the slight changes induced by ES-62 reflected the anti-inflammatory effects of this glycoprotein in the joints. Consistent with this, it has been suggested that synoviocyte cytokine production may give a more accurate indication of arthritis severity in human RA and murine models [207]. Thus, examination of cytokine profiles in synovial samples from these mice might provide more conclusive information.

3.3.5 The immunostimulatory action of rES-62 in CIA model mice

Treatment of CIA model mice with rES-62 did not inhibit CIA inflammation and this was attributed to a lack of PC within the structure of rES-62. However, in addition, prophylactic treatment of CIA model mice with rES-62 appeared to promote prematurely enhanced collagen-induced inflammation and onset of arthritis. This observation indicated that rES-62 exhibits pro-inflammatory properties, consistent with the elevated serum IL-12 concentration observed in these mice. Indeed, it has been shown that mice treated with IL-12 developed significantly higher incidence and more severe collagen-induced arthritis [292]. Furthermore, it has been demonstrated that serum IL-12 levels reflect disease activity in humans and that IL-12 is involved in the production of pro-inflammatory cytokines in RA [293]. In addition, blockade of IL-12 has been shown to reduce the severity, but not the incidence, of CIA [294]. Interestingly, another study demonstrated that IL-12 has a stimulatory role in early arthritis expression, (accelerated onset and enhanced severity of CIA were provoked when IL-12 was administered around the time of arthritis onset), whereas it has a suppressive role in the established phase of collagen arthritis [295]. Thus, rES-62-induced promotion of CIA inflammation may be attributed to its IL-12-inducing action. However, it is not yet known how it induces IL-12.

Recombinant ES-62 was generated using the protein synthesis machinery of the yeast *P. pastoris*. Thus, several candidates may be responsible for the immunostimulatory action of rES-62 exhibited in the CIA model. Firstly, it might be considered that if PC is responsible for dampening inflammatory immune responses to native ES-62, the absence of PC may confer immunostimulatory properties on the rES-62 molecule. Indeed it has been identified that removal of PC from parasite-derived ES-62 (by treating with 1-deoxy-mannojirimycin (dMM) or hemicholinium-3 (HC-3)) prevented PC-dependent blockade of IgG2a antibody production against non-PC epitopes of ES-62 [187]. Thus, it is possible that lack of PC prevents masking of ES-62 antigenicity, resulting in an immunostimulatory substance that appears pathogenic and therefore stimulates an inflammatory immune response (and IL-12 production) in the CIA model.

Secondly, it has been recently identified that yeast-derived rES-62 exhibits increased mannose content compared to parasite-derived ES-62 [199]. Mannose is a common pathogen-associated carbohydrate and, therefore, activates immune responses *in vivo*. Mannose receptors are commonly expressed by cells of the innate immune response (such as macrophages and dendritic cells) as part of their repertoire of pattern-recognition receptors and upon ligation of these receptors the cells become activated. For example, the mannose receptor has been shown to recognise and bind components of mannose-containing *T. spiralis* muscle larvae [296], leading to cytokine production by macrophages. Therefore, the enhanced mannose content of rES-62 may induce an additional immune

response in CIA model mice, leading to activation of innate immune cells, increased IL-12 production and enhanced severity of CIA.

Finally, another factor to consider is that yeast-derived rES-62 also exhibits altered secondary and tertiary structures to parasite-derived ES-62 [199]. Using biophysical analysis methods it was recently determined that the secondary structure of rES-62 exhibits a reduced proportion of α-helices and increased proportions of β-sheets and β-turns compared with native, parasite-derived ES-62 [181, 199], indicating that rES-62 does not completely mimic the conformation of ES-62. Furthermore, using small angle X-ray scattering, it was demonstrated that the shape of rES-62 was most likely elongated [199], which was in contrast to the previously determined globular tertiary structure of parasite-derived ES-62 [181]. Thus, the refolded secondary and altered tertiary structures of rES-62 (compared with parasite derived ES-62) may influence the way in which the epitopes of rES-62 are presented to cells of the immune response in CIA model mice. For example, rES-62 may be recognized as pathogenic and, hence, activate additional immune responses. Ultimately the enhanced severity of CIA in rES-62 treated mice may be a result of a combination of any of the three factors mentioned above. The mechanism underlying this immunostimulatory action of rES-62 will only be conclusively identified upon further investigation.

3.3.6 Dendritic cells from CIA model mice exhibit a mature phenotype

Dendritic cells are integral and fundamental for organisation and initiation of immune responses *in vivo* [3]. Therefore, disruption of DC signalling and onward communication would be an efficient method by which immune responses could be modulated. Compared with DC cultured from bone marrow of naïve mice, DC cultured from CIA model mice exhibited a matured phenotype, demonstrated by enhanced expression levels of cell surface markers required for activation of T cells and increased spontaneous cytokine production levels. Therefore, cells which differentiated from bone marrow progenitors in the CIA model mice were pre-activated as a result of the collagen immunisation protocol (depicted in Figure 3.33). The activated TH1-promoting status of cells such as DC would help to accelerate the development of the TH1-type immune response required for establishing inflammation in this model.

3.3.7 The effects of treatment of CIA model mice with ES-62 on DC phenotype: prophylactic vs therapeutic treatment

As described above, ES-62 treatment of CIA model mice significantly inhibited articular inflammation. In addition, it has previously been demonstrated that a modulated DC phenotype results following *in vivo* exposure of bone marrow (in otherwise untreated mice) to ES-62 [191]. Thus it has been suggested that ES-62 is able to modulate

development of an immune response *in vivo* via rewiring of bone marrow DC progenitors. To determine whether ES-62 employed this mechanism to ameliorate disease in the CIA system, the phenotypes of DC cultured from PBS- and ES-62-treated CIA model mice were compared.

Prophylactic treatment of CIA model mice with ES-62 induced differentiation of bone marrow-derived DC that exhibited a subtly modulated phenotype compared with DC derived from control CIA model mice. In more detail, ES-62 treatment *in vivo* induced differentiation of DC that exhibited enhanced IL-12 production. Considering IL-12 is a TH1-promoting cytokine, this action of ES-62 might appear counterintuitive to the anti-inflammatory action of ES-62 in this model of TH1-mediated inflammation, however, as mentioned above it has been demonstrated that the action of IL-12 can change during the course of CIA inflammation development. For example, IL-12 has been identified as immunosuppressive after the onset of inflammation in CIA [295], thus, enhanced production of IL-12 by DC at this stage in the model could represent an anti-inflammatory effect of ES-62. It must also be considered that in this investigation production of only the p40 subunit of IL-12 has been measured. Bioactive IL-12 exists as a heterodimer composed of p40 and p35 subunits. Furthermore, it is known that IL-12 can also exist as a homodimer of two identical p40 subunits (IL-12p40/p40). This p40 homodimer has very different immunological activity to the heterodimer IL-12p40/p35 [297, 298]. Indeed, it has been demonstrated that treatment with IL-12p40 homodimer (in low concentrations) inhibits inflammation exhibited in a model of colitis by blocking the IL-12 receptor and antagonising the action of heterodimeric IL-12 [299]. Thus, it might be proposed that, via this mechanism, the enhanced IL-12p40 secreted by DC derived from ES-62-treated mice could act in an anti-inflammatory manner *in vivo* in the CIA model. Conclusive elucidation of this theory might be permitted following further investigation.

Nevertheless, the modulation of phenotype induced by ES-62 was minimal relative to that induced by the collagen administration protocol. Interestingly, the modulatory action of ES-62 on DC phenotype was more obvious when ES-62 was administered in the therapeutic treatment model. Such difference in the potency of ES-62 action indicated that the timing and quantity of ES-62 treatment *in vivo* influenced its action on resultant DC phenotype.

To explain, during the prophylactic treatment model, ES-62 was administered only three times, on days –2, 0 and 21, therefore the last exposure to ES-62 was administered 12 days before sacrifice. In contrast, in the therapeutic treatment model, ES-62 was administered to CIA model mice daily for 14 days immediately prior to sacrifice of the mice and subsequent culture of bone marrow-derived DC. Thus bone marrow cells of CIA

model mice in the therapeutic treatment model were exposed to a greater number of ES-62 applications, which were administered immediately prior to the start of the DC culture period. Therefore, the potency of the modulatory effect of ES-62 on DC derived from CIA model mice was most likely directly related to the duration and quantity of ES-62 exposure *in vivo*. Indeed, significant modulatory effects of ES-62 on DC phenotype *ex vivo* have been demonstrated when bone marrow cells are exposed *in vivo* to ES-62 continually, via the use of osmotic pumps, which continuously release ES-62 at a concentration similar to that produced during active infection with filarial nematodes [191]. In more detail, DC derived from such 'ES-62 pump' mice exhibited an immature phenotype and an inhibited response to LPS stimulation *in vitro*. By comparison, the modulation of bone marrow-derived cell phenotype induced by this type of continuous ES-62 exposure was more pronounced that that observed in response to daily boluses of therapeutic ES-62 treatment in the CIA model. This difference helps to confirm that the nature and duration of ES-62 treatment *in vivo* may be directly related to the extent of modulation of DC phenotype that results.

Nevertheless, it must be considered that the modulation of DC phenotype by ES-62 treatment of CIA model mice *in vivo* was modest, even in DC cultured from the therapeutic treatment model. Moreover, the subtle effects of ES-62 on DC phenotype were largely overshadowed by the powerful modulatory effect of the collagen administration protocol on this cell type. Therefore, in future investigations of this model, it would be interesting to examine the phenotype of DC cultured from bone marrow taken at different time-points throughout development of the CIA model and determine any change over time. Similarly, it may be informative to extract mature peripheral tissue DC from CIA model mice and compare phenotype with DC cultured from bone marrow taken at the same time-point. The results of such investigations may help to clarify the in vivo effects of ES-62 on DC in developing and established CIA.

3.3.8 PC-dependent modulation of DC phenotype

It appears likely that the small additional changes to DC phenotype induced by ES-62 treatment *in vivo* may not reflect the marked reduction in inflammation exhibited by the ES-62 treated CIA model mice. Nevertheless, in an attempt to determine the PC-dependency of ES-62-mediated modulation of DC phenotype in this model, DC derived from ES-62- rES-62- and OVA-PC treated CIA model mice were compared. Despite concluding that the changes to DC phenotype induced by prophylactic ES-62 treatment of CIA model mice were modest, a few effects were particularly interesting. Firstly, DC from ES-62 treated mice exhibited a trend of reduced TNFα production, which was more significantly exhibited by DC from OVA-PC treated mice, but not observed in DC from rES-62 treated mice. Therefore DC with reduced TNFα production were promoted only by

the treatments that contained PC, suggesting that this was a PC-dependent effect. TNFα blockade has been used as an effective treatment for arthritis [255] therefore, if this TNFα-reducing effect was more potent, it might have an anti-inflammatory effect in this model. Indeed, an effect such as this may be more apparent *in vivo*, and may be lost *in vitro* due to the required culture time for differentiation of DC.

Secondly, ES-62 treatment of CIA model mice *in vivo* promoted the DC inflammatory cytokine response to LPS *in vitro*. This was unexpected because it was the reverse effect to that induced by ES-62 when administered directly to DC *in vitro* or when administered to bone marrow progenitors *in vivo* via osmotic pumps [191]. Nevertheless, this effect was additionally observed in DC from OVA-PC treated mice, but not DC from rES-62 treated mice, suggesting that this was also a PC-dependent effect. It is unclear how this PC-dependent action of ES-62 and OVA-PC on DC cytokine production would be related to ES-62 or OVA-PC induced anti-inflammatory action in this model, however it must be reiterated that these modulatory effects of ES-62 and OVA-PC, whilst significant, were relatively small.

In summary, it was concluded that whilst ES-62 clearly was capable of modulation of DC phenotype, its action was mild in this disease model. Nevertheless, ES-62 treatment after the onset of established inflammation did inhibit inflammation, therefore ES-62 must be targeting other cell types to induce this significant anti-inflammatory action, in established disease.

3.3.9 Cell-cell communication in the inflammatory site

Of the several cell types commonly found in close proximity in the arthritic synovium, macrophages and T cells are major players [300]. Furthermore, communication between macrophages and T cells is thought to be of central importance in propagation of inflammation in the joint. For example TNFα production in RA synovium is T-cell dependent, as removal of CD3-positive T cells from RA synovial mononuclear cells resulted in significant reduced macrophage-derived TNFα production [301].

It was suggested by McInnes et al [245] that contact-dependent communication between activated T cells and adjacent macrophages in the arthritic synovium is fundamental for development and amplification of inflammation in arthritis. More specifically, T cells activated by pro-inflammatory cytokines or components of the extracellular matrix, but not via ligation of the antigen receptor (i.e. bystander activation), promote pro-inflammatory cytokine (e.g. TNFα) and inflammatory mediator (e.g. matrix metalloproteinases and prostaglandins) production by macrophages [302]. Since cytokines generated by this method could in turn activate T cells, positive feedback and amplification of inflammation

may be initiated (Figure 3.1). Therefore, disruption of this method of cell-cell communication would represent an efficient mechanism by which inflammation in arthritis could be reduced locally.

Indeed, support for this theory was provided by co-culture of fixed, activated human peripheral blood T cells (from healthy or RA donors) and human macrophage-like THP-1 cells, which induced TNFαproduction by the THP-1 cells (Figure 3.28). Furthermore, TNFα production by the THP-1 cells in response to contact with the T cells was inhibited when the THP-1 cells were pre-treated with ES-62. Confirming that, via modulation of macrophage function, T cell contact-induced cytokine production was a viable target for ES-62 in mediation of anti-inflammatory effects.

Macrophages are innate cells possessing important roles in maintenance and progression of inflammation at the inflammatory site. As mentioned previously, by secreting chemotactic factors and cytokines, macrophages function to recruit other immune cells to the inflammatory site and promote their survival and activation [285]. Furthermore, it has been clearly established that macrophages are important cells for maintenance of inflammation in the arthritic joint. For example, this cell type represents a primary source of TNFα, a cytokine known to be integral to arthritis pathogenesis [207]. It has previously been demonstrated that ES-62 treatment of macrophages, *in vivo* or *in vitro* inhibits the normal inflammatory cytokine secretion response of this cell type [190, 191]. Furthermore, this action has subsequently been found to be PC-dependent. Recently, another study has demonstrated similar macrophage inhibiting properties of a PC-containing phospholipid, which inhibited TNFα production by macrophages and endothelial cells [303], indicating that this action of ES-62 might also be employed by other PC-containing parasite derived substances. Considering that macrophages confer a significant contribution to local inflammation in arthritis and that ES-62 is capable of inhibiting macrophage inflammatory responses, macrophages represented a promising target for ES-62 mediated anti-inflammatory action in the CIA model, especially in the therapeutic treatment protocol.

3.3.10 ES-62 disrupts T cell contact induced macrophage activation

In accordance with the McInnes theory of RA pathogenesis [245], it was proposed that the anti-inflammatory action of ES-62 treatment in the CIA model might be mediated by disruption of contact-dependent T cell-macrophage communication. Therefore, analysis of T cell contact-induced macrophage cytokine production was conducted. Using a specially designed *in vitro* system that employed murine cells it was possible to demonstrate T cell contact-induced macrophage cytokine production (Figure 3.29). Furthermore it was clear that prior ES-62 treatment of T cells or macrophages *in vitro*, or both cell types *in vivo*,

acted to disrupt this method of communication, demonstrated by inhibition of macrophage cytokine production. Therefore it was concluded that this represented a potential method by which ES-62 might inhibit established inflammation in the CIA model (illustrated in Figure 3.34). In future experiments it would be interesting to investigate the PC-dependency of this action of ES-62, however, due to time constraints this was not possible during this study.

A particularly interesting observation concerning regulation of IL-12 and TNFα production was made during analysis of this *in vitro* system. Following *in vivo* treatment of T cells with ES-62, it was demonstrated that macrophage TNFα, but not IL-12 production (in response to T cell contact) was inhibited. This difference indicated that T cell contact-induced production of IL-12 and TNFα by macrophages might be differentially regulated. Ultimately treatment of macrophages or T cells with ES-62 consistently targeted TNFα production. Considering TNFα is an integral cytokine for development of inflammation in CIA, this action of ES-62 *in vivo* would be selectively anti-inflammatory.

In summary, ES-62 treatment disrupts contact-dependent communication between T cells and macrophages, evidenced by inhibition of macrophage cytokine production levels. It has previously been identified that ES-62 modulates macrophage cytokine production in response to LPS by suppressing the activation of mitogen-activated protein (MAP) kinases, p38 and JNK [304], which are required for production of cytokines, such as IL-12 and TNFα. Furthermore, ES-62 promotes the activation of Erk MAP kinase [305], which functions as a negative regulator of IL-12 in macrophages. Thus, it was planned to investigate expression of MAP kinases in macrophage-like THP-1 cells, in response to treatment with ES-62 alone. However, preliminary experiments examining expression and activation of Erk, JNK, p38 and Akt MAP kinases in THP-1 cells following ES-62 treatment did not provide conclusive data (results not shown). Furthermore, previous investigations conducted in this laboratory have concluded that whilst the direct action of ES-62 on T cell cytokine production is minimal, ES-62 treatment of T cells inhibits their proliferation in response to ligation of the antigen receptor [186]. Such ES-62-mediated disruption of TCR signalling (leading to abrogated proliferation) is associated with disruption of TCR coupling to phospholipase D, protein kinase C, phosphatidylinositol-3-kinase and Ras Erk MAP kinases [304]. Furthermore, it has previously been demonstrated that ES-62 treatment alone induces Erk phosphorylation in T cells [186], before desensitizing these cells to activation by anti-CD3. However, as yet it has not been determined whether ES-62 can modulate T cell signalling when they are activated in a bystander mechanism, such as that described in McInnes' theory [245]. In accordance with the previously documented effects of ES-62 treatment alone, the results of a preliminary experiment conducted during study of ES-62 action in the CIA model indicated that ES-62 treatment

of CIA model mice *in vivo* induced transient Erk phosphorylation *ex vivo* in lymph node T cells from CIA model mice. This was not observed in T cells from PBS-treated CIA model mice (results not shown). Therefore, via modulation of Erk MAP kinase activation in T cells, ES-62 treatment of CIA model mice might induce desensitisation of T cells to bystander activation, resulting in reduced expression of co-stimulatory (e.g. CD154 and CD28) and adhesion molecule (e.g. LFA-1 and ICAM-1) receptors on the surface. As these surface molecules are required for mediation of contact-dependent communication between T cells and macrophages [245], thus the action of ES-62 on T cell activation might disrupt T cell contact-mediated macrophage pro-inflammatory cytokine production and therefore progression of inflammation in the CIA model. However, as yet, the signalling mechanisms employed by ES-62 for mediation of inhibitory action on T cell contact-induced macrophage cytokine production are not known. Following further investigation, it will be possible to elucidate these mechanisms.

3.3.11 Conclusion

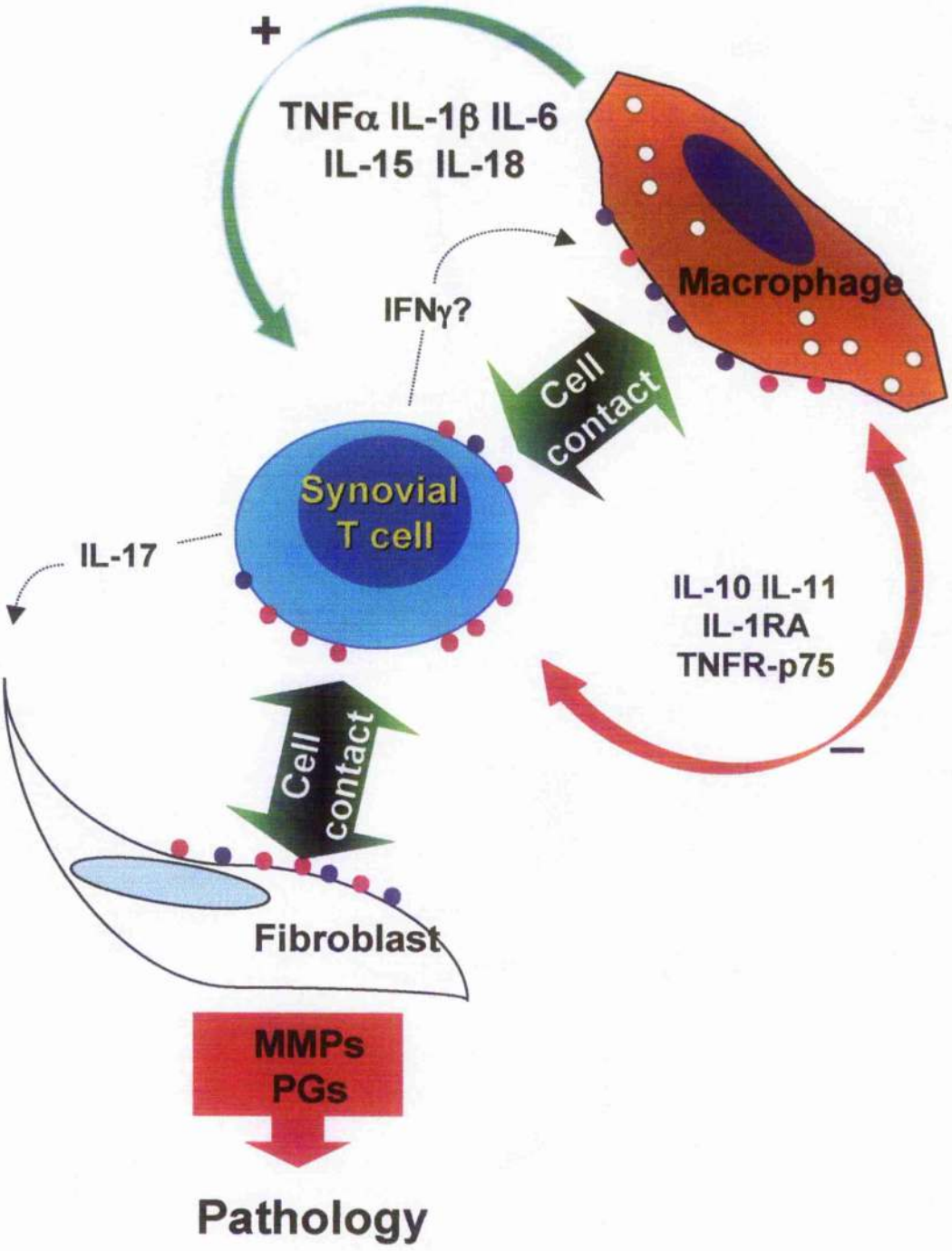
It has previously been demonstrated that ES-62 inhibits inflammation exhibited in murine collagen-induced arthritis, a well-established model of human RA [192]. Data presented in this chapter has provided evidence to extend this previous study and examine more closely the mechanisms of anti-inflammatory action induced by ES-62 in CIA.

First and foremost, the major finding described in this chapter has been that PC largely mimics the anti-inflammatory action of ES-62 in CIA. This finding can be added to the wealth of actions of ES-62 that have been found to be PC-dependent [167, 185, 190, 191, 281]. However, not all of the effects of ES-62 on this model were PC-dependent. As previously published, ES-62-mediated inhibition of the inflammation exhibited in CIA was associated with reduction of both antigen-specific TH1 cytokine production and TH1-type antibody (IgG2a) production. The results presented in this chapter have confirmed that although ES-62 does inhibit development of the collagen-specific IgG2a response in the CIA model, this particular action on antibody levels is not directly associated with or necessary for reduction of inflammation. Furthermore, this action of ES-62 appears to be PC-independent. Interestingly, the collagen immunisation protocol appeared to modulate bone marrow progenitor cells, in order that resultant bone marrow-derived DC (CIA DC) exhibited a matured phenotype. The effect of ES-62 exposure *in vivo* was modestly reflected in the phenotype of the CIA DC, however the phenotype was not significantly altered by ES-62 to such an extent that it appeared responsible for the anti-inflammatory action observed.

It is well-established that the communication between the cells present in the inflamed synovium is integral to amplification and maintenance of articular inflammation in arthritis [245, 306]. By employing an *in vitro* cell-contact system, it became clear that ES-62 is

capable of significantly disrupting T cell contact induced macrophage cytokine production. Thus, it is postulated that ES-62 mediated anti-inflammatory action in this model of arthritis might be, at least partially, mediated by inhibition of contact-dependent macrophage activation and cytokine production. The potential anti-inflammatory action of ES-62 is illustrated in Figure 3.35.

As mentioned in Chapter 1, ES-62 has several homologues secreted during infection with human filarial parasites. Thus, it might be postulated that the reduced incidence of inflammatory autoimmune diseases, such as RA, in countries endemic for filariasis could be attributed to the anti-inflammatory action of filarial nematode ES product homologues of ES-62.



(McInnes et al, 2000)

Figure 3.1 Contact-dependent mechanisms by which T cells contribute to synovial inflammation

Through cell contact, using membrane-bound cytokines and/or co-stimulatory and adhesion molecules, T cells can stimulate macrophages to produce pro-inflammatory cytokines such as TNFα, IL-1, IL-15 and IL-18. In turn, these cytokines re-stimulate the T cell to further activate the macrophage, by cell contact or perhaps via the production of IFNγ, another pro-inflammatory cytokine. In this way a positive feedback system is set up. The synovial T cell interacts in a similar manner with the fibroblast-like synoviocytes and an inflammatory environment is created. This interaction initiates the production of and activates matrix metalloproteinases (MMPs) and prostaglandins (PGs), which facilitate cartilage destruction and inflammation exhibited in RA respectively [245].

Figure 3.2 ES-62 treatment delays onset and reduces severity of collagen-induced arthritis

A and B, DBA/1 mice were immunised on days 0 and 21 with collagen, and were treated with ES-62 on days –2, 0 and 21 (n = 9) or PBS (n = 9). Clinical score and incidence of arthritis were monitored daily. Data are presented from day 16 to day 28, expressed as mean ± SEM and are representative of at least 10 independent experiments. For mean articular index (A), *, $p < 0.05$ (ES-62), compared to PBS (Student's t-test). Although also reduced by prior exposure to ES-62, no statistical difference was observed for incidence of arthritis (B) between ES-62 treated and control mice.

C-F Pooled draining lymph node cells were collected from ES-62 treated or PBS control mice (n = 3) on day 28 and cultured in medium alone (open bars) or with 50 μg/ml collagen (filled bars). T cell proliferation (C) and cytokine production (D-F) are expressed as mean ± SEM and are representative of at least 10 independent experiments. *, $p < 0.05$ compared with PBS control.

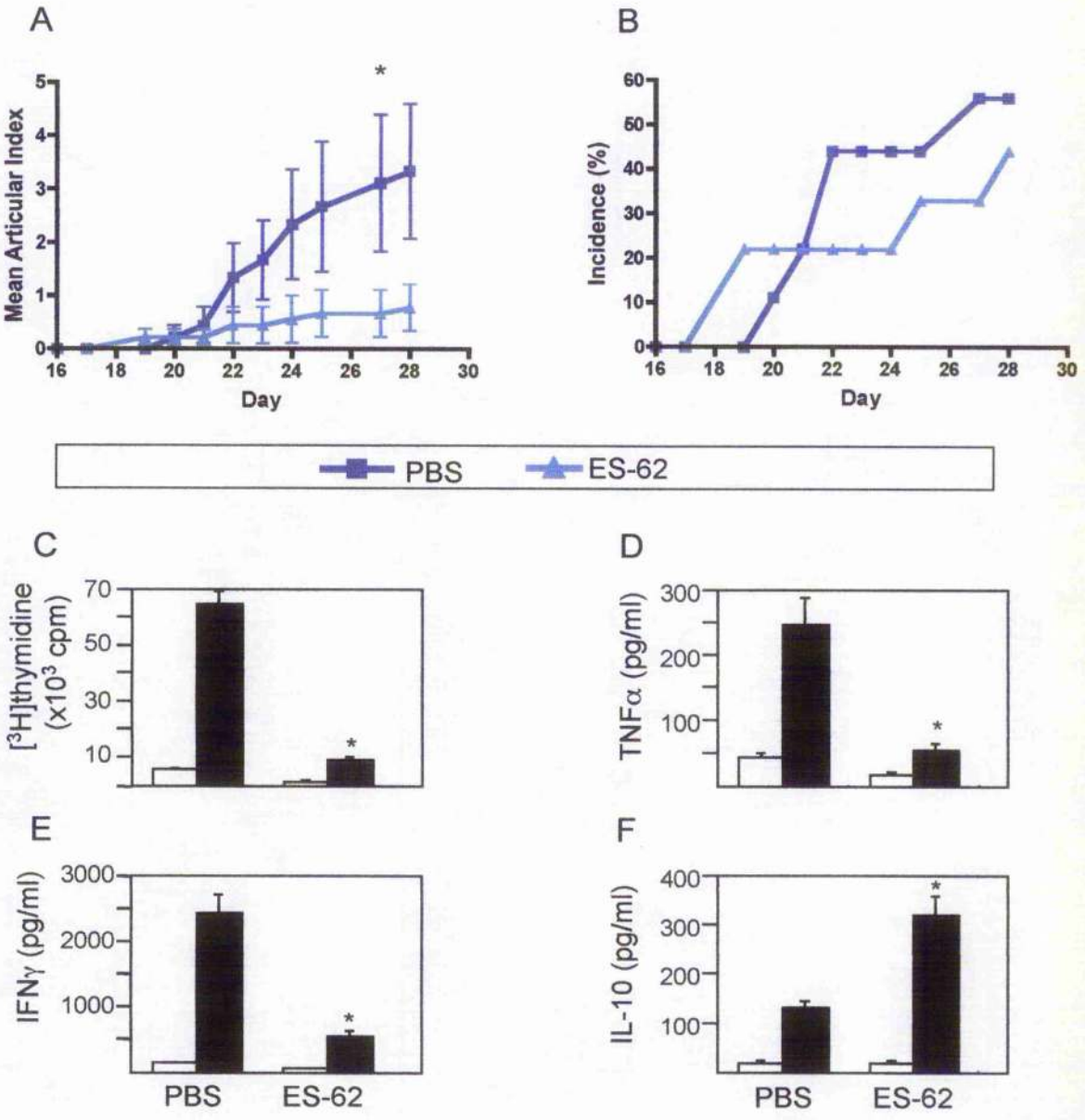

A
B
Mean Articular Index
Incidence (%)
Day
Day
PBS
ES-62
C
D
E
F
[3H]thymidine (x10³ cpm)
TNFα (pg/ml)
IFNγ (pg/ml)
IL-10 (pg/ml)
PBS
ES-62
PBS
ES-62

Figure 3.3 ES-62 reduces serum antigen-specific IgG2a in collagen-induced arthritis

Serum samples were obtained from mice in each treatment group on day 28 and diluted accordingly with FCS. Collagen-specific IgG1, IgG2a, IgG3 and IgM levels in serum samples from PBS and ES-62 treated mice were measured by ELISA. Data are individual measurements (n = 9/group), expressed as mean concentration (pg/ml) ± SEM and are representative of at least 5 independent experiments. *, $P < 0.05$ versus ES-62 (Student's t-test).

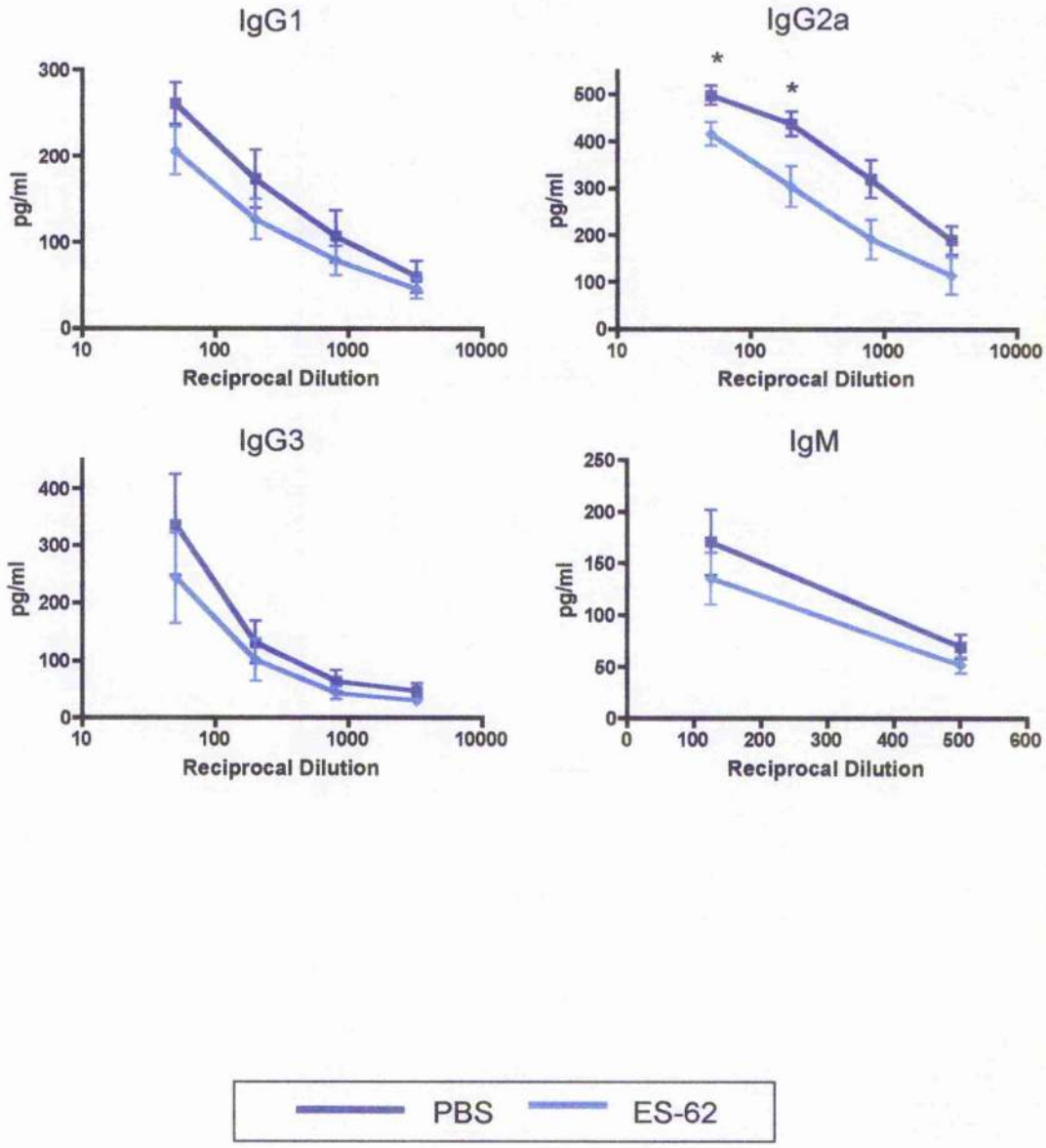

IgG1
pg/ml
300
200
100
0
10
100
1000
10000
Reciprocal Dilution
IgG2a
*
*
pg/ml
500
400
300
200
100
0
10
100
1000
10000
Reciprocal Dilution
IgG3
pg/ml
400
300
200
100
0
10
100
1000
10000
Reciprocal Dilution
IgM
pg/ml
250
200
150
100
50
0
0
100
200
300
400
500
600
Reciprocal Dilution
PBS
ES-62

Figure 3.4 ES-62 treatment inhibits the severity of collagen-induced arthritis when administered therapeutically

DBA/1 mice were immunised on days 0 and 21 with collagen and arthritic animals were treated daily one day after detectable clinical CIA with ES-62 (n = 6) or PBS (n = 5).

A, Mean articular index (A) was monitored daily and data are presented from day 18 to day 28, expressed as mean ± SEM and are representative of at least 10 experiments. *, $P < 0.05$ (ES-62), compared to PBS (Student's t-test).

B-F At sacrifice, arthritic paws from the therapeutic study were removed and sections stained with hematoxylin and eosin (B & C), or toluidine blue (D & E). **B** and **D** Profound infiltration, cartilage surface erosion and loss of proteoglycan was observed in PBS control as indicated by the arrows, whereas treatment with ES-62 exhibited significantly reduced histologic evidence of destruction (**C** & **E**). **F**, Histological appearances were scored for the presence of synovial bone erosion, hyperplasia, and cellular infiltration in ES-62 (open bars) or PBS (filled bars)-treated paws. *, $P < 0.05$ versus PBS control (Mann-Whitney *U* test). Data are mean ± SEM (n = 10 / group). Original magnification, *(A & B)*, X 25 ; *(C & D)*, X 50.

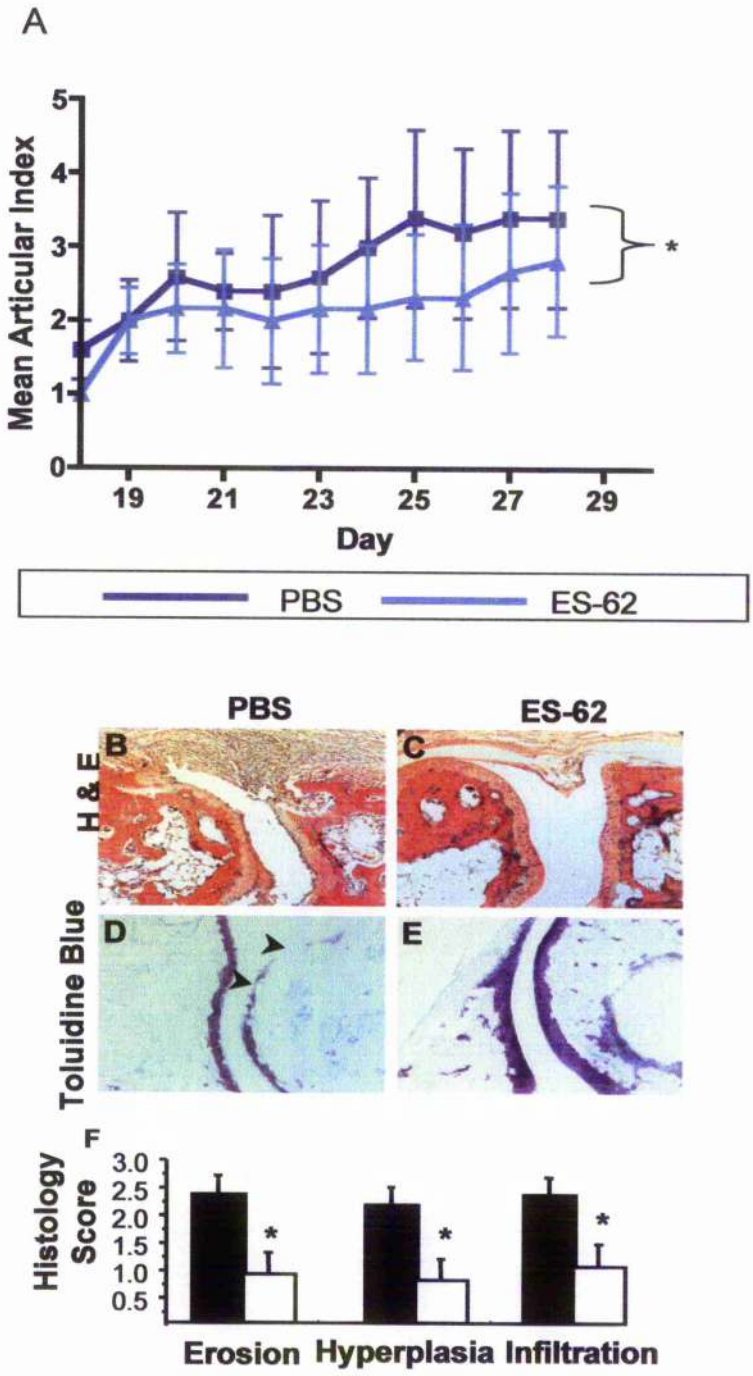

A
Mean Articular Index
0
1
2
3
4
5
19
21
23
25
27
29
Day
*
PBS
ES-62
PBS
ES-62
H & E
B
C
Toluidine Blue
D
E
F
Histology Score
3.0
2.5
2.0
1.5
1.0
0.5
*
*
*
Erosion
Hyperplasia
Infiltration

Figure 3.5 Suppression of established disease by ES-62 is associated with reduction in pro-inflammatory cytokine production *in vitro*

Draining lymph node cells from arthritic mice (n = 3) treated with either ES-62 or PBS were cultured with medium (open bars) or collagen (filled bars) and T cell proliferation (A) and cytokine production (IFNγ, (A), TNFα (B) and IL-6 (D)) were assessed as previously described. Data are expressed as means ± SEM and are representative of at least 5 experiments . *, $P < 0.05$ ES-62 versus PBS (Student's t test).

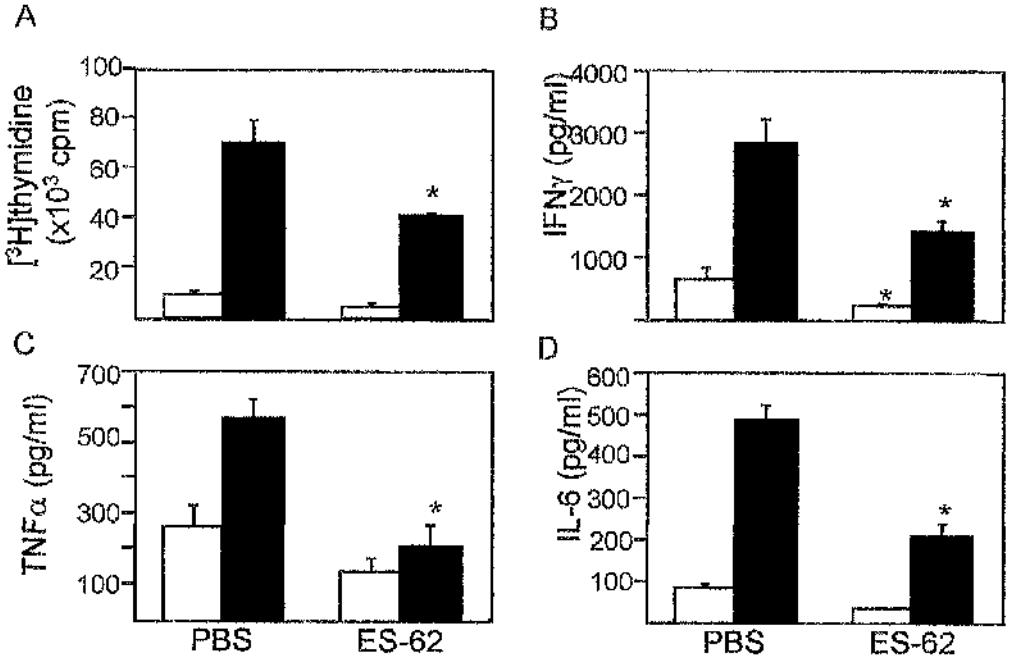

A
B
C
D
[³H]thymidine (x10³ cpm)
IFNγ (pg/ml)
TNFα (pg/ml)
IL-6 (pg/ml)
100
80
60
40
20
4000
3000
2000
1000
700
500
300
100
600
500
400
300
200
100
*
PBS
ES-62

Figure 3.6 ES-62 does not reduce serum antigen-specific IgG2a in established collagen-induced arthritis

Serum samples were obtained from mice in each treatment group on day 28 and diluted accordingly with FCS. Collagen-specific IgG1, IgG2a, IgG3 and IgM levels in serum samples from PBS and ES-62 treated mice were measured by ELISA. Data are individual measurements (n = 5 / group), expressed as mean absorbance (OD630nm) ± SEM and are representative of at least 3 experiments. *, $P < 0.05$ versus ES-62 (Student's t-test).

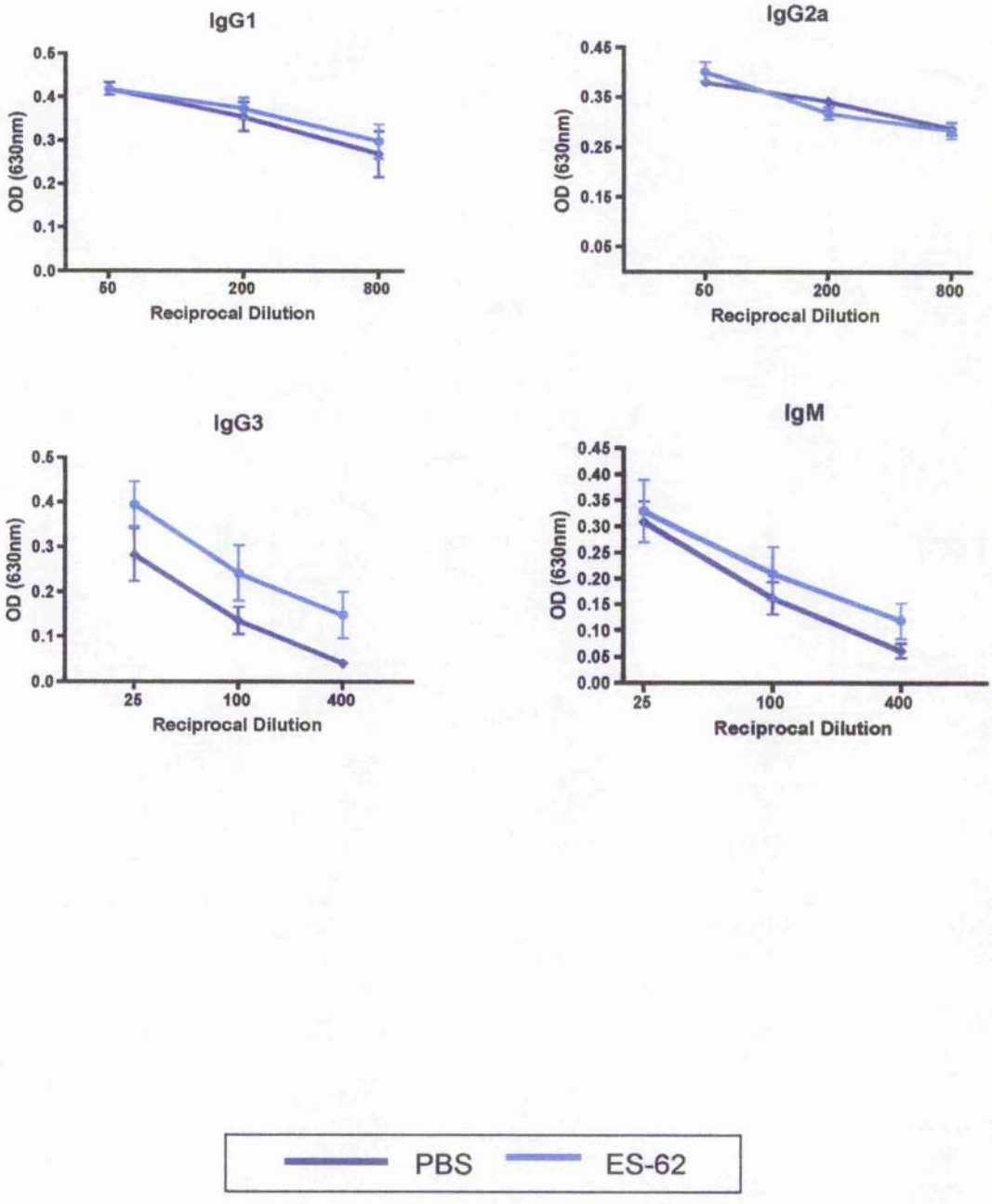

IgG1
OD (630nm)
0.6
0.4
0.3
0.2
0.1
0.0
50
200
800
Reciprocal Dilution
IgG2a
OD (630nm)
0.45
0.35
0.25
0.15
0.05
50
200
800
Reciprocal Dilution
IgG3
OD (630nm)
0.5
0.4
0.3
0.2
0.1
0.0
25
100
400
Reciprocal Dilution
IgM
OD (630nm)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
25
100
400
Reciprocal Dilution
PBS
ES-62

Figure 3.7 Prophylactic treatment with recombinant ES-62 does not inhibit onset or reduce severity of collagen-induced arthritis

A and **B**, DBA/1 mice were immunised on days 0 and 21 with collagen, and were treated with ES-62 (n = 9), recombinant ES-62 (n=9) or PBS (n = 9) on days –2, 0 and 21. Clinical score and incidence of arthritis were monitored daily. Data are presented from day 16 to day 28, expressed as mean ± SEM and are representative of at least 2 independent experiments. For mean articular index (*A*), ***, $p < 0.001$ rES-62 compared to PBS. For incidence of arthritis (B) **, $P<0.01$ rES-62 compared to ES-62 (Student's t-test).

C-F Serum samples were obtained from mice in each treatment group on day 28 and diluted accordingly with FCS. Collagen-specific IgG1 (C), IgG2a (D), IgG3 (E) and IgM (F) levels in serum samples from PBS, ES-62 and rES-62-treated mice were measured by ELISA. Data are individual measurements (n = 9 / group), expressed as mean concentration (pg/ml) ± SEM and are representative of 2 experiments. *, $p< 0.05$ rES-62 versus PBS (Student's t-test).

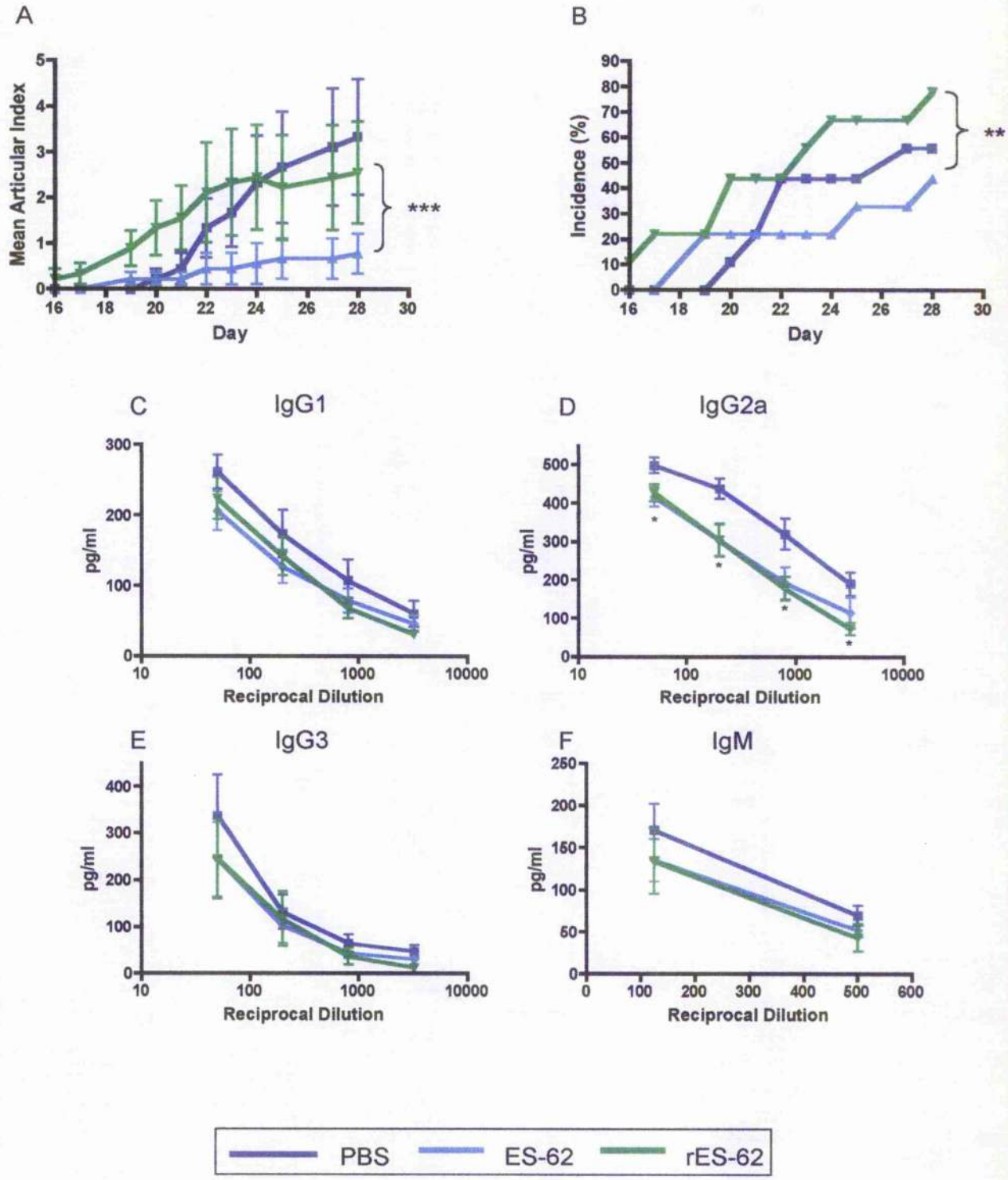

A
Mean Articular Index
Day
***
B
Incidence (%)
Day
**
C
IgG1
pg/ml
Reciprocal Dilution
D
IgG2a
pg/ml
Reciprocal Dilution
E
IgG3
pg/ml
Reciprocal Dilution
F
IgM
pg/ml
Reciprocal Dilution
PBS
ES-62
rES-62

Figure 3.8 Recombinant ES-62 treatment does not inhibit the severity of established collagen-induced arthritis

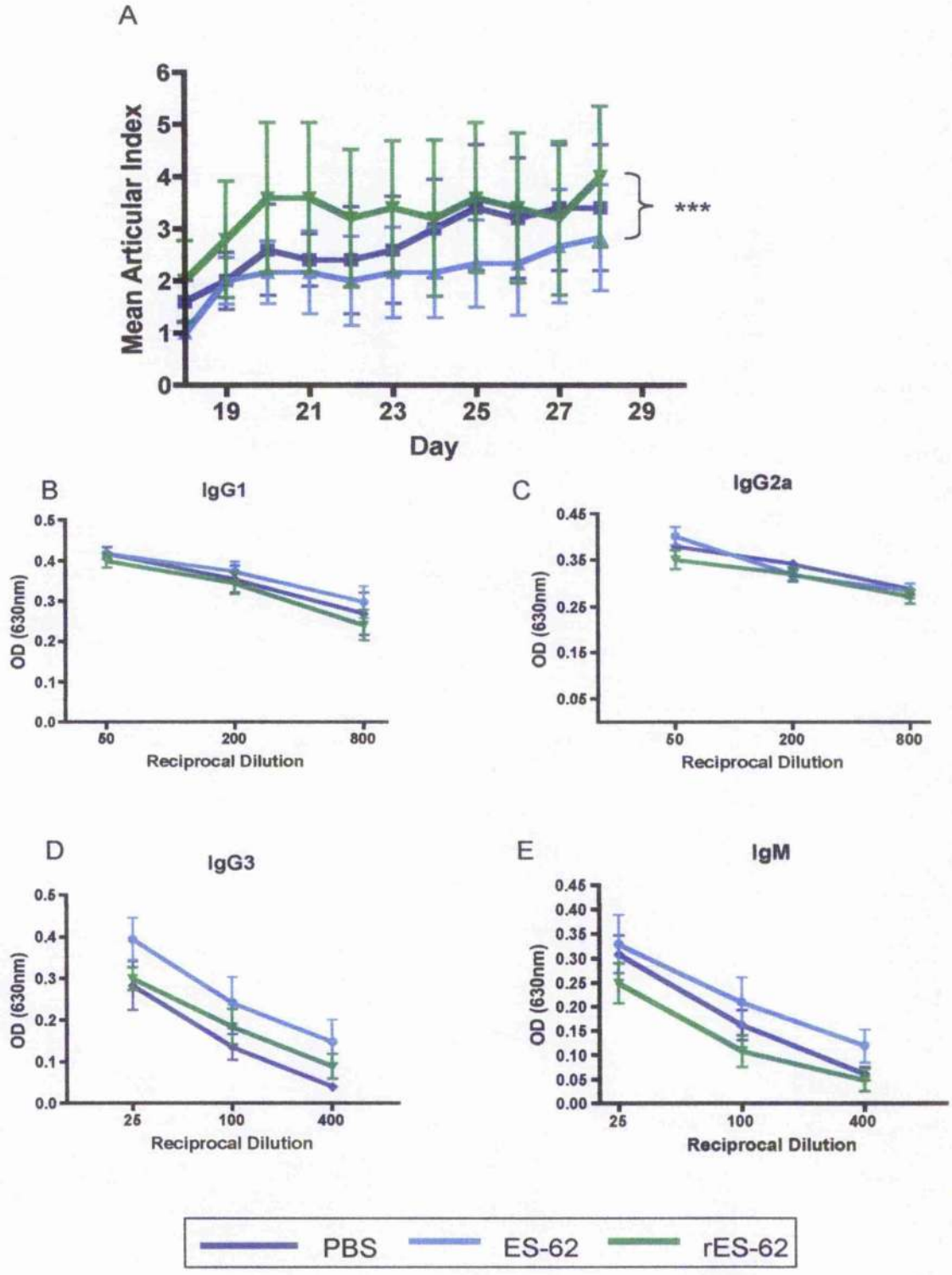
A, DBA/1 mice were immunised with collagen and arthritic animals were treated daily one day after detectable clinical CIA with rES-62 (n=5), ES-62 (n = 6) or PBS (n = 5). Mean articular index was monitored daily and data are presented from day 18 to day 28, expressed as mean ± SEM. Data are representative of 2 independent experiments. ***, $p < 0.001$, rES-62 compared to ES-62 (Student's t-test).

B-E Serum samples were obtained from mice in each treatment group on day 28 and diluted accordingly with FCS. Collagen-specific IgG1 (B), IgG2a (C), IgG3 (D) and IgM (E) levels in serum samples from PBS, ES-62 and rES-62 treated mice were measured by ELISA. Data are individual measurements (n = 5 / group), expressed as mean absorbance (OD630nm) ± SEM and are representative of 2 independent experiments. No significant differences in Ig levels were observed between serum samples from PBS, ES-62 or rES-62 treated mice (Student's t-test).

Figure 3.9 OVA-PC treatment delays onset and reduces severity of collagen-induced arthritis

A, B DBA/1 mice were immunised on days 0 and 21 with collagen, and were treated with ES-62 (n = 9), PBS (n = 9), OVA-PC (n = 9) or OVA (n = 9) on days –2, 0 and 21. Clinical score and incidence of arthritis were monitored daily. Data are presented from day 16 to day 28 (ES-62 and PBS) or 30 (OVA and OVA-PC), expressed as mean ± SEM and are representative of at least 2 independent experiments.. For mean articular index (*A*) and incidence of arthritis (B) **, $P < 0.01$ OVA compared to OVA-PC (Student's t-test).

C-F Serum samples were obtained from mice in each treatment group at sacrifice and diluted accordingly with FCS. Collagen-specific IgG1 (C), IgG2a (D), IgG3 (E) and IgM (F) levels in serum samples from PBS, OVA and OVA-PC treated mice were measured by ELISA. Data are individual measurements (n = 9 / group) and expressed as mean concentration (pg/ml) ± SEM. Data are representative of 2 independent experiments. *, $p<0.05$ OVA vs OVA-PC treated mice (Student's t-test).


A
Mean Articular Index
0
1
2
3
4
5
6
19
21
23
25
27
29
Day
***
B
IgG1
OD (630nm)
0.0
0.1
0.2
0.3
0.4
0.5
50
200
800
Reciprocal Dilution
C
IgG2a
OD (630nm)
0.05
0.15
0.25
0.35
0.45
50
200
800
Reciprocal Dilution
D
IgG3
OD (630nm)
0.0
0.1
0.2
0.3
0.4
0.5
25
100
400
Reciprocal Dilution
E
IgM
OD (630nm)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
25
100
400
Reciprocal Dilution
PBS
ES-62
rES-62

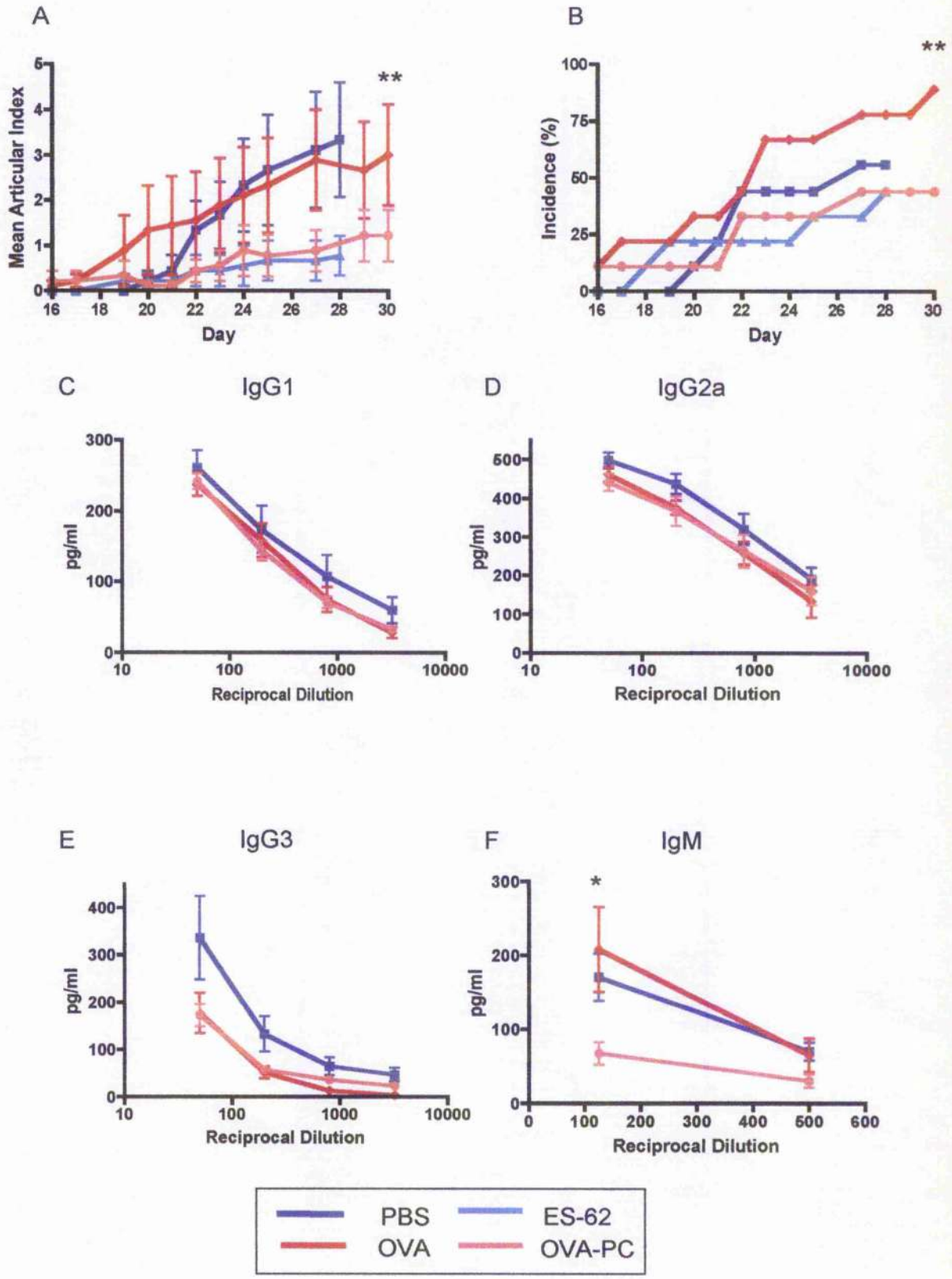

A
Mean Articular Index
Day
**
B
Incidence (%)
Day
**
C
IgG1
pg/ml
Reciprocal Dilution
D
IgG2a
pg/ml
Reciprocal Dilution
E
IgG3
pg/ml
Reciprocal Dilution
F
IgM
pg/ml
Reciprocal Dilution
*
PBS
OVA
ES-62
OVA-PC

Figure 3.10 OVA-PC treatment inhibits the severity of established collagen-induced arthritis

A, DBA/1 mice were immunised with collagen and arthritic animals were treated daily one day after detectable clinical CIA with OVA-PC (n=5) or OVA (n = 6). Mean articular index was monitored daily and data are presented from day 18 to day 28, expressed as mean ± SEM. Data are representative of 2 independent experiments. ***, $P < 0.001$ (OVA) compared to OVA-PC (Student's t-test).

B-E Serum samples were obtained from mice in each treatment group at sacrifice and diluted accordingly with PBS. Collagen-specific IgG1 (B), IgG2a (C), IgG3 (D) and IgM (E) levels in serum samples from OVA and OVA-PC treated mice were measured by ELISA. Data are individual measurements (n = 5 / group) and expressed as mean absorbance (OD630nm) ± SEM. Data are representative of 2 independent experiments. No significant differences were observed between serum samples from OVA and OVA-PC treated mice (Student's t-test).

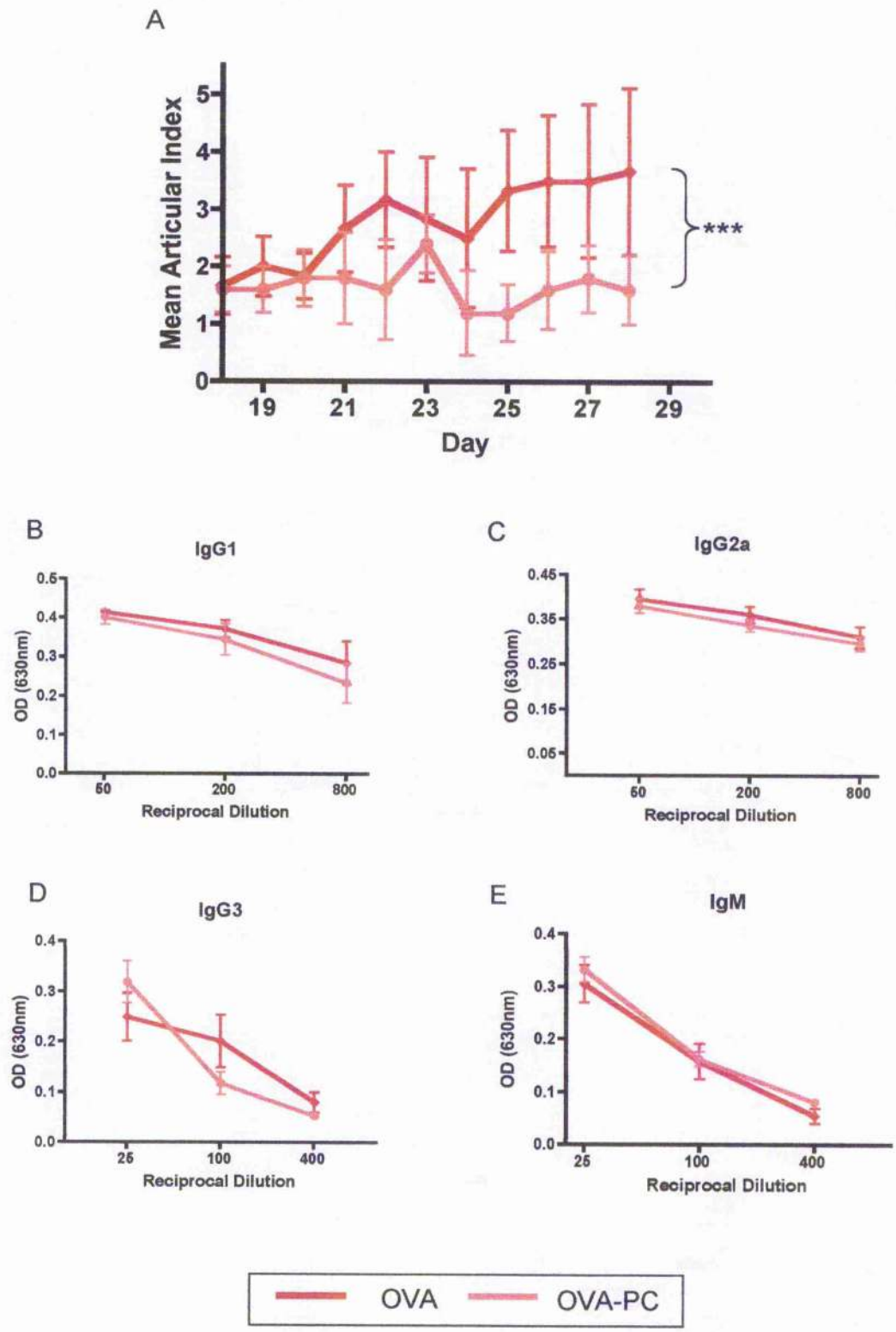
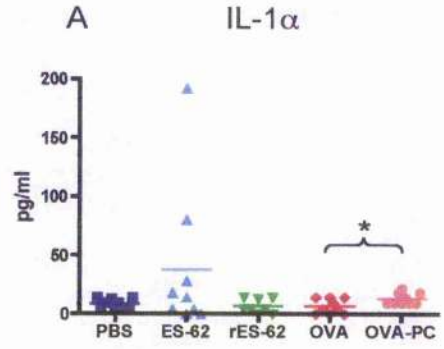
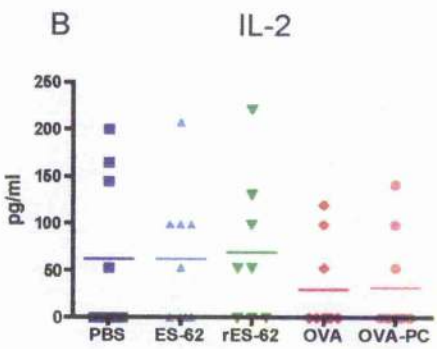
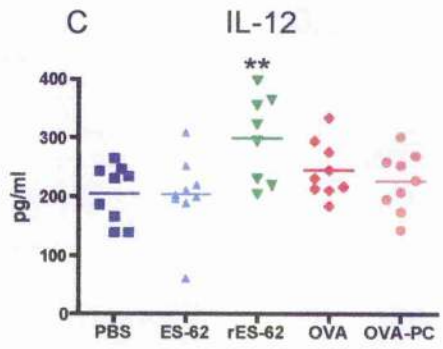

A
Mean Articular Index
0
1
2
3
4
5
19
21
23
25
27
29
Day
***
B
IgG1
OD (630nm)
0.0
0.1
0.2
0.3
0.4
0.6
50
200
800
Reciprocal Dilution
C
IgG2a
OD (630nm)
0.05
0.15
0.25
0.35
0.45
50
200
800
Reciprocal Dilution
D
IgG3
OD (630nm)
0.0
0.1
0.2
0.3
0.4
25
100
400
Reciprocal Dilution
E
IgM
OD (630nm)
0.0
0.1
0.2
0.3
0.4
25
100
400
Reciprocal Dilution
OVA
OVA-PC

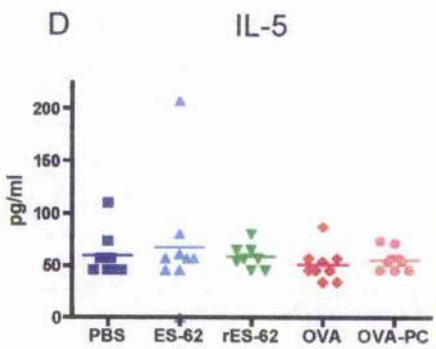
Figure 3.11 Comparison of serum cytokines in CIA model mice

Serum samples were obtained from mice in each treatment group at sacrifice and analysed, using the Luminex system, to detect IL-1α (A), IL-2 (B), IL-12 (C), IL-5 (D) and IL-10 (E). Data are plotted as the mean of duplicate serum samples from individual PBS, ES-62, rES-62, OVA and OVA-PC (n=9/group) treated mice; the bar represents the group mean value. Data are representative of 2 independent experiments. **, $p<0.05$ (rES-62 versus ES-62 treatment group) and *, $p<0.05$ (OVA versus OVA-PC treatment group) (Students t-test)


A
IL-1α
pg/ml
200
150
100
50
0
*
PBS
ES-62
rES-62
OVA
OVA-PC


B
IL-2
pg/ml
250
200
150
100
50
0
PBS
ES-62
rES-62
OVA
OVA-PC


C
IL-12
pg/ml
400
300
200
100
0
**
PBS
ES-62
rES-62
OVA
OVA-PC


D
IL-5
pg/ml
200
150
100
50
0
PBS
ES-62
rES-62
OVA
OVA-PC

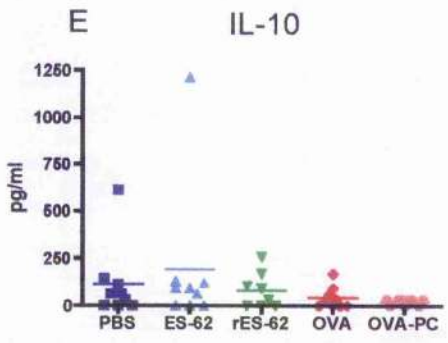

E
IL-10
pg/ml
1250
1000
750
500
250
0
PBS
ES-62
rES-62
OVA
OVA-PC

Figure 3.12 Comparison of serum chemokines and growth factors in CIA model mice

Serum samples were obtained from mice in each treatment group at sacrifice and analysed, using the Luminex system, to detect MIP-1α (A), KC (B), IP-10 (C), MIG (D) and FGF (E). Data are plotted as the mean of duplicate serum samples from individual PBS, ES-62, rES-62, OVA and OVA-PC (n=9/group) treated mice; the bar represents the group mean value. Data are representative of 2 independent experiments. There were no significant differences between mean concentrations of serum chemokines detected in samples from any of the treatment groups (Students t-test).

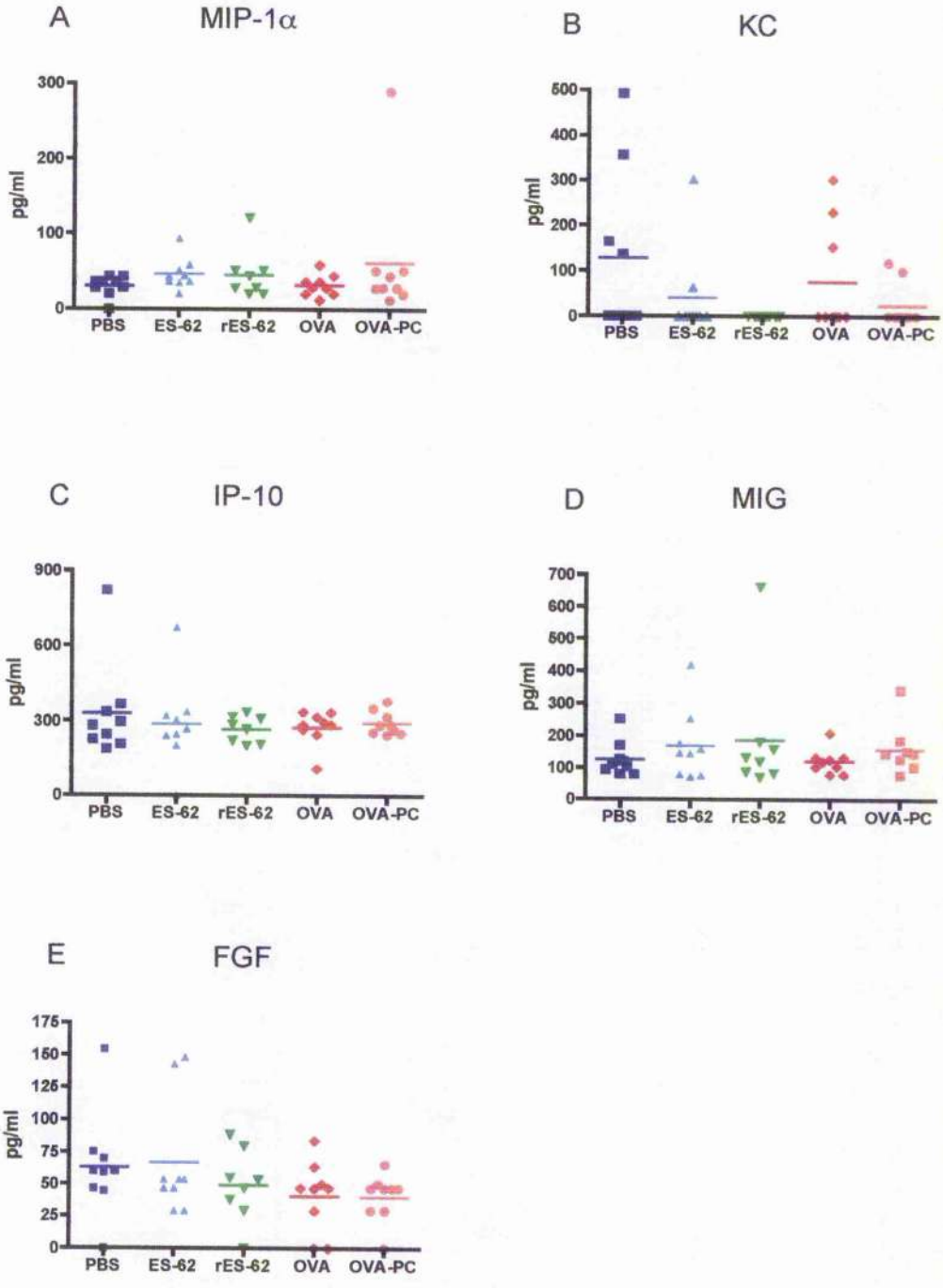

A
MIP-1α
pg/ml
300
200
100
0
PBS
ES-62
rES-62
OVA
OVA-PC
B
KC
pg/ml
500
400
300
200
100
0
PBS
ES-62
rES-62
OVA
OVA-PC
C
IP-10
pg/ml
900
600
300
0
PBS
ES-62
rES-62
OVA
OVA-PC
D
MIG
pg/ml
700
600
500
400
300
200
100
0
PBS
ES-62
rES-62
OVA
OVA-PC
E
FGF
pg/ml
175
150
125
100
75
50
25
0
PBS
ES-62
rES-62
OVA
OVA-PC

Figure 3.13 Comparison of serum cytokines in established CIA

Serum samples were obtained from mice in each treatment group at sacrifice and analysed, using the Luminex system, to detect IL-2 (A) and IL-12 (B). Data are plotted as the mean of duplicate serum samples from individual PBS (n=5), ES-62 (n=6), rES-62 (n=5), OVA (n=5) and OVA-PC (n=3) treated mice; the bar represents the group mean value. Data are representative of 2 independent experiments. There were no significant differences between mean concentrations of serum cytokines detected in samples from any of the treatment groups (Students t-test).

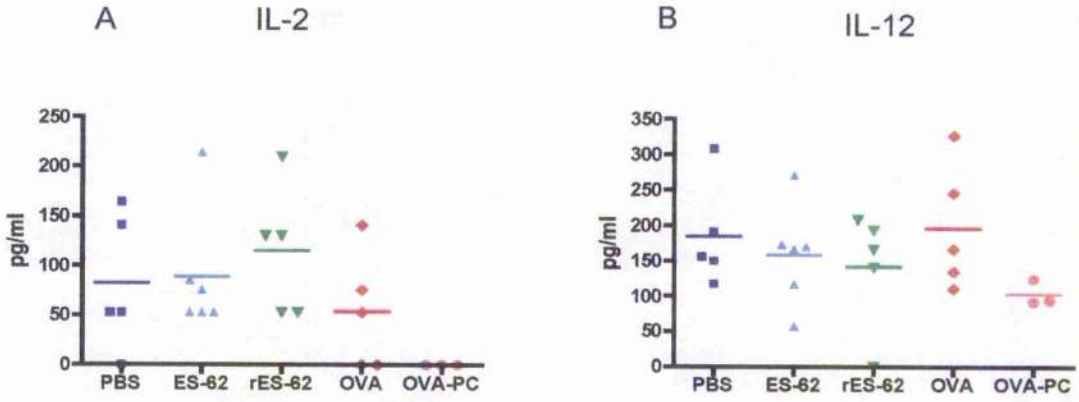

A
IL-2
pg/ml
250
200
150
100
50
0
PBS
ES-62
rES-62
OVA
OVA-PC
B
IL-12
pg/ml
350
300
250
200
150
100
50
0
PBS
ES-62
rES-62
OVA
OVA-PC

Figure 3.14 Comparison of serum chemokines in established CIA

Serum samples were obtained from mice in each treatment group at sacrifice and analysed, using the Luminex system, to detect IP-10 (A), MIG (B) and MIP-1α (C). Data are plotted as the mean of duplicate serum samples from individual PBS (n=5), ES-62 (n=6), rES-62 (n=5), OVA (n=5) and OVA-PC (n=3) treated mice; the bar represents the group mean value. Data are representative of 2 independent experiments. There were no significant differences between mean concentrations of serum chemokines detected in samples from any of the treatment groups (Students t-test).

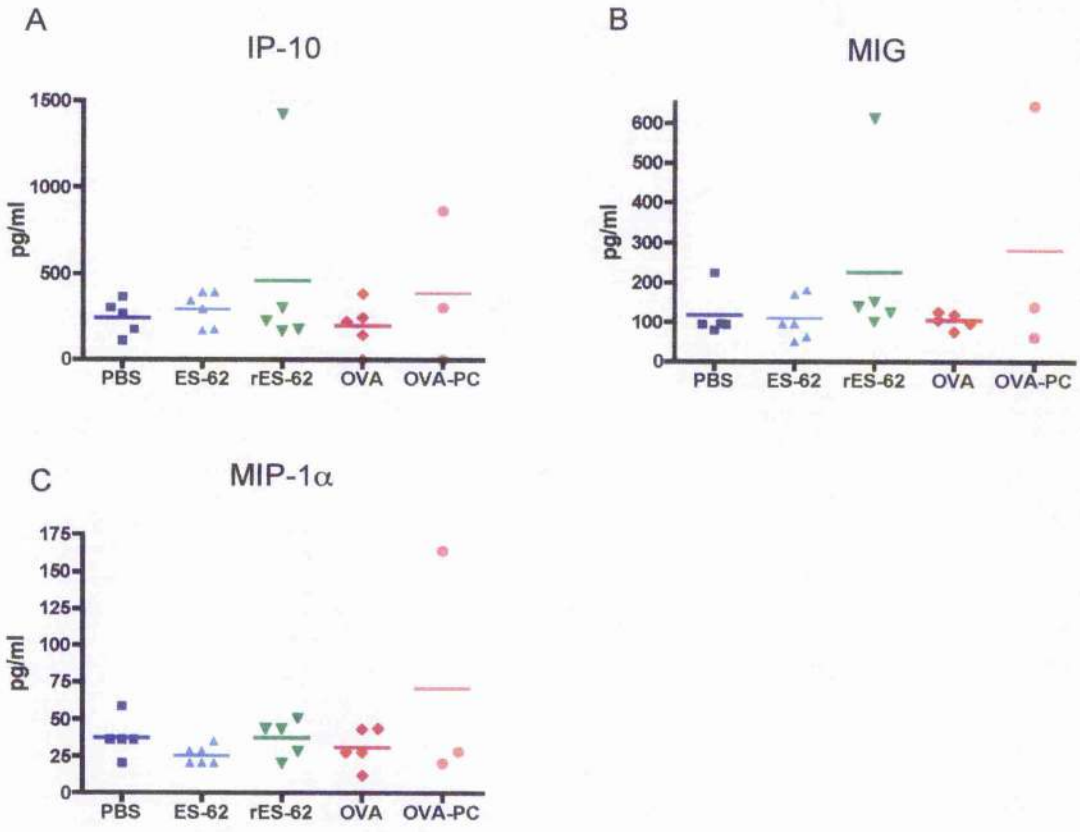

A
IP-10
pg/ml
1500
1000
500
0
PBS
ES-62
rES-62
OVA
OVA-PC
B
MIG
pg/ml
600
500
400
300
200
100
0
PBS
ES-62
rES-62
OVA
OVA-PC
C
MIP-1α
pg/ml
175
150
125
100
75
50
25
0
PBS
ES-62
rES-62
OVA
OVA-PC

Figure 3.15 Comparison of serum growth factors in established CIA

Serum samples were obtained from mice in each treatment group at sacrifice and analysed, using the Luminex system, to detect growth factors FGF (A) and VEGF (B). Data are plotted as the mean of duplicate serum samples from individual PBS (n=5), ES-62 (n=6), rES-62 (n=5) OVA (n=5) and OVA-PC (n=3) treated mice; the bar represents the group mean value. Data are representative of 2 independent experiments. *, $p<0.05$ PBS vs rES-62 treated mice (Students t-test).

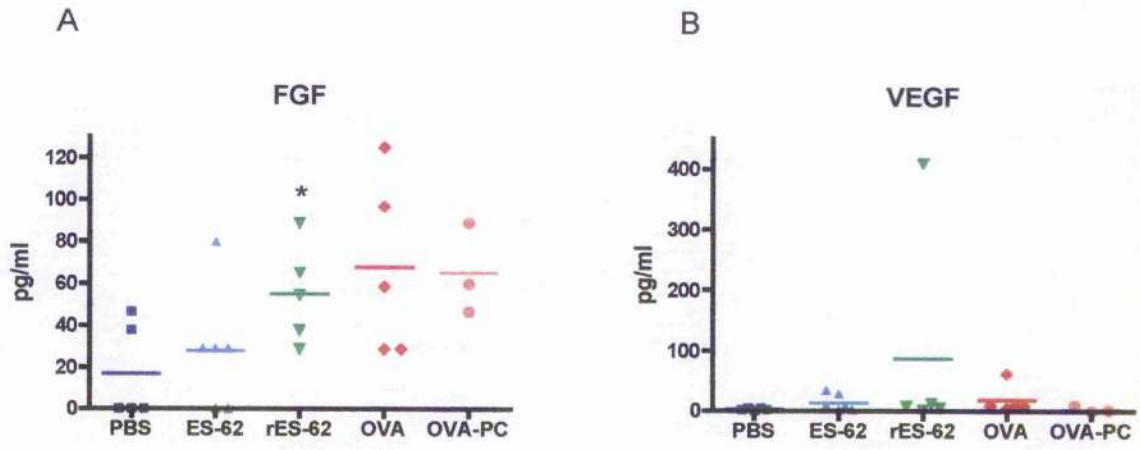

A
FGF
pg/ml
120
100
80
60
40
20
0
*
PBS
ES-62
rES-62
OVA
OVA-PC
B
VEGF
pg/ml
400
300
200
100
0
PBS
ES-62
rES-62
OVA
OVA-PC

Figure 3.16 Effects of ES-62 pre-exposure on LPS-induced bone marrow-derived DC pro-inflammatory cytokine production

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 days. These bmDC were then either stimulated with GM-CSF alone or with ES-62 (2 μg/ml) for 24 hours on day 6 and subsequently in the absence or presence of LPS (1 μg/ml) for an additional 24 hours on day 7. On day 8, IL-12p40 (A) and TNF-α (B) in culture supernatants were measured by ELISA. Data are presented as means ± SD and are representative of over 5 independent experiments. *** $p<0.005$ ES-62 compared to none, ES-62+LPS compared to LPS.

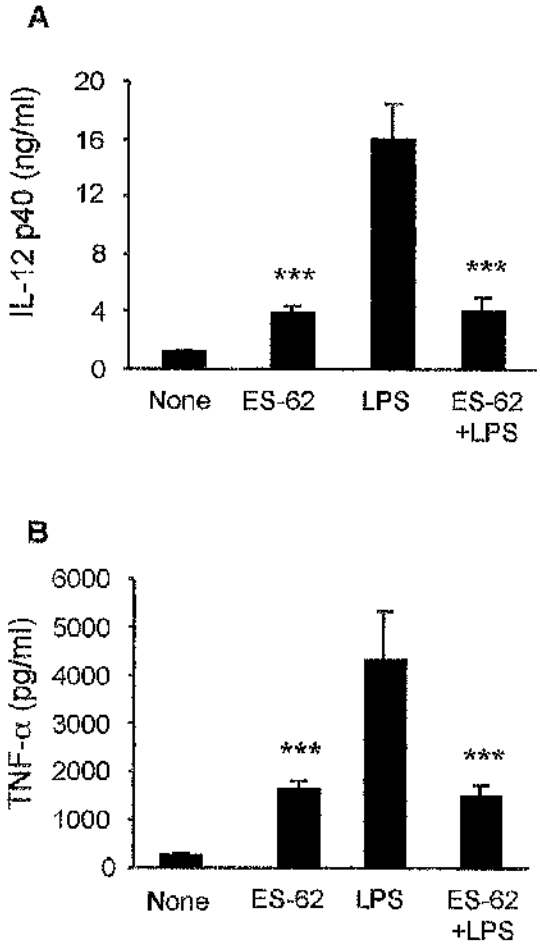

A
IL-12 p40 (ng/ml)
20
16
12
8
4
0
***
***
None
ES-62
LPS
ES-62 +LPS
B
TNF-α (pg/ml)
6000
5000
4000
3000
2000
1000
0
***
***
None
ES-62
LPS
ES-62 +LPS

Figure 3.17 Effects of ES-62 and/or LPS on the costimulatory molecule expression by murine bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 days. These bmDC were then either stimulated with GM-CSF or ES-62 (2 μg/ml) for 24 hours on day 6 and subsequently in the absence or presence of LPS (1 μg/ml) for an additional 24 hours on day 7. On day 8, cells were harvested and cell surface expression of costimulatory molecule expression was analysed by flow cytometry as described in Materials and Methods. Data are presented as dotplots and the % of cells contained within each quadrant is located in the corners of the dotplots. Additionally, the mean fluorescent intensity (MFI) (in red) of the double positive population in shown in the upper right quadrant. The data presented are from a single experiment representative of at least 5 other independent experiments.

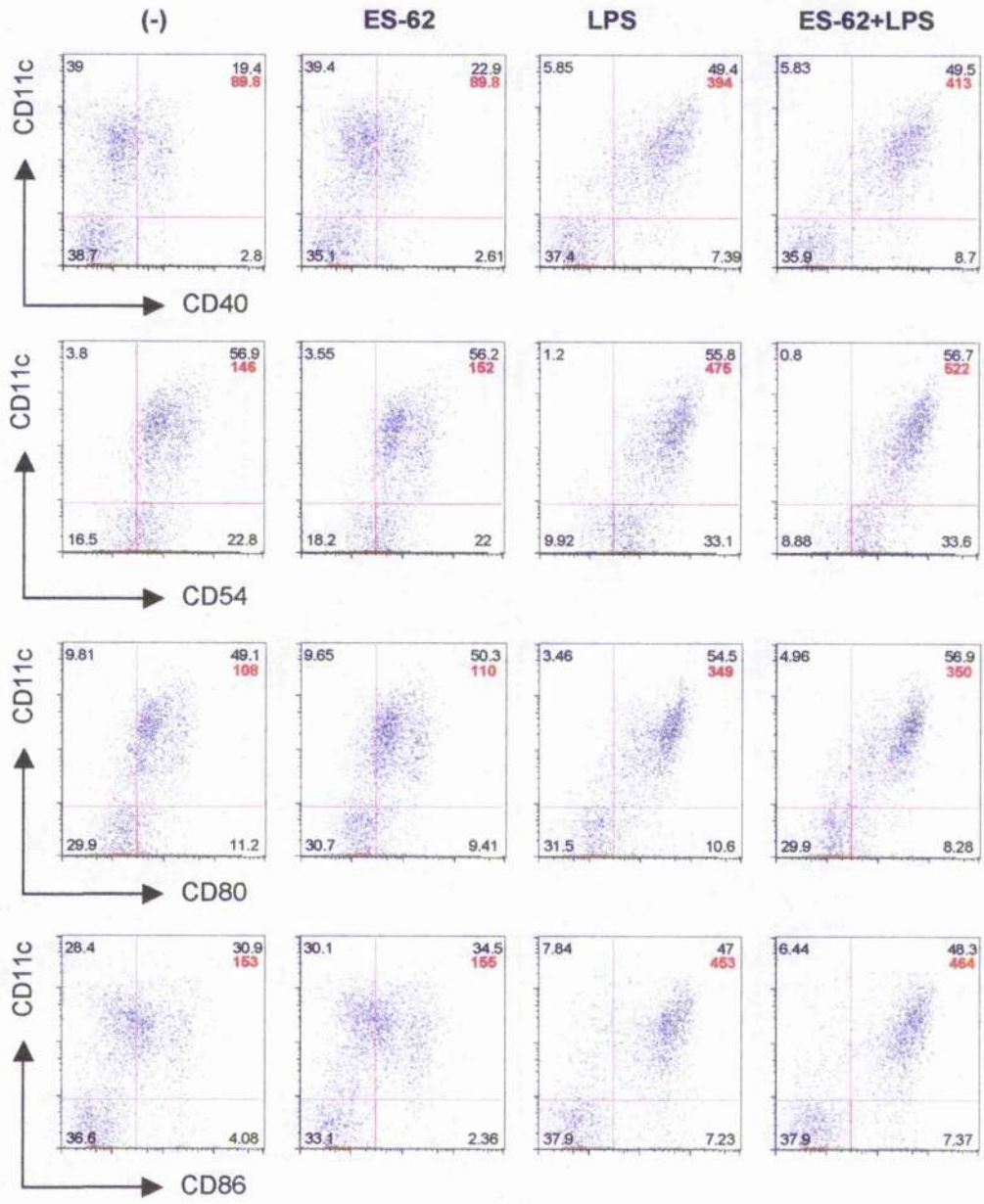

(-)
ES-62
LPS
ES-62+LPS
CD11c
CD40
39
19.4
89.8
38.7
2.8
39.4
22.9
89.8
35.1
2.61
5.85
49.4
394
37.4
7.39
5.83
49.5
413
35.9
8.7
CD11c
CD54
3.8
56.9
146
16.5
22.8
3.55
56.2
152
18.2
22
1.2
55.8
475
9.92
33.1
0.8
56.7
522
8.88
33.6
CD11c
CD80
9.81
49.1
108
29.9
11.2
9.65
50.3
110
30.7
9.41
3.46
54.5
349
31.5
10.6
4.96
56.9
350
29.9
8.28
CD11c
CD86
28.4
30.9
153
36.6
4.08
30.1
34.5
155
33.1
2.36
7.84
47
453
37.9
7.23
6.44
48.3
464
37.9
7.37

Figure 3.18 Bone marrow-derived DC from CIA mice exhibit a modulated phenotype

DBA/1 CIA model mice were treated and sacrificed as described in the legend to Figure 3.2. Femoral bone marrow from CIA model mice and naïve mice was removed and pooled by treatment group. Bone marrow-derived DC were cultured *in vitro* for 7 days and stimulated with media (panel **A**) or LPS (1μg/ml; panel **B**) for 24h. Expression of MHCII, CD40, CD54, CD80 and CD86 on DC was analysed by flow cytometry. Data were gated on the $CD11c^+$ population and expressed as histograms depicting the expression level of the surface marker versus the number of cells, as a percentage of the maximum $CD11c^+$ cell number. Results depicted are from one experiment representative of two independent experiments.

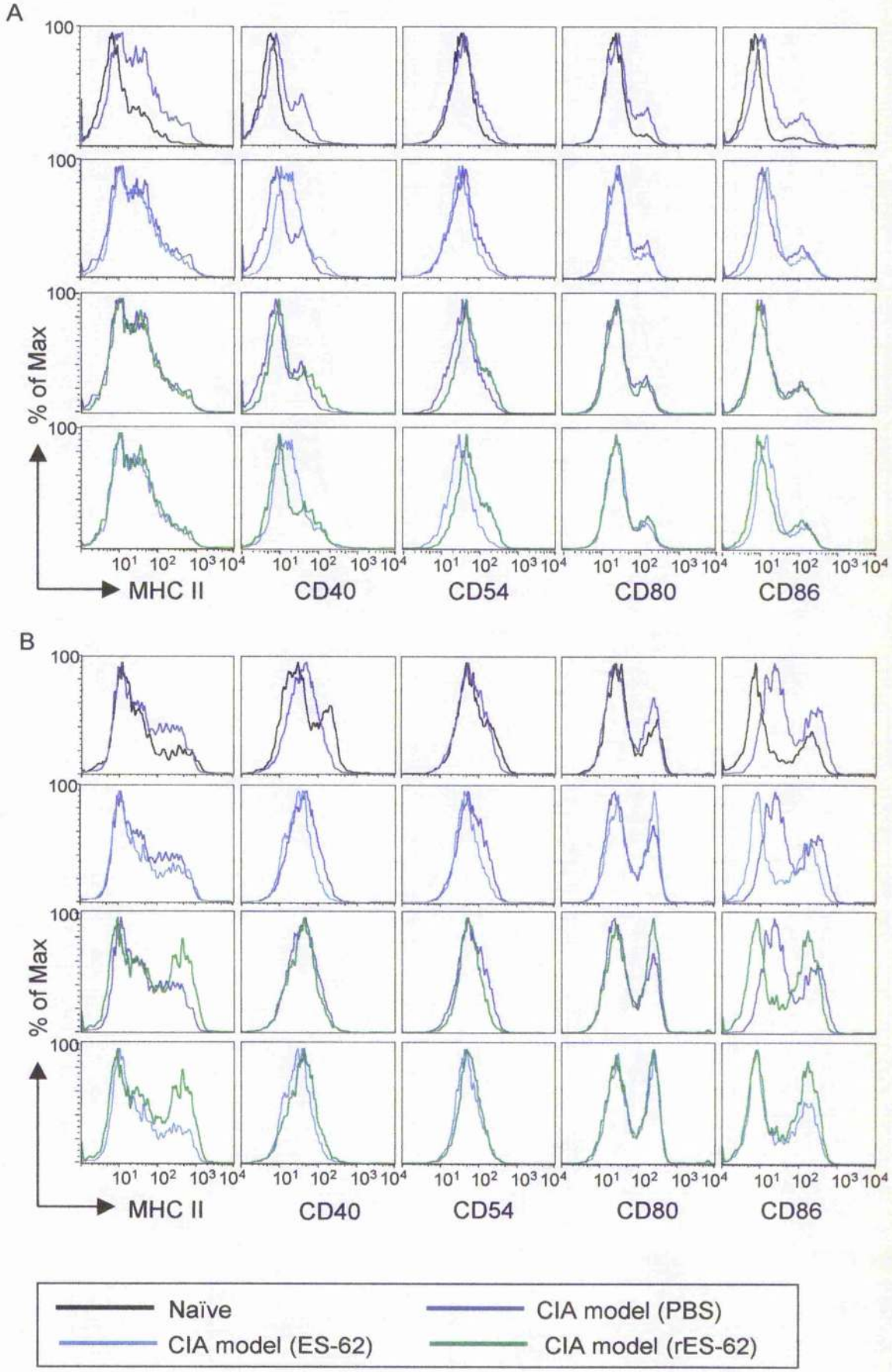

A
B
100
% of Max
MHC II
CD40
CD54
CD80
CD86
10^1 10^2 10^3 10^4
Naïve
CIA model (PBS)
CIA model (ES-62)
CIA model (rES-62)

Figure 3.19 ES-62 treatment of CIA model mice modulates cytokine production by bone marrow derived DC

DBA/1 CIA model mice were treated and sacrificed as described in the legend to Figure 3.2. Femoral bone marrow from CIA model mice and naïve DBA/1 mice was removed and pooled by treatment group. Bone marrow-derived DC were cultured *in vitro* for 7 days and stimulated with media (panel **A**) or LPS (1μg/ml; panel **B**) for 24h. Culture supernatants were analysed for IL-12, TNFα, and IL-10 by ELISA. ELISA data are expressed as mean concentration (pg/ml) ± SD (n=3) and are representative of at least 2 independent experiments. ***, p<0.001; **, p<0.01 and *, p<0.05 vs DC from naïve mice; +, p<0.05, ++, p<0.01, +++, p<0.001 vs PBS DC by Student's t-test.

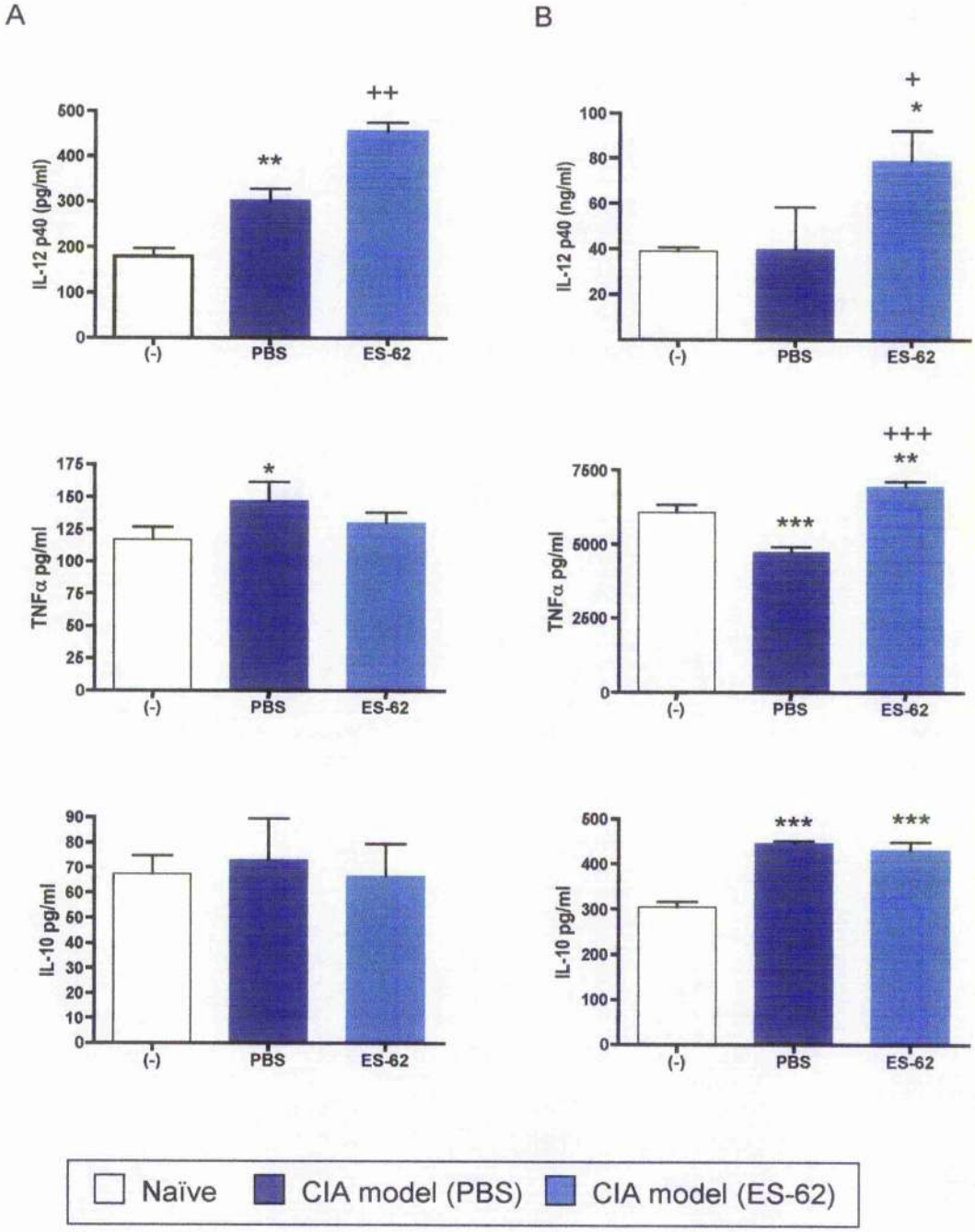

A
B
IL-12 p40 (pg/ml)
IL-12 p40 (ng/ml)
TNFα pg/ml
IL-10 pg/ml
(-)
PBS
ES-62
Naïve
CIA model (PBS)
CIA model (ES-62)

Figure 3.20 Recombinant ES-62 treatment of CIA model mice modulates cytokine production by bone marrow derived DC

DBA/1 CIA model mice were treated and sacrificed as described in the legend to Figure 3.2. Femoral bone marrow from CIA model mice and naïve DBA/1 mice was removed and pooled by treatment group. Bone marrow-derived DC were cultured *in vitro* for 7 days and stimulated with media (panel **A**) or LPS (1μg/ml; panel **B**) for 24h. Culture supernatants were analysed for IL-12, TNFα, and IL-10 by ELISA. Naïve mouse, PBS and ES-62 treatment group data from Figure 3.19 are re-plotted in this figure in addition to data from rES-62 treatment group and are expressed as mean ± SD (n=3) and are representative of at least 2 independent experiments. *, $p<0.05$ versus ES-62 DC and +, $p<0.05$, ++, $p<0.01$, +++, $p<0.001$ versus PBS DC by student's t-test.

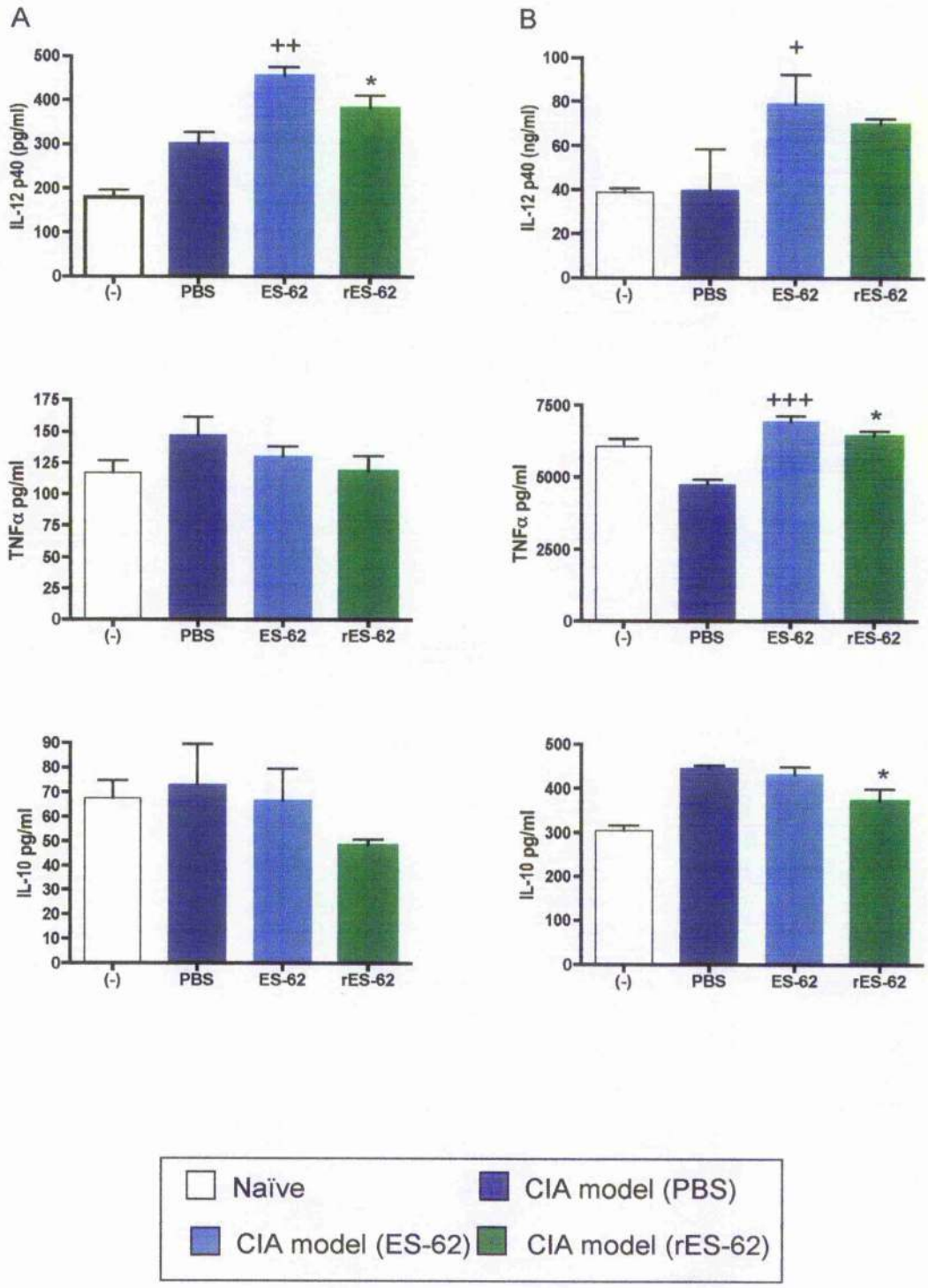

A
B
IL-12 p40 (pg/ml)
IL-12 p40 (ng/ml)
TNFα pg/ml
TNFα pg/ml
IL-10 pg/ml
IL-10 pg/ml
(-)
PBS
ES-62
rES-62
++
*
+
+++
Naïve
CIA model (PBS)
CIA model (ES-62)
CIA model (rES-62)

Figure 3.21 The effect of OVA-PC treatment of CIA model mice on bone marrow-derived DC phenotype

DBA/1 CIA model mice were treated and sacrificed as described in the legend to Figure 3.2. Femoral bone marrow from CIA model mice and naïve mice was removed and pooled by treatment group. Bone marrow-derived DC were cultured in vitro for 7 days and stimulated with media (panel **A**) or LPS (1μg/ml; panel **B**) for 24h. Expression of MHCII, CD40, CD54, CD80 and CD86 on DC was analysed by flow cytometry. Data were gated on the $CD11c^+$ population and expressed as histograms depicting the expression level of the surface marker versus the number of cells, as a percentage of the maximum $CD11c^+$ cell number. Results depicted are from one experiment representative of two independent experiments.

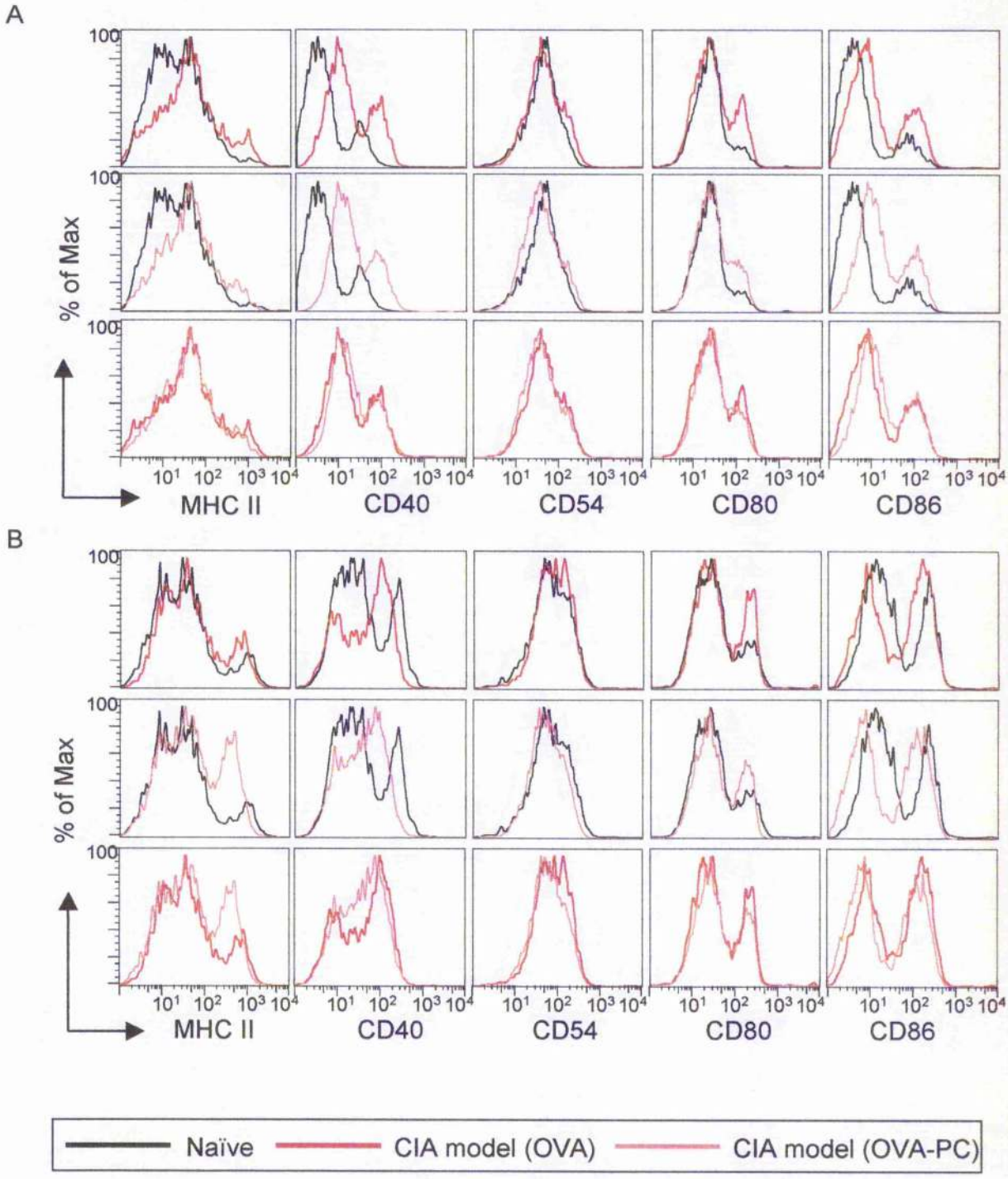

A
B
% of Max
100
10^1 10^2 10^3 10^4
MHC II
CD40
CD54
CD80
CD86
Naïve
CIA model (OVA)
CIA model (OVA-PC)

Figure 3.22 OVA-PC treatment of CIA model mice modulates cytokine production by bone marrow derived DC

DBA/1 CIA model mice were treated and sacrificed as described in the legend to Figure 3.2. Femoral bone marrow from CIA model mice and naïve DBA/1 mice was removed and pooled by treatment group. Bone marrow-derived DC were cultured *in vitro* for 7 days and stimulated with media (panel **A**) or LPS (1μg/ml; panel **B**) for 24h. Culture supernatants were analysed for IL-12, TNFα, and IL-10 by ELISA. Data are expressed as mean ± SD (*n*=3) and are representative of at least 2 independent experiments. ***, $p<0.001$ and **, $p<0.01$ versus OVA DC by student's t-test.

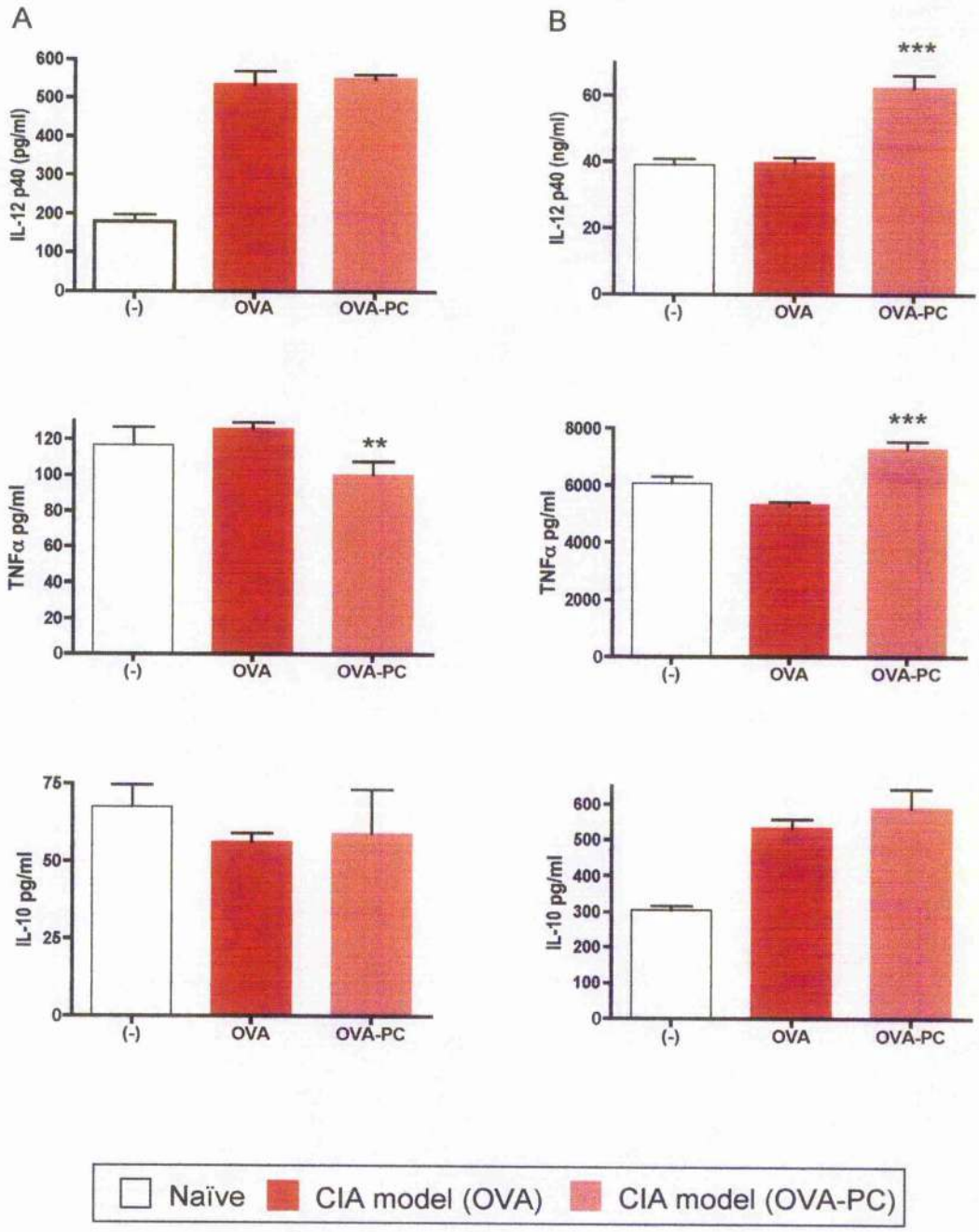

A
B
IL-12 p40 (pg/ml)
600
500
400
300
200
100
0
(-)
OVA
OVA-PC
IL-12 p40 (ng/ml)
60
40
20
0
***
TNFα pg/ml
120
100
80
60
40
20
0
**
8000
6000
4000
2000
0
***
IL-10 pg/ml
75
50
25
0
Naïve
CIA model (OVA)
CIA model (OVA-PC)

Figure 3.23 Analysis of the phenotype of bone marrow-DC derived from CIA model mice that have been treated therapeutically with ES-62, rES-62 or OVA-PC

DBA/1 CIA model mice were treated as described in the legend to Figure 3.4. Femoral bone marrow from CIA model mice was removed at sacrifice and pooled by treatment group. Bone marrow-derived DC were cultured in vitro for 7 days and stimulated with media (panels **A, C** & **E**) or LPS (1μg/ml; panels **B, D** & **F**) for 24h. Expression of MHCII, CD40, CD54, CD80 and CD86 on DC was analysed by flow cytometry. Data were gated on the $CD11c^+$ population and expressed as histograms depicting the expression level of the surface marker versus the number of cells, as a percentage of the maximum $CD11c^+$ cell number. Results depicted are from one experiment representative of two independent experiments.

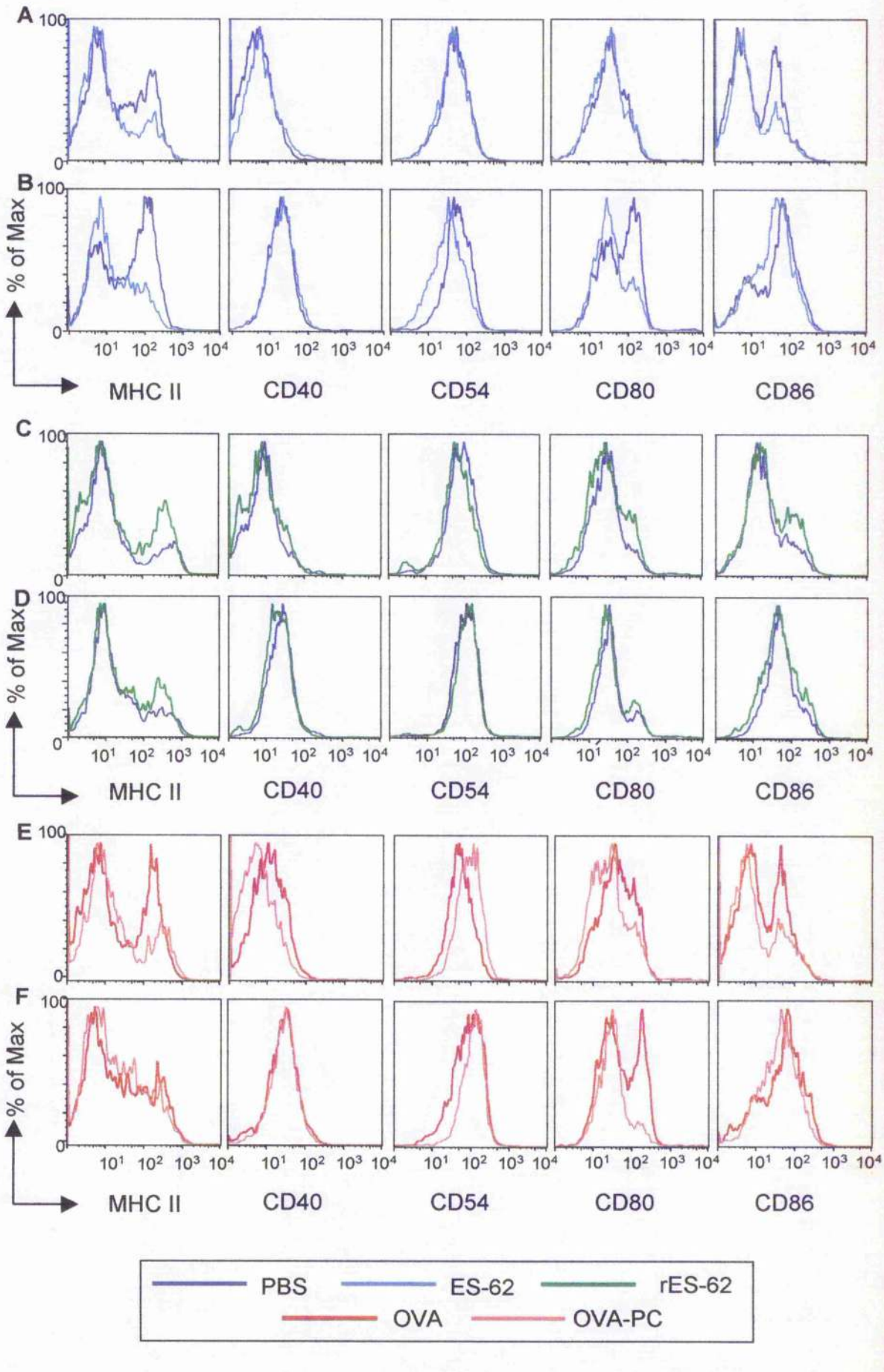

A
B
C
D
E
F
100
0
% of Max
10^1 10^2 10^3 10^4
MHC II
CD40
CD54
CD80
CD86
PBS
ES-62
rES-62
OVA
OVA-PC

Figure 3.24 Bone marrow-derived DC from CIA model mice that have been treated therapeutically with ES-62 exhibit modulated cytokine production

DBA/1 CIA model mice were treated as described in the legend to Figure 3.4 and femoral bone marrow was removed at sacrifice. Bone marrow-derived DC from each treatment group were cultured in vitro for 7 days and stimulated with media (panel **A**) or LPS (1μg/ml; panel **B**) for 24h. Culture supernatants were analysed for IL-12, TNFα, and IL-10 by ELISA. ELISA data are expressed as treatment group mean ± SD (n=3) and are representative of at least 2 independent experiments. ***, $p<0.001$ vs control by student's t-test.

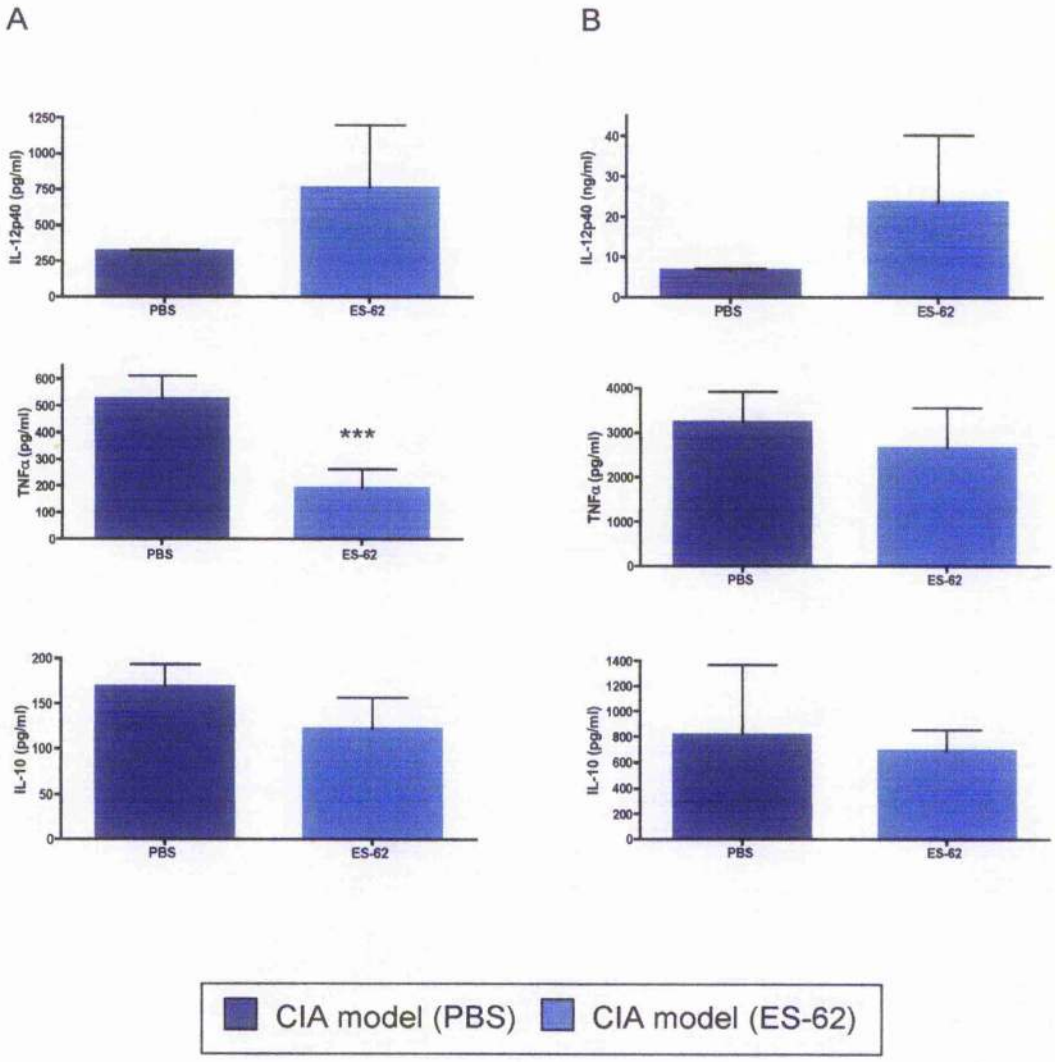

A
B
IL-12p40 (pg/ml)
1250
1000
750
500
250
0
PBS
ES-62
IL-12p40 (ng/ml)
40
30
20
10
0
PBS
ES-62
TNFα (pg/ml)
600
500
400
300
200
100
0
***
PBS
ES-62
TNFα (pg/ml)
4000
3000
2000
1000
0
PBS
ES-62
IL-10 (pg/ml)
200
150
100
50
0
PBS
ES-62
IL-10 (pg/ml)
1400
1200
1000
800
600
400
200
0
PBS
ES-62
CIA model (PBS)
CIA model (ES-62)

Figure 3.25 Cytokine production by bone marrow-derived DC from CIA model mice that have been treated therapeutically with recombinant ES-62

DBA/1 CIA model mice were treated as described in the legend to Figure 3.4 and femoral bone marrow was removed at sacrifice. Bone marrow-derived DC from each treatment group were cultured in vitro for 7 days and stimulated with media (panel **A**) or LPS (1μg/ml; panel **B**) for 24h. Culture supernatants were analysed for IL-12, TNFα, and IL-10 by ELISA. Data are expressed as treatment group mean ± SD (n=3) and are representative of at least 2 independent experiments. No statistical differences were observed in cytokine production levels between DC from control CIA model mice and DC from CIA model mice treated with recombinant ES-62.

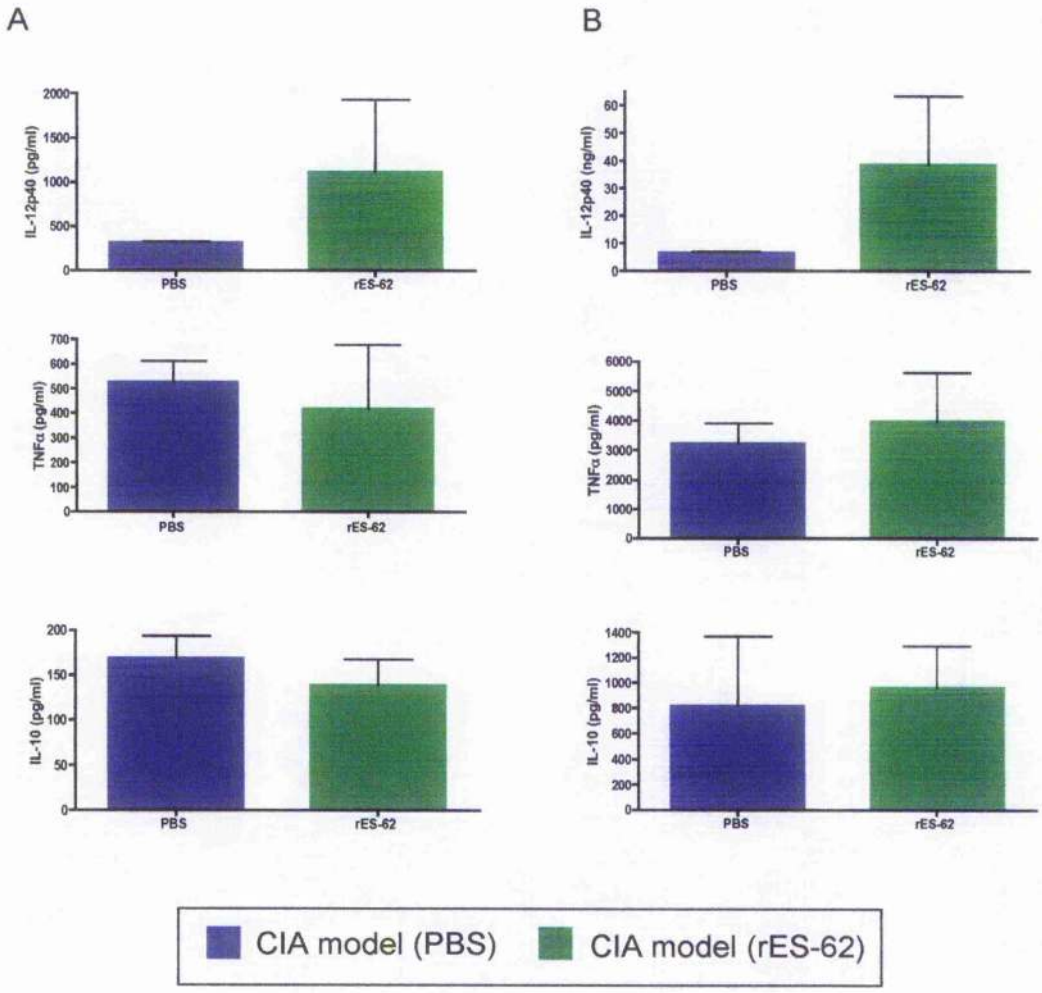

A
B
IL-12p40 (pg/ml)
2000
1500
1000
500
0
PBS
rES-62
IL-12p40 (ng/ml)
60
50
40
30
20
10
0
PBS
rES-62
TNFα (pg/ml)
700
600
500
400
300
200
100
0
PBS
rES-62
TNFα (pg/ml)
6000
5000
4000
3000
2000
1000
0
PBS
rES-62
IL-10 (pg/ml)
200
150
100
50
0
PBS
rES-62
IL-10 (pg/ml)
1400
1200
1000
800
600
400
200
0
PBS
rES-62
CIA model (PBS)
CIA model (rES-62)

Figure 3.26 Bone marrow-derived DC from CIA mice that have been treated therapeutically with OVA-PC exhibit a modulated phenotype

DBA/1 CIA model mice were treated as described in the legend to Figure 3.4 and femoral bone marrow was removed at sacrifice. Bone marrow-derived DC from each treatment group were cultured in vitro for 7 days and stimulated with media (panel **A**) or LPS (1μg/ml; panel **B**) for 24h. Culture supernatants were analysed for IL-12, TNFα, and IL-10 by ELISA. Data are expressed as treatment group mean ± SD and are representative of at least 2 independent experiments. ***, $p<0.001$; **, $p<0.01$ vs DC from OVA treated mice by student's t-test.

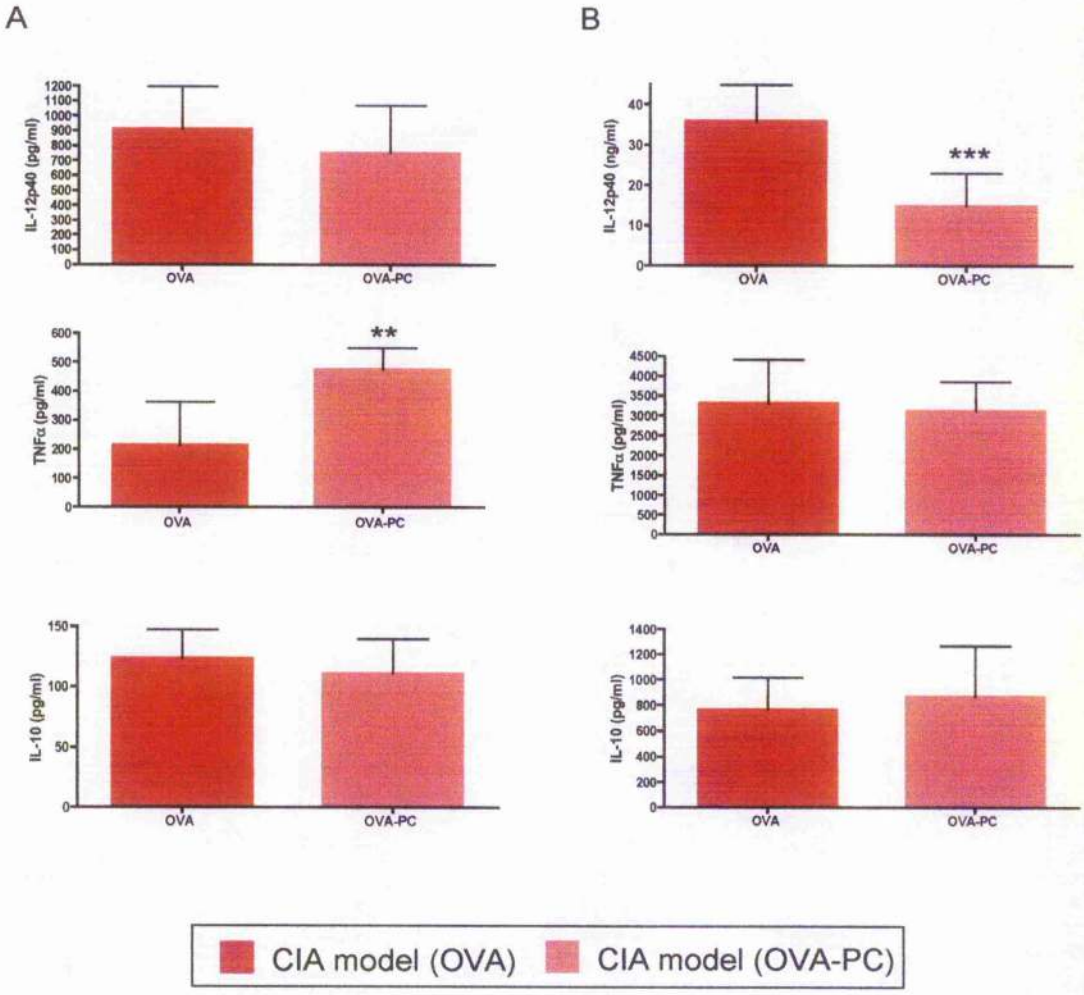

A
B
IL-12p40 (pg/ml)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
OVA
OVA-PC
TNFα (pg/ml)
600
500
400
300
200
100
0
**
OVA
OVA-PC
IL-10 (pg/ml)
150
100
50
0
OVA
OVA-PC
IL-12p40 (ng/ml)
40
30
20
10
0
***
OVA
OVA-PC
TNFα (pg/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
OVA
OVA-PC
IL-10 (pg/ml)
1400
1200
1000
800
600
400
200
0
OVA
OVA-PC
CIA model (OVA)
CIA model (OVA-PC)

Figure 3.27 Effects of ES-62 pre-exposure on LPS-induced bone marrow-derived macrophage pro-inflammatory cytokine production

Bone marrow-derived macrophages were grown from BALB/c mice *in vitro* in the presence of CSF-1 for 6 days. These bm-macrophages were then either cultured with CSF-1 alone or with ES-62 (2 μg/ml) for 24 hours on day 6 and subsequently in the absence or presence of LPS (1 μg/ml) for an additional 24 hours on day 7. On day 8, IL-12p40 (**A**) and TNF-α (**B**) were measured in culture supernatants, by ELISA. Data are presented as mean ± SD and are representative of over 5 independent experiments. * $p<0.05$, ** $p<0.01$.

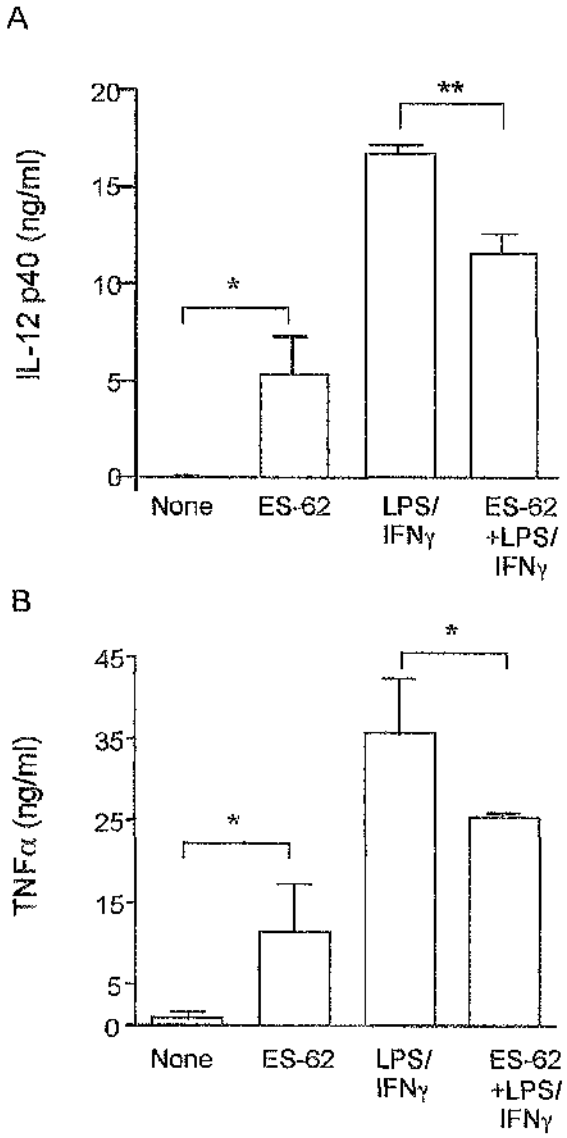
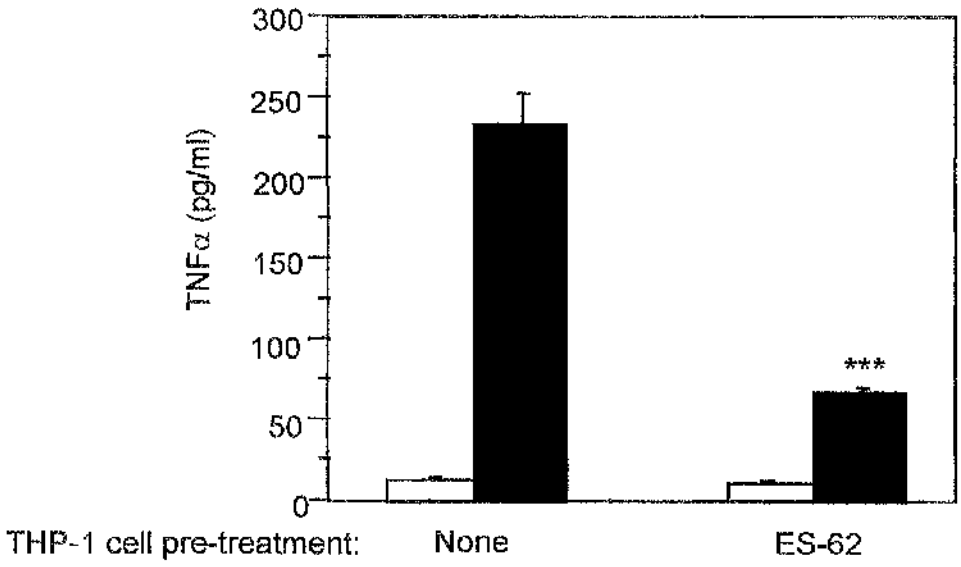

A
IL-12 p40 (ng/ml)
20
15
10
5
0
*
**
None
ES-62
LPS/ IFNγ
ES-62 +LPS/ IFNγ
B
TNFα (ng/ml)
45
35
25
15
5
0
*
*
None
ES-62
LPS/ IFNγ
ES-62 +LPS/ IFNγ

Figure 3.28 ES-62 inhibits TNFα production from human activated T cell-macrophage cell-contact cultures

Purified human peripheral blood T cells were cultured unstimulated (open bars), or activated by PHA and PMA (filled bars), then fixed in paraformaldehyde to preserve membrane structural integrity. THP-1 cells were pre-treated with media or ES-62 for 18h prior to co-culture with fixed T cells at a ratio of 1:8. Harvested culture supernatants were measured for TNFα release by ELISA. Results are depicted as mean ± SD (n=3) and are representative of at least 3 independent experiments. ***, $p<0.01$ vs untreated THP-1 cells, determined by student's t-test.


300
250
200
150
100
50
0
TNFα (pg/ml)
***
THP-1 cell pre-treatment:
None
ES-62


T cell pre-treatment:
None
PHA/PMA

Figure 3.29 T cell contact promotes macrophage pro-inflammatory cytokine production

Bone marrow-derived macrophages were grown *in vitro* from bone marrow of BALB/c mice in the presence of CSF-1 for 7 days. On day 4 of macrophage culture, lymph node T cells from BALB/c mice were cultured *in vitro* and activated with PMA (10 ng/ml) and Con A (5 μg/ml) for 3 days. On day 7, T cells were fixed by incubating in formaldehyde, before co-culture in fresh media with the bm-macrophages for 24 hours. No cytokine release was detected following culture of fixed T cells alone. Culture supernatants were analysed for TNFα (A) and IL-12 p40 (B) by ELISA. Data are expressed as mean ± SD (n=3) and are representative of 3 independent experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ vs macrophages alone (ANOVA)

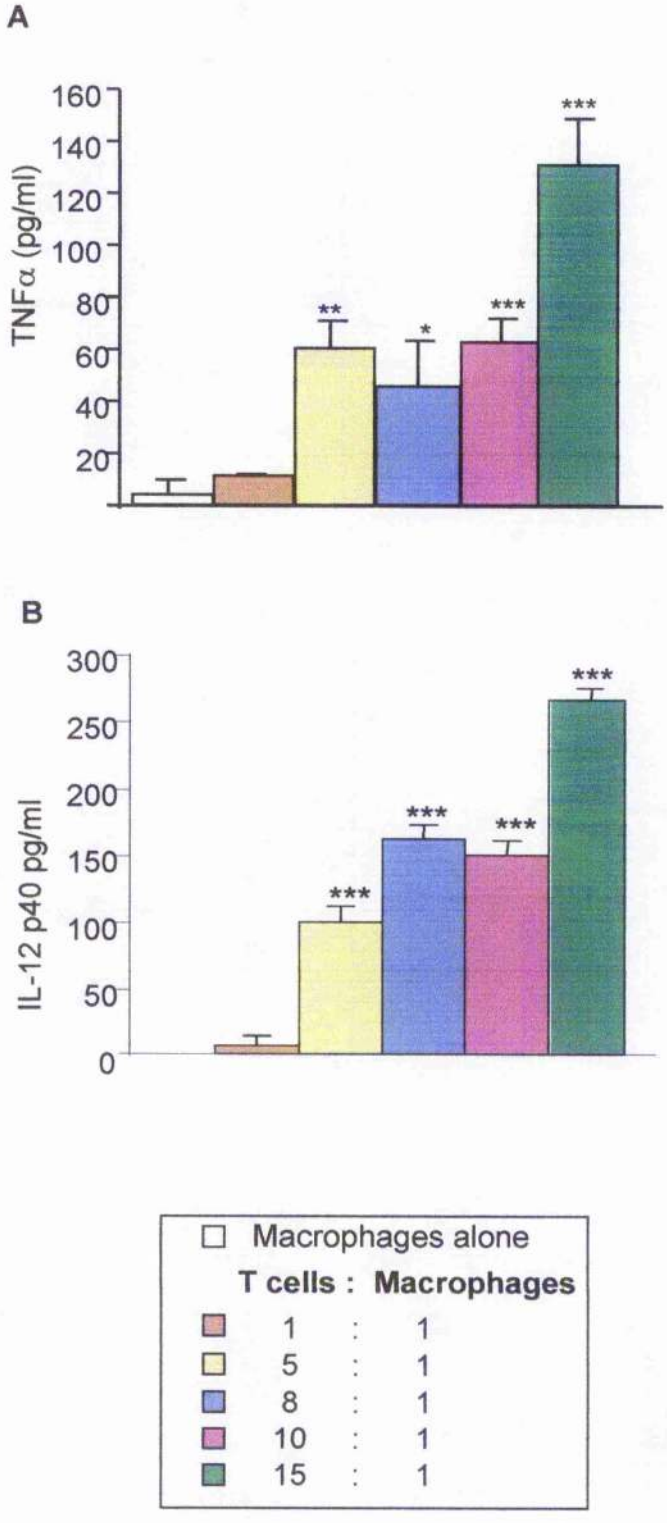
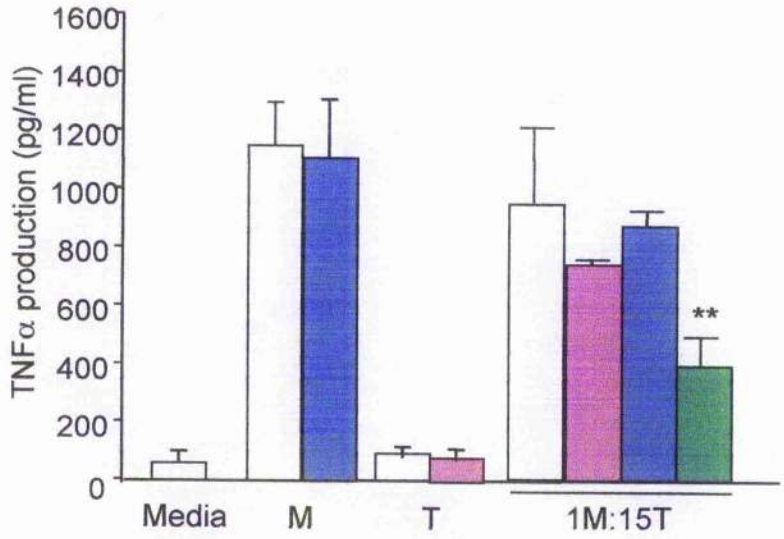

A
TNFα (pg/ml)
160
140
120
100
80
60
40
20
**
*
***
***
B
IL-12 p40 pg/ml
300
250
200
150
100
50
0
***
***
***
***
Macrophages alone
T cells : Macrophages
1 : 1
5 : 1
8 : 1
10 : 1
15 : 1

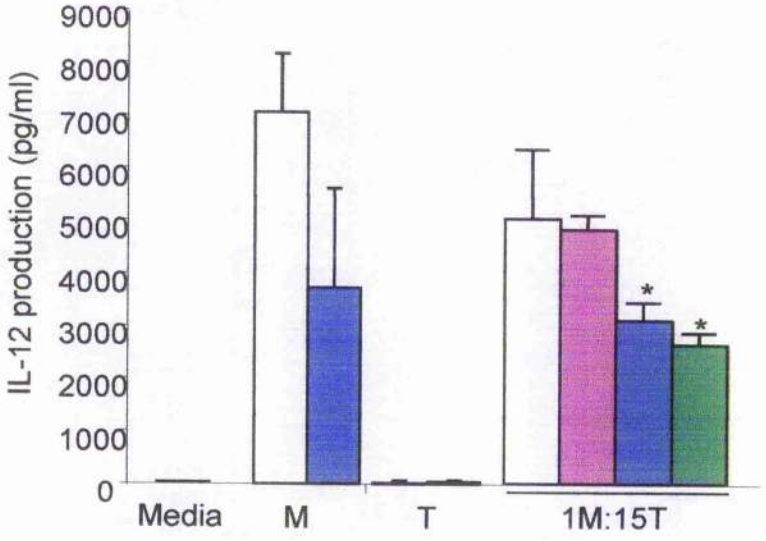
Figure 3.30 ES-62 pre-treatment of T cells or macrophages disrupts T cell contact mediated macrophage pro-inflammatory cytokine production

Bone marrow-derived macrophages were grown *in vitro* from bone marrow of BALB/c mice in the presence of CSF-1 for 6 days. On day 4 of macrophage culture, lymph node T cells from BALB/c mice were cultured *in vitro* and activated with PMA (10 ng/ml) and Con A (5 μg/ml) for 2 days. On day 6, both cell types were re-plated and stimulated with media or ES-62 for 18h. On day 7, T cells were fixed by incubating in formaldehyde, before co-culture in fresh media with the bm-macrophages, in the ratio 15:1, for 24 hours. Culture supernatants were analysed for TNFα (A) and IL-12 p40 (B) by ELISA. Data are expressed as mean ± SD (*n*=3) and are the results of one experiment *, $p<0.05$ and **, $p<0.01$ vs control (ANOVA).

A



B



	Macrophages	T cells	
Pre-treatment	None	None	□
	None	ES-62	■
	ES-62	None	■
	ES-62	ES-62	■

Figure 3.31 ES-62 treatment of BALB/c mice disrupts T cell contact mediated macrophage pro-inflammatory cytokine production

Bone marrow-derived macrophages were grown *in vitro* from bone marrow of BALB/c mice in the presence of CSF-1 for 6 days. On day 4 of macrophage culture, lymph node T cells from BALB/c mice that had previously been treated with ES-62 (2 μg) *in vivo* were cultured *in vitro* and activated with PMA (10 ng/ml) and Con A (5 μg/ml) for 3 days. On day 7, T cells were fixed by incubating in formaldehyde, before co-culture in fresh media with the bm-macrophages, in different ratios, for 24 hours. Culture supernatants were analysed for TNFα (A) and IL-12 p40 (B) by ELISA. Data are expressed as mean ± SD (n=3) and are the results of one experiment. **, $p<0.01$ and ***, $p<0.001$ vs macrophages alone (ANOVA).

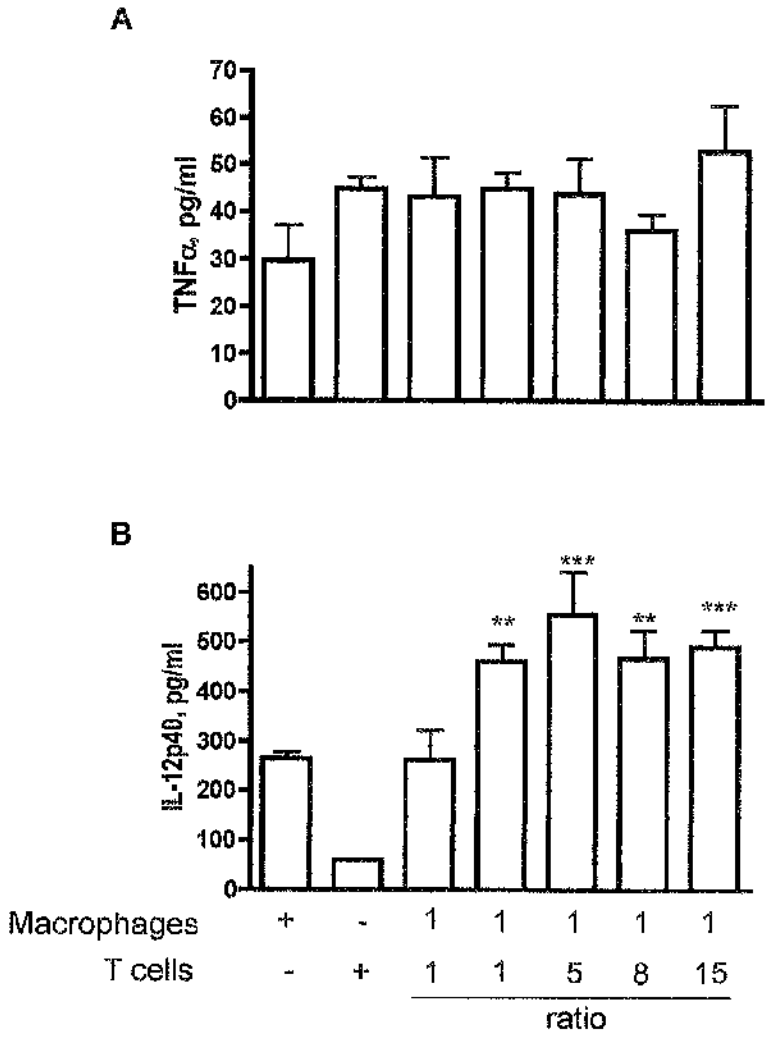

A
70
60
50
40
30
20
10
0
TNFα, pg/ml
B
600
500
400
300
200
100
0
IL-12p40, pg/ml
**
***
**
***
Macrophages + - 1 1 1 1 1
T cells - + 1 1 5 8 15
ratio

Figure 3.32 ES-62 treatment of BALB/c mice disrupts macrophage pro-inflammatory cytokine production in response to T cell contact

BALB/c mice were administered subcutaneously with ES-62 (2 μg). After 9 days bone marrow-derived macrophages from the ES-62 treated mice were grown *in vitro* in the presence of CSF-1 for 6 days. On day 4 of macrophage culture, lymph node T cells from untreated BALB/c mice were cultured *in vitro* and activated with PMA (10 ng/ml) and Con A (5 μg/ml) for 3 days. On day 7, T cells were fixed by incubating in formaldehyde, before co-culture in fresh media with the bm-macrophages, in different ratios, for 24 hours. Culture supernatants were analysed for TNFα (A) and IL-12 p40 (B) by ELISA. Data are expressed as mean ± SD (n=3) and are the results of one experiment. No significant differences between co-cultures and macrophages cultured alone were detected.

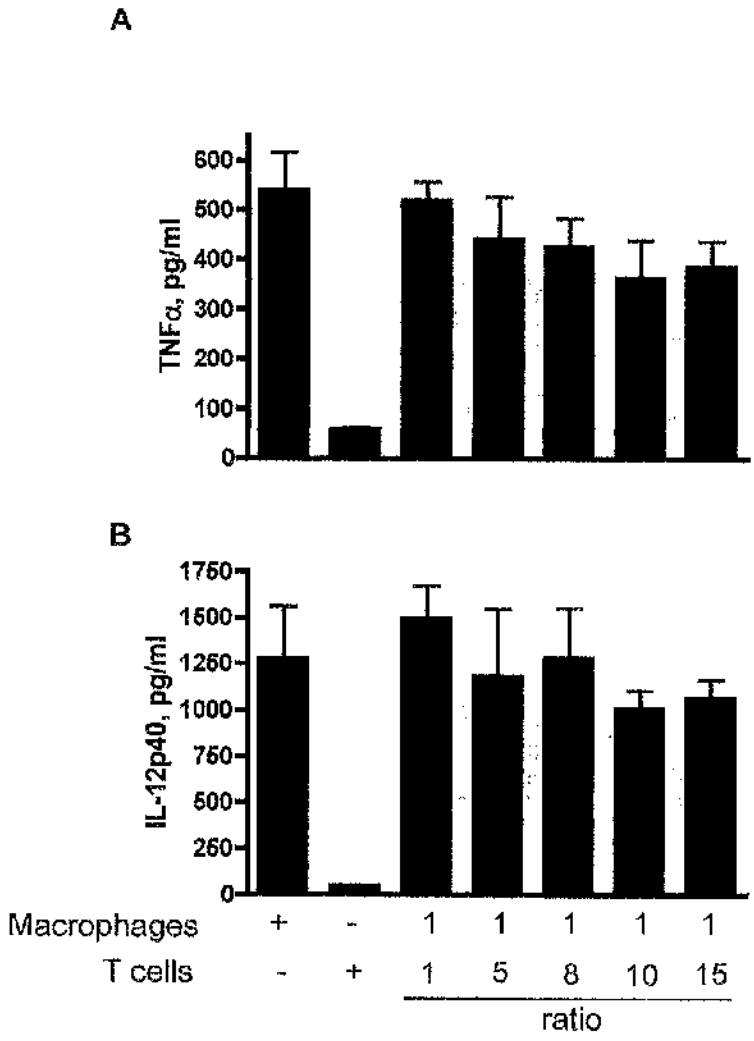

A
TNFα, pg/ml
600
500
400
300
200
100
0
B
IL-12p40, pg/ml
1750
1500
1250
1000
750
500
250
0
Macrophages + - 1 1 1 1 1
T cells - + 1 5 8 10 15
ratio

Figure 3.33 The collagen administration protocol induces differentiation of bone marrow derived DC with a mature phenotype

In naïve DBA/1 mice, in vitro culture of bm progenitor cells in the presence of GM-CSF induces differentiation of immature DC exhibiting low levels of e.g. antigen-presentation molecule, MHCII, co-stimulatory molecules, CD40, CD80 and CD86 and adhesion molecule CD54. Stimulation of these immature DC with LPS in vitro induces activation of the DC, evidenced by increased expression of antigen presentation, co-stimulation and adhesion molecules and release of pro-inflammatory cytokines, IL-12 and TNFα. In addition, maturation of DC involves a morphological change, with mature DC exhibiting dendrites ideally suited for activation of naïve T cells. In contrast, in vitro culture of bone marrow progenitor cells from CIA model mice, in the presence of GM-CSF, results in differentiation of DC that are 'pre-matured', evidenced by their spontaneous secretion of pro-inflammatory cytokines and expression of high levels of co-stimulatory, adhesion and antigen-presentation molecules.

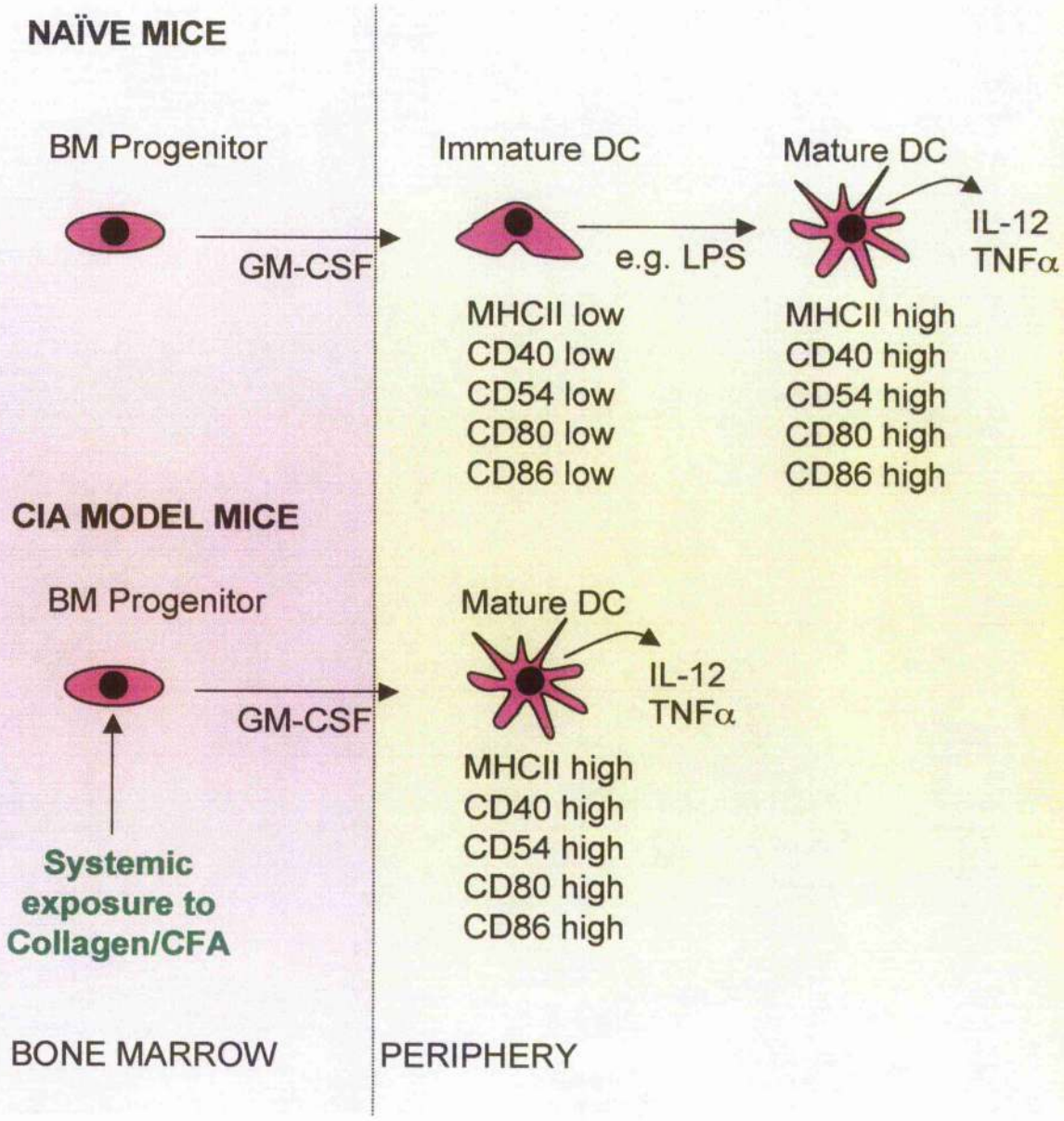

NAÏVE MICE
BM Progenitor
GM-CSF
Immature DC
e.g. LPS
Mature DC
IL-12
TNFα
MHCII low
CD40 low
CD54 low
CD80 low
CD86 low
MHCII high
CD40 high
CD54 high
CD80 high
CD86 high
CIA MODEL MICE
BM Progenitor
GM-CSF
Systemic exposure to Collagen/CFA
Mature DC
IL-12
TNFα
MHCII high
CD40 high
CD54 high
CD80 high
CD86 high
BONE MARROW
PERIPHERY

Figure 3.34 ES-62 mediated inhibition of synovial inflammation via disruption of T cell- macrophage communication

(Figure adapted from [245])

Activated TH1 cells present in the synovial tissues of CIA model mice act to prime macrophages in a contact independent manner, by producing TH1 cytokine, IFNγ. In addition, contact-dependent macrophage cytokine production is permitted by T cell and macrophage expression of membrane bound cytokines, co-stimulatory molecules and adhesion molecules. In response to contact-dependent or independent activation by T cells, macrophages release pro-inflammatory cytokines, such as TNFα and IL-12. Furthermore, activation of macrophages in this way may lead to production of matrix metalloproteinases (MMPs) and prostaglandins (PGs), which help to mediate the inflammation, tissue destruction and hence, the pathology exhibited in CIA.

The results of this investigation, and previous investigations by this laboratory, have demonstrated that ES-62 acts to inhibit pro-inflammatory cytokine production by macrophages in response to contact-dependent and contact-independent stimulation. It is not currently known what mechanism is employed by ES-62 for disruption of T cell contact-mediated macrophage cytokine production, however it is possible that it may act via modulation of the expression of cytokines, co-stimulatory molecules and adhesion molecules on T cells, macrophages, both. Ultimately, disruption of T cell-induced macrophage activation may also lead to inhibited production of tissue-damaging MMPs and PGs.

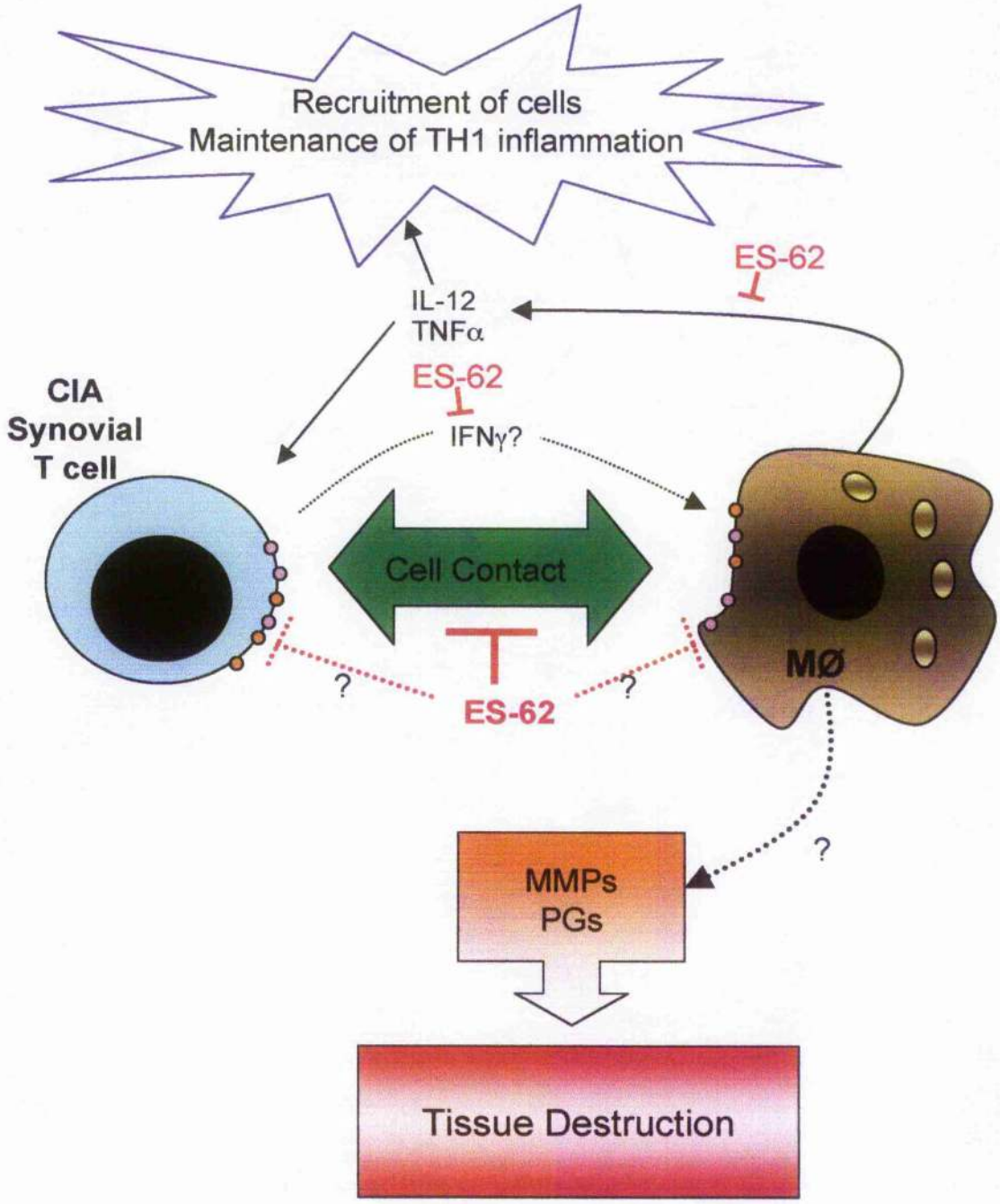
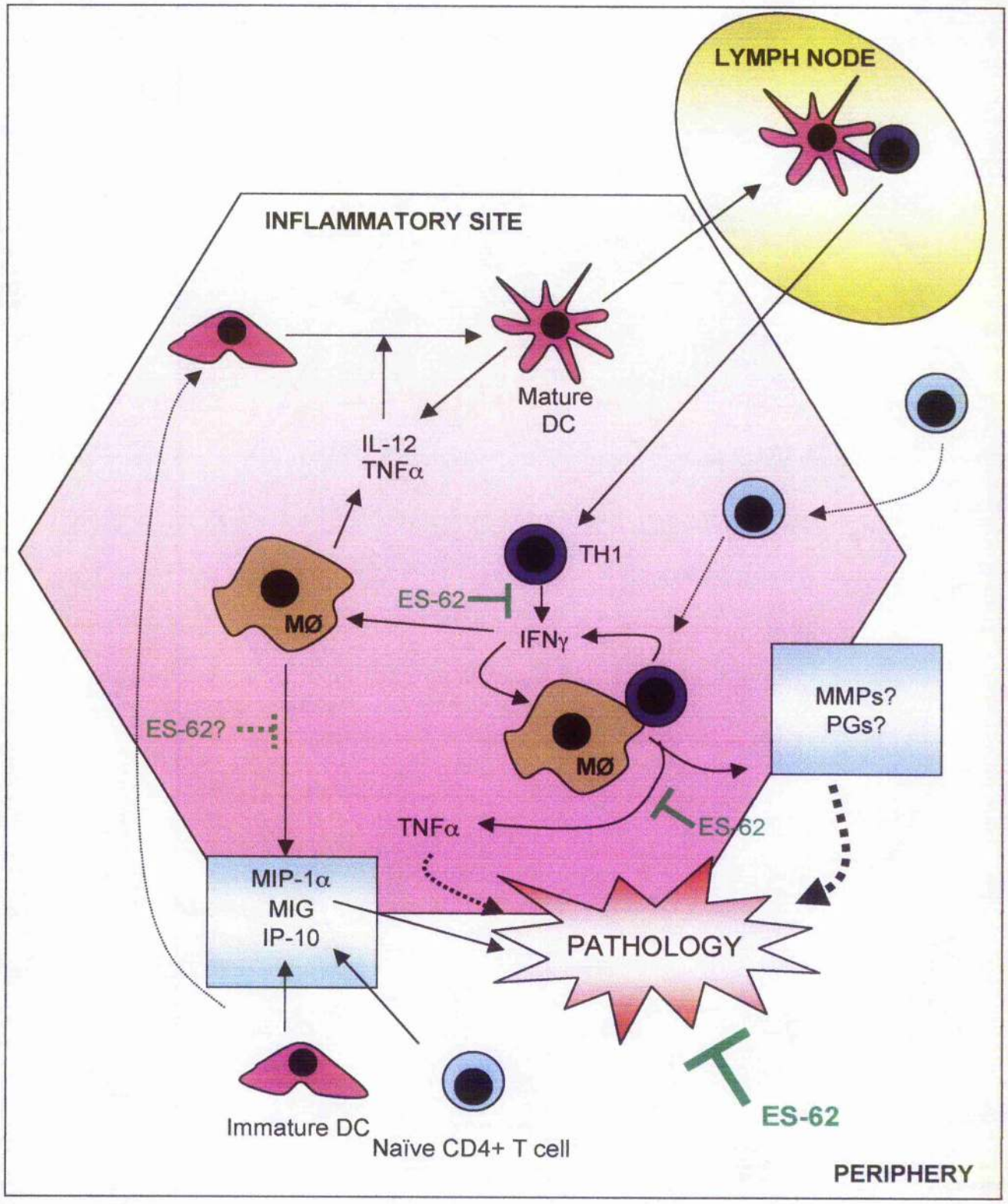

Recruitment of cells
Maintenance of TH1 inflammation
ES-62
IL-12
TNFα
ES-62
IFNγ?
CIA
Synovial
T cell
Cell Contact
MØ
?
?
ES-62
?
MMPs
PGs
Tissue Destruction

Figure 3.35 A model of ES-62 mediated inhibition of inflammation in collagen-induced arthritis

In CIA model mice pre-matured DC, expressing collagen antigens and secreting pro-inflammatory cytokines (IL-12 and TNFα), induce priming of naïve $CD4^+$ T cells into collagen-specific TH1 effector cells in the lymph nodes. Such TH1 effector cells migrate to the inflammatory site (the synovium) and, in turn, release IFNγ in response to antigen (collagen) stimulation. In response to IFNγ stimulation, macrophages in the inflammatory site are activated to release pro-inflammatory cytokines (e.g. IL-12 and TNFα) and inflammatory chemokines (e.g. MIP-1α, MIG and IP-10). These inflammatory chemokines induce recruitment of immature DC and naïve $CD4^+$ T cells from the periphery to the inflammatory site. Once recruited, immature DC become activated by the inflammatory milieu and antigens present and help to activate recruited naïve $CD4^+$ T cells. In addition, T cell contact-induced macrophage activation contributes further pro-inflammatory cytokines and mediators of tissue destruction (e.g. matrix metalloproteinases and prostaglandins) to the existing inflammatory milieu. Production of TH1-promoting cytokines promotes survival and activation of the cell types present and chemokines recruit further cells. Thus, a positive feedback cycle of inflammation is set up.

ES-62 has previously been demonstrated to inhibit antigen-specific production of IFNγ by TH1 cells in CIA and pro-inflammatory cytokine production by macrophages and DC in response to inflammatory stimuli. In addition, it has been demonstrated in this chapter that ES-62 disrupts T cell contact-induced macrophage activation, evidenced by pro-inflammatory cytokine production. Therefore via these mechanisms at least, ES-62 could mediate anti-inflammatory action in this system.


LYMPH NODE
INFLAMMATORY SITE
Mature
DC
IL-12
TNFα
TH1
ES-62
MØ
IFNγ
MØ
MMPs?
PGs?
ES-62?
TNFα
ES-62
MIP-1α
MIG
IP-10
PATHOLOGY
ES-62
Immature DC
Naïve CD4+ T cell
PERIPHERY

4 ES-62 inhibits TH2-like inflammation in an ovalbumin-induced model of asthma

4.1 Introduction

Asthma is a disease of developed, westernised countries. At this time, Scotland is home to more childhood asthma sufferers than any other country in the world and is the country with the third highest prevalence for adult asthma patients [307]. Asthma in humans is a chronic pulmonary disease characterised by persistent airway inflammation, periods of acute airway obstruction and bronchial hyperresponsiveness [308]. Whilst it is known that the inflammation exhibited in asthma occurs as a consequence of a dysregulated TH2-mediated immune response, current understanding of the precise aetiology of asthma is unclear [103]. It has been suggested that genetic pre-disposition to development of TH2-type inflammation [309] in combination with environmental exposures; for example with allergenic substances, during infancy has significant roles to play [310]. Indeed, in a Greek study it was demonstrated that the prevalence of asthma varies with the level of exposure to house dust mite, a common allergen to which asthmatics can become sensitised [311]. However, the role of exposure to allergens from pets in asthma development has fuelled conflicting arguments [312, 313]. Furthermore, the roles of home central heating methods and exposure to cigarette smoke or gas cooking fumes have also been investigated [314-319]. It has even been suggested that the frequency of moving home contributes to asthma development [320]. As yet, the key conditions necessary for development of asthma have not been conclusively defined and, therefore, it is most likely that asthma is result of a number of inter-related factors.

4.1.1 Animal models of asthma

To dissect the immunological mechanisms by which asthma is initiated in humans, researchers have employed a number of murine models. In antigen-induced asthma models, inflammation is generated by sensitising TH2-prone mice with antigen (co-administered intraperitoneally with a TH2-skewing adjuvant e.g. aluminium hydroxide), to induce development of an antigen-specific memory immune response, and then subsequently challenging with aerosolised antigen intranasally, to induce an TH2-mediated inflammatory immune response in the lungs [321, 322]. One of the most common antigen-induced models of asthma is the ovalbumin (OVA)-induced airway inflammation model generated in BALB/c mice (Figure 2.2), where inflammation generated in response to the OVA administration protocol is induced over a relatively short period of 28 days. This short-term model of airway inflammation induces TH2-mediated airway inflammation and bronchial hyper-reactivity, similar to that observed in human asthma and has been widely used to study the pathogenesis and investigate potential therapies for this inflammatory condition. [321, 323]. However, it is well-established that the short-term ovalbumin-induced asthma model does not precisely

mimic all of the pathology exhibited in human asthma and hence, is normally used to examine only the acute inflammatory component of asthmatic disease. In human asthmatics, it has been identified that exposure to chronic long-term reversible pulmonary inflammation, leads to irreversible tissue damage within the lungs and eventually, airway remodelling [84]. Airway remodelling normally involves hyperplasia and hypertrophy of airway smooth muscle and deposition of collagen. These changes modulate airway integrity and elasticity, thus inhibiting efficient gas exchange, which contributes to the pathology exhibited in asthma. Therefore, to determine the secondary effects of exposure to long-term asthmatic inflammation, a model of chronic airway inflammation and pulmonary remodelling can be generated. This model [324] is simply an extension of the ova-induced asthma model (Figure 2.3) and enables investigation of the physiological and immunological effects associated with long-term pulmonary disease in humans. Using this model it was previously demonstrated that longer-term pulmonary inflammation induced irreversible structural changes to the airways, such as smooth muscle and goblet cell hyperplasia and deposition of collagen. Thus, it was possible that this model exhibits a more accurate representation of the effects of chronic inflammation in human asthma, nevertheless, the majority of studies investigating the immunological basis of the pathogenesis of asthma have been conducted in the shorter-term model.

4.1.2 The immunopathology of asthma

As mentioned previously, it has been well documented that the inflammation exhibited in asthma is associated with action of a TH2-mediated immune response. Using procedures such as broncho-alveolar lavage (BAL), the major inflammatory cell infiltrates of the airways have been identified and shown to include allergy-associated leukocytes such as eosinophils, macrophages, mast cells and TH2 lymphocytes. Moreover, it has been demonstrated that all these cells have a role to play in the pathogenesis of asthma. In particular, TH2 lymphocytes, mast cells and eosinophils secrete TH2 cytokines, such as IL-5, IL-4 and IL-13, which recruit further infiltration of inflammatory cells and maintain or amplify the TH2 inflammatory immune response [91].

Of the many different classifications of asthmatic disease exhibited by patients, possibly the best known is allergic asthma, triggered by exposure to environmental allergen [325]. In allergic asthma, allergen inhalation and recognition by cells resident in the airways activates onset of an asthma 'attack'. Not all allergic asthmatics are atopic (a genetic predisposition to allergy) but, like atopic individuals, asthma patients exhibit increased levels of serum IgE, the signature antibody isotype in allergy. IgE is centrally involved in the pathogenesis of asthma and the onset of an acute attack of inflammation [326, 327]. Although IgE can exist as a soluble molecule in the airway, it is usually found bound to the high affinity IgE receptor, FcεRI on the surface of mast cells (or the low affinity IgE

receptor, FcεRII/CD23 on other leukocytes). When allergen is inhaled, it is recognised specifically by IgE and the resulting antigen-IgE complex cross-links the FcεRI-IgE receptor complexes on the mast cells [328] and degranulation ensues [329]. The degranulation process results in release of inflammatory mediators such as leukotrienes and TH2 cytokines such as IL-4 and IL-5. It is understood that inflammatory mediators and granule proteins liberated from mast cells facilitate the inflammatory and degradative changes observed in the asthmatic epithelium [91]. TH2 cytokines (IL-4, IL-5 and IL-13), produced by mast cells and other TH2 effector cells, activate a cascade of inflammation-generating activity [330]. IL-4 is a key promoter of the TH2-mediated immune response and can induce differentiation of eosinophils from bone marrow progenitor cells, which expand to form an IL-4 producing innate cell population, thus amplifying the TH2 cytokine environment and hence the TH2 immune response *in vivo* [331]. IL-4 also promotes B cell antibody isotype switching, to increase IgE production [332]. Facilitated by the action of IL-5 and chemokines like eotaxin, eosinophils are recruited to the airways and activated [91]. Furthermore, IL-13 can also ligate the IL-4 receptor and has been shown to be required for allergen-induced airway hyper-responsiveness in mouse models of allergic airway inflammation [333]. In particular, IL-4 and IL-13 have been demonstrated to play prominent roles in the initiation and effector mechanisms of asthmatic inflammation, respectively [84].

Current treatment strategies for asthma involve long-term administration of corticosteroids, to reduce general inflammation and therapeutic use of broncho-dilator agents (beta(2)-adrenoceptor agonists) [334, 335]. Interestingly, blockade of TH2 cytokines (IL-4 or IL-5), as a treatment strategy for asthma (in a parallel manner to anti-TH1 cytokine treatment in RA) has generally proved unsuccessful in amelioration of all asthma-associated pathologies, probably because the development of inflammation in asthma is a result of the action of complex cytokine networks and pathways. Thus, disruption of mechanisms that initiate the TH2-type inflammation (i.e. upstream of TH2 cytokine production) would most likely be a more successful method of therapy. As mentioned previously, IgE levels are consistently elevated in atopic and non-atopic asthma patients and have integral roles to play in asthma pathogenesis, therefore, anti-IgE therapy has recently been proposed for treatment of asthmatic inflammation [336]. This type of treatment prevents IgE binding to FcεRI receptors on mast cells and FcεRII on other leukocytes, therefore disrupting the inflammatory cascade initiated by IgE ligation and mast cell degranulation. It has yet to be determined whether this type of treatment will help to reduce inflammation associated with asthma long-term.

4.1.3 ST2

The ST2 gene (also known as T1, Fit-1, or DER4) encodes a protein (ST2L) selectively expressed on the surface of $CD4^+$ TH2, but not TH1 cells, whose expression is regulated by TH2 transcription factor, GATA-3 [337-339]. In addition to membrane-bound ST2L, the ST2 gene also encodes a shorter soluble protein, ST2. ST2L has been identified as helping to initiate the TH2 immune response [340] therefore it has been suggested that ST2 expression/signalling is important for the function of TH2 cells, however evidence to support or disprove this has been contradictory. For example, in a TH1-mediated CIA model, blockade of ST2 exacerbated the severity of the disease whilst TH1-mediated resistance to *Leishmania major* infection was promoted [337], indicating that blockade of ST2 protein inhibited development of a functional TH2 immune response. Furthermore, knock-out of the ST2 gene inhibited infiltration of eosinophils and production of TH2 cytokines in a TH2-mediated pulmonary granuloma model [341]. Soluble ST2 has been shown to inhibit the inflammatory cytokine response to LPS and down-regulate TLR4 expression on the surface of macrophages, thereby reducing TH1-mediated LPS-shock in mice. [342]. However, in contrast, Hoshino et al [343] have demonstrated that ST2-deficient mice maintain their capacity for development of normal TH2 cell responses in TH2-mediated helminth parasite infection and a model of allergic airway inflammation. Likewise, it has been observed that T1 (ST2)-deficient and ST2 receptor transgenic mice develop normal TH2 responses to parasite infection [344]. Thus, conclusive demonstration of the role ST2 is yet to be established. If achieved, this knowledge may provide further understanding of pathogenesis, and represent a potential therapeutic target, in TH2-mediated inflammatory conditions such as asthma.

4.1.4 Is infection with parasites protective against development of asthma?

As mentioned in Chapter 1, the hygiene hypothesis aims to explain the increased prevalence of allergy and allergic disease in developed countries (and increasing incidence in developing countries as they become more affluent) as a result of increased sanitation and reduced exposure to pathogens in early childhood [102]. In particular, exposure to helminth parasite infections in developing countries is common. Indeed, it has been suggested that helminth infection protects individuals from atopic asthma, prevalent in developed countries. [110, 131, 345]. Moreover, it has been suggested that immunomodulatory excretory-secretory (ES) products, generated by parasites, modulate the host immune response, to favour survival of the parasite and longevity of infection. Immunomodulatory ES products are continuously released into the host blood stream [161] during infection with filarial nematodes. Therefore it has been postulated that they might facilitate inhibition of asthma and other inflammatory atopic diseases in parasite-infected individuals.

4.1.5 Aims

It has previously been established that ES-62, an immunomodulatory ES product of rodent filarial nematode, *A. viteae*, inhibits TH1-mediated inflammation exhibited in a murine model of arthritis (Chapter 3 and [192]). This inhibitory action was associated with reduction of antigen-specific TH1 cytokine production and possible modulation of contact-dependent communication between activated T cells and macrophages. Indeed, amelioration of TH1-type inflammation by ES-62 treatment was in accordance with the previously demonstrated actions of ES-62 *in vitro*. In more detail, via modulatory action on dendritic cells, ES-62 has been demonstrated to reduce a TH1-promoting immune response, whilst simultaneously promoting a TH2-type immune response [189]. Thus, it might be predicted that ES-62 treatment of TH2-type inflammation, in a murine model of asthma, would induce exacerbation of disease. However, as part of extensive *in vitro* investigations, it has been concluded that ES-62 induces *anti-inflammatory* action in several cell types *in vitro* [193]. Therefore, the core aim of this chapter was to determine whether ES-62 treatment mediates anti-inflammatory action in a model of acute TH2-mediated allergic inflammation such as OVA-induced asthma.

As mentioned above, changes to airway physiology are often exhibited in patients with long-term pulmonary inflammation. For example, narrowing of airways and loss of elasticity can occur as a result of airway smooth muscle proliferation. Changes in smooth muscle contractility in murine models can be measured *in vitro* using a well-established laboratory technique, myography. It has not previously been determined whether the short-term model of OVA-induced airway inflammation exhibits associated airway smooth muscle changes or indeed, whether treatment of the model with ES-62 affects airway smooth muscle physiology. Thus, it was planned to investigate the contractility of airway smooth muscle obtained from the OVA-induced asthma model mice. As mentioned previously, the short-term OVA-induced asthma model can be adapted to develop a model of chronic airway inflammation and remodelling, which exhibits physiological features similar to those displayed by chronic asthmatics, for example, fibrosis. In particular, it was of interest to determine whether the immunology underlying the physiological changes to airway integrity induced by chronic airway inflammation were different from those responsible for acute airway inflammation, such as in the short-term model of OVA-induced asthma. Thus, it was planned to investigate the immunology of the longer-term model of airway inflammation, to compare and contrast with the immunology of the shorter-term model. In addition, the effect of ES-62 treatment on a model of long-term airway inflammation had not previously been determined. Thus, it was additionally planned to determine the immunological effects of treatment of the longer-term airway inflammation model with ES-62.

It is well established that asthma-like pulmonary inflammation is mediated by a TH2-type immune response. TH2-associated factor, ST2, has previously been analysed in a murine model of acute airway inflammation [343], but not in a longer-term airway-remodelling model. Therefore, the immunological role of ST2 in initiation of long-term pulmonary inflammation is currently unknown. Thus, it was additionally proposed to determine whether the model of longer-term airway inflammation can be induced in ST2-deficient mice and to investigate the role of ST2 in the immunology underlying inflammation in this model. Furthermore, it has previously been demonstrated that administration of soluble ST2 ameliorates inflammation in CIA [346]. Similarly, ES-62 treatment has also been shown to mediate anti-inflammatory action in the CIA model (Chapter 3 and [192]). However, it is not currently known whether the ST2 protein or ST2 downstream signalling bears any correlation or relationship to the *in vivo* actions of ES-62. Thus, assuming it was possible to induce the long-term pulmonary inflammation model in ST2-deficient mice, it was planned to determine the role of ST2 in the action of ES-62 treatment of the long-term pulmonary inflammation model.

In summary, the individual aims of this investigation were:

1. To determine and characterise the effect of ES-62 treatment (prophylactically and therapeutically) on the inflammation exhibited in the short-term ova-induced asthma model.
2. To determine whether the ova-induced asthma model exhibits accompanying changes in airway smooth muscle physiology similar to that exhibited in human asthma patients by analysing the contractility of airway smooth muscle and, furthermore, to determine any action of ES-62 on airway smooth muscle contractility.
3. To extend immunological analysis of ES-62 action on pulmonary inflammation, by examining a model of longer-term pulmonary inflammation and airway remodelling.
4. To determine the immunological role of TH2 marker gene ST2 in a model of longer- term pulmonary inflammation.
5. To determine any relationship or correlation between ST2 and the effects of ES-62 treatment in the long-term pulmonary inflammation model.

4.2 Results

4.2.1 OVA-induced airway inflammation model

The OVA-induced airway inflammation model was set up as described previously and in (figure 2.2). On day 28 of the OVA administration protocol, mice were sacrificed, broncho-alveolar lavage (BAL) was performed and the cytokine and cell profile of the lavage fluid was analysed. Macrophages were the predominant cell type detected in the lavage from all treatment groups (Figure 4.1). However, significantly enhanced levels of eosinophils were identified in the BAL fluid of Asthma group mice, when compared with control mice (Figure 4.1). In support of these findings, histological analysis of lung tissue from Asthma group mice revealed profuse peribronchial inflammation, mucosal hyperplasia and eosinophil infiltration (Figure 4.1), compared with lung tissue from control mice (Figure 4.1).

Consistent with inflammation of a TH2 phenotype, the BAL fluid from asthma group mice contained enhanced levels of TH2 cytokines, IL-4 and IL-5 and reduced levels of TH1 cytokine, IFNγ (Figure 4.2). Satisfied that the ovalbumin administration protocol induced hallmark features associated with asthma in humans, this model was considered appropriate for determination of the effects of ES-62 on TH2-mediated inflammation.

4.2.2 Decreased OVA-induced airway eosinophilia and IL-4 in mice treated with ES-62.

Ovalbumin sensitised and challenged mice, which had been treated prophylactically with ES-62 displayed significantly reduced levels of airway eosinophils compared to mice that had not received ES-62 treatment. In contrast, there were no significant effects on the proportion of airway macrophages, epithelial cells, lymphocytes or neutrophils (Figure 4.1), indicating that this action of ES-62 was eosinophil-specific. Furthermore, development of peribronchial inflammation as displayed by Asthma group mice was reduced by treatment with ES-62 (Figure 4.1). As mentioned above, the TH2 nature of the airway inflammation was demonstrated by elevated IL-4 and IL-5 levels in the BAL fluid of mice from the *Asthma* group (Figure 4.2), the former of which was significantly reduced by ES-62. The very low levels of BAL IFN-γ production (Figure 4.2) were not significantly modulated by ES-62 treatment. In summary, the action of ES-62 on airway cytokine profile was targeted specifically for reduction of TH2 cytokine production.

4.2.3 Effect of ES-62 treatment on antigen-specific responses by draining lymph node cells

It has been demonstrated that the pulmonary inflammation exhibited in asthma is the result of an activated antigen-specific immune response, under the direction of TH2 cells. During asthma pathogenesis TH2 cells become activated in lung-draining lymph nodes

before migration to the site of inflammation. To begin dissection of the underlying mechanisms of pulmonary inflammation in this model, thoracic, cervical and pulmonary draining lymph nodes (DLN) were obtained. DLN cells from each treatment group were pooled and cultured *in vitro*, stimulated with antigen (OVA) or mitogen (Con A). *Ex vivo* DLN cells from mice that had received the airway inflammation protocol (*Asthma* and *Asthma + ES-62* groups) displayed elevated spontaneous proliferation compared to those from *control* or *ES-62* groups (Figure 4.3a). Re-stimulation with OVA *in vitro*, induced antigen-specific proliferation in Asthma group DLN cells, which was not modulated by prior treatment with ES-62 *in vivo*. This result indicated that targeting proliferation of lymph node cells was not a method employed by ES-62 for mediation of anti-inflammatory effects *in vivo*. However, ES-62 significantly inhibited antigen-specific IL-4, IL-5 and IL-10 production by such DLN cells *in vitro* (Figure 4.3b-d). By contrast, Ag-specific production of TH1/pro-inflammatory cytokines such as IFNγ and TNFα were not reduced by prior exposure to ES-62 *in vivo* (indeed the latter was elevated) (Figure 4.3e,f). These effects of ES-62 were antigen-specific because mitogen (ConA)-induced proliferation and cytokine responses were unaffected by ES-62 treatment (Figure 4.4). Therefore, it appeared that ES-62 inhibited antigen-specific TH2 cytokine production as a method of preventing development of asthma-like airway inflammation in this model.

4.2.4 The effects of ES-62 treatment on antigen-specific responses by splenocytes.

To determine any difference between local and systemic lymphocyte responses, splenocytes from each treatment group were cultured and stimulated in a similar manner to DLN cells from this model. Consistent with the findings obtained in DLN cells, splenocytes from the *Asthma* group showed antigen-specific enhancement of proliferation when re-stimulated with OVA *in vitro* (Figure 4.5a), which was not modulated by prior exposure to ES-62 (*Asthma + ES-62*) *in vivo*. Likewise, ES-62 treatment *in vivo* inhibited antigen-specific IL-4 production by splenocytes following *ex vivo* re-stimulation with OVA (Figure 4.5b), however antigen-specific IL-5 and IL-10 levels were unaffected (Figure 4.6c-d). Furthermore, whilst the level of antigen-specific IFNγ was unaffected by ES-62 (as also witnessed with DLN cells), TNFα release was enhanced by prior exposure to ES-62 *in vivo* (Figure 4.5f). As with DLN, the *ex vivo* proliferative and cytokine responses of splenocytes to mitogenic Con A were unaffected by ES-62 treatment (Figure 4.6). Collectively, the decrease in TH2 responses and maintenance or increase of Th1/pro-inflammatory responses observed in DLN cells and splenocytes derived from the *Asthma + ES-62*, relative to the *Asthma*, groups of mice suggests that ES-62 mediates its anti-inflammatory action on airways by diverting the immune response away from the pathological TH2 bias.

4.2.5 The effect of ES-62 on asthma model lymph node cells is dependent upon anatomical site.

With the aim of obtaining a full body profile of lymphocyte responses, *ex vivo* analysis of lymph node cells was extended to additional anatomical sites. Thus, peripheral lymph nodes (PLN), obtained from anatomical areas distal to the lungs, were pooled in treatment groups. PLN cells were isolated, cultured and stimulated *in vitro*, using the same methods as have been described for DLN cells. It was observed that the effects of prior *in vivo* exposure to ES-62 were quite different in cells derived from peripheral lymph nodes (PLN) compared to draining lymphoid organs. PLN cells from mice that had been treated with ES-62 (*ES-62* and *Asthma + ES-62* groups) showed increased levels of spontaneous proliferation *ex vivo,* relative to those from *control* or *Asthma* groups (Figure 4.7a). However, stimulation of PLN cells with OVA *in vitro* did not significantly modulate the rates of proliferation in cells from any of the treatment groups, indicating that the recall antigen specific responses displayed by splenocytes and DLN cells were absent in the PLN cell population. Cytokine analysis indicated the overall *in vitro* cytokine production by PLN cells, where detectable, was very low. Whilst the control and Asthma groups did not produce any increase in IL-4, IL-5 or IL-10 following stimulation with OVA *ex vivo*, cells from those groups, which had been exposed to ES-62 *in vivo*, showed low but increased levels of IL-5 and IL-10 when challenged with Ag (Figure 4.7b-d). In contrast, prior exposure to ES-62 had negligible effects on the very low production of IFNγ or TNFα (Figure 4.7e,f). The lack of responses to OVA in the *control* and *Asthma* groups are therefore perhaps consistent with the secondary immune response to aerosolised Ag being generally restricted to the appropriate DLN and spleen whereas, because of the subcutaneous delivery of ES-62, some weak, yet significant responses can be detected in cells derived from ES-62-treated groups. Moreover, the overall polarisation of such responses towards a TH2/anti-inflammatory phenotype reflects our previous *in vivo* studies on the immune response to ES-62 itself [193, 347].

4.2.6 ES-62 treatment of Ova-induced airway inflammation inhibits Ova-specific IgE production

Inflammatory signals are communicated around the body using various methods. One method of communication between different immune action sites is through the use of immunoglobulin (Ig) isotypes. IgE is the signature TH2 antibody isotype and is an important mediator in the pathogenesis of allergic inflammation in asthma. Therefore it was necessary to determine whether the anti-inflammatory action of ES-62 on TH2 cytokine production in this model was reflected by a change in the pattern of IgE antibody secretion *in vivo*. Serum from all groups of mice was thus analysed *in vitro* for total IgE and OVA-specific IgE content. Analysis of IgE levels in the serum showed that whilst OVA-specific and total IgE levels were elevated in the *Asthma* relative to the *control* and

ES-62 groups (Figure 4.8a,b;), this elevation of antibody production was significantly inhibited by prophylactic treatment (*Asthma + ES-62* group) with ES-62.

Serum samples were also analysed for OVA-specific IgG of TH1 AND TH2 subclasses, IgG2a and IgG1 respectively. Both OVA- specific IgG2a and IgG1 were detected in the serum from mice which had received the ova administration protocol, however prophylactic treatment of these mice with ES-62 did not significantly alter the production of either Ig isotype (Figure 4.8c,d), indicating that modulation of antigen-specific IgG subclasses was not a method employed by ES-62 for preventing development of inflammation in this model.

4.2.7 In situ IgE expression in airway inflammation model lymph nodes

It is well documented that B cells found in the lymph nodes are the primary generators of Ig. Following stimulation with TH2 cytokine, IL-4, B cell antibody production is switched to TH2-type isotype, IgE. Sections of LN were therefore analysed for IgE and B220 (a marker of B cells) expression. Following examination of Figure 4.8, it can be concluded that LN from mice that had undergone the OVA-induced asthma protocol expressed increased levels of IgE, due to more brightly stained areas of the lymph node sections, than LN from control mice. Furthermore, it was obvious, from examination of the section pictures, that IgE staining was present in areas that appeared follicle-shaped within the lymph node section, suggesting that IgE was predominantly restricted to the B cell follicular areas in the LN.

4.2.8 ES-62 mediates anti-inflammatory action in established airway inflammation

The results described above indicate that ES-62 can inhibit the pulmonary inflammation that develops in a model of asthma, when administered prophylactically, before onset of inflammation. In addition, it was necessary to determine whether ES-62 could mediate anti-inflammatory action in this model, when administered therapeutically (i.e. after the onset of pulmonary inflammation). Therefore, a second model of OVA-induced pulmonary inflammation was set up, identical to that previously described and ES-62 was administered *only* on days 25 and 27 of the OVA administration protocol (Figure 2.3). Administered therapeutically, ES-62 maintained its inhibition of OVA-induced pulmonary inflammation, as demonstrated by reduction of eosinophil numbers in the BAL fluid (Figure 4.9). Furthermore, this eosinophilia-reducing action of ES-62 was found to be dose-dependent.

4.2.9 The effects of ES-62 treatment on antigen-specific DLN cell responses in established airway inflammation

Following confirmation of the therapeutic effects of ES-62 in the OVA-induced asthma model, it was logical to determine whether the anti-inflammatory action of ES-62 was associated with modulation of DLN cell antigen-specific responses. Using the method adopted for analysis of the prophylactic treatment model, DLN cells, obtained from mice in the therapeutic treatment model, were cultured *ex vivo* and re-stimulated with OVA. Although not significant, it was identified that therapeutic treatment with ES-62 appeared to inhibit antigen-specific IL-4 and IL-5 cytokine secretion in response to re-stimulation with OVA *in vitro*. Furthermore, the modest inhibitory effect of ES-62 appeared to be dose-dependent (Figure 4.9). These findings indicated that ES-62-mediated inhibition of inflammation in the therapeutic model might be, at least partially, facilitated by inhibition of antigen-specific TH2 cytokine production. This effect was similar to, but less potent than the observed action of ES-62 in this model when administered prophylactically.

4.2.10 ES-62 treatment of established airway inflammation does not modulate serum immunoglobulin.

To determine whether therapeutic ES-62 treatment modulated serum antibody levels in this model, analysis of serum samples was conducted. Interestingly, it was observed that therapeutic treatment with ES-62 did not significantly modulate total or OVA-specific IgE levels detected in the serum (Figure 4.10). These findings indicated that ES-62 was capable of preventing development of enhanced serum IgE, as had been determined in the prophylactic treatment model, but not reduction of existing IgE levels after establishment of disease. To obtain a more complete picture of the phonotype of serum Ig in this model, OVA- specific IgG1 and IgG2a were also analysed in the serum from the therapeutic treatment model mice. Consistent with the findings in the prophylactic treatment model, therapeutic treatment with ES-62 did not significantly modulate the production of either IgG subclass (Figure 4.10c, d).

4.2.11 The OVA-induced airway inflammation model does not display augmented airway smooth muscle contractility

It is well established that an irreversible remodelling of airway structure accompanies the reversible inflammation exhibited in asthma [84]. This remodelling includes fibrosis and narrowing of the airways, reduced elasticity and increased contractility (or tightening) of the pulmonary smooth muscle. An experiment was designed to determine whether there was any difference in airway smooth muscle contractility in the model of airway inflammation and if so, could ES-62's inhibitory capacity extend to inhibition of these structural changes.

In a preliminary experiment a four-group ova-induced pulmonary inflammation model was set up as before, with a prophylactic ES-62 treatment protocol and the tracheal smooth muscle was analysed. Using a large vessel myograph, the force of contraction of tracheal rings from mice in both treatment groups in response to methacholine (MCh) was measured. Comparison of the cumulative concentration-response curves, plotted as a percentage of the maximum response to potassium chloride (Figure 4.11), indicated negligible difference between the smooth muscle from each model treatment group. These findings were consistent with those of previous investigators, using a porcine model of airway hyper-responsiveness [348].

4.2.12 An OVA-induced model of chronic airway inflammation.

The results of the myograph experiments highlighted the short-term nature of the pulmonary inflammation set up in the ova-induced inflammation model. As an extension of the airway inflammation study, analysis of the immunological parameters in a model of irreversible pulmonary inflammation was proposed. To accomplish this the previously used airway inflammation model was adapted by maintaining the ovalbumin administration protocol and extending the time of inflammation to ensure the model acquired adequate features of longer-term pulmonary disease (Figure 2.3). It has previously been identified that this prolonged OVA-administration protocol induces fibrosis of the airways, e.g. deposition of collagen [324], therefore groups administered with long-term ovalbumin were termed, "Fibrosis". There were three treatment groups in this model: Control, Fibrosis and Fibrosis + ES-62. ES-62 was administered in a prophylactic manner to the latter treatment group.

Due to the physiological severity of this extended inflammation induction protocol, 4 out of the 6 mice in the wild-type fibrosis treatment group did not survive until the end of the experimental protocol (day 56) and therefore, this model was not further pursued. Interestingly, all mice in the Fibrosis + ES-62 and control treatment groups did survive, which immediately indicated that ES-62 treatment might be, in some way, counteracting the severity of the extended ovalbumin administration protocol. Nevertheless, as a result of this unexpected loss samples from the Fibrosis group mice were limited, therefore the *in vitro* analysis was adapted to make best use of the samples available. For example, due to limited resources, insufficient lymph node cells were available for culture, however, sufficient quantities of splenocytes (which responded in a similar manner to DLN cells in the short term model) from all three experimental groups were obtained to allow comparison of treatment groups *ex vivo*.

4.2.13 Splenocyte cytokine production in the chronic airway inflammation model

To determine the antigen-specific responses of lymphocytes *ex vivo* splenocytes from this model were cultured *in vitro* and their responses to antigen and mitogen analysed. Splenocytes isolated from fibrosis model mice were cultured *in vitro* and stimulated with antigen (OVA) or mitogen (Con A). After 72h the culture supernatants were analysed for TH2 cytokines; IL-4, IL-5 and IL-10 and TH1-promoting cytokines; IL-12 and TNFα. Splenocytes from mice in each treatment group displayed spontaneous IL-12 secretion *ex vivo* (Figure 4.12e). Consistent with a TH2 phenotype, splenocytes from fibrosis mice produced less TH1-promoting IL-12 than control cells (from mice that had not received OVA *in vivo*). Prophylactic ES-62 treatment of fibrosis mice appeared to promote the level spontaneous IL-12 production by splenocytes (Figure 4.12e). This pattern of *ex vivo* IL-12 production between splenocytes from the different treatment groups was repeated following stimulation with OVA or Con A *in vitro*, indicating that splenocyte IL-12 production in this model was constitutive, not antigen-specific and independent of culture conditions. However, antigen-specific production of TH2 cytokines was displayed by this cell type, in response to re-stimulation with OVA *in vitro*. TH2 cytokines, IL-5, IL-4 and IL-10 (Figure 4.12a-c) were produced only by splenocytes from mice that had undergone the ova-administration protocol *in vivo* (i.e. fibrosis and fibrosis+ES-62 mice). Findings consistent with this inflammation model exhibiting a TH2 phenotype. Interestingly, in response to OVA treatment, low levels of the pro-inflammatory cytokine, TNFα, were also produced by splenocytes from the three *in vivo* treatment groups (Figure 4.12d). Considering that low levels of TNFα were secreted by splenocytes from control mice, it was concluded that this cytokine secretion was not antigen-specific. Thus, similar to the findings in the previous model, antigen-specific cytokine production by splenocytes from this chronic airway inflammation model was predominantly TH2 in phenotype.

Surprisingly, prophylactic ES-62 treatment of fibrosis group mice *in vivo* increased antigen-specific IL-5, IL-4 and IL-10, but not TNFα production by splenocytes *in vitro*. This TH2-promoting action of ES-62 was opposite to that observed in splenocytes in the short-term asthma model described above and is interesting in view of the survival-promoting effects of ES-62 in this model. However, in addition to its TH2-promoting functions, IL-10 also functions as an anti-inflammatory cytokine, thus, it is possible that ES-62-mediated promotion of IL-10 production is anti-inflammatory in effect. As a polyclonal stimulation for comparison, splenocytes were additionally cultured with mitogenic Con A, but the response was modest. More specifically, Con A treatment *in vitro* induced low levels of IL-5, IL-4 and IL-10 by splenocytes (Figure 4.12a-c) from all treatment groups. In addition, as mentioned above, spontaneous *ex vivo* IL-12 production levels were not significantly modulated further (figure 4.18e). However, splenocytes from control mice appeared to

produce elevated quantities of TNFα in response to Con A, indicating that the TNFα-producing cell population had been targeted by this mitogen in this group (Figure 4.12d).

4.2.14 ES-62 treatment of chronic airway inflammation inhibits production of antigen-specific immunoglobulin isotypes associated with a TH2 phenotype.

It has been well documented that IgE is an immunoglobulin isotype that promotes TH2-driven inflammatory conditions. As described above, prophylactic ES-62 treatment inhibits antigen-specific and total IgE levels in the serum of asthma model mice. Therefore, in this model of TH2-driven inflammation, serum levels of IgE and IgG were analysed. Concurrent with the findings of the asthma model, the chronic airway inflammation induction protocol induced markedly elevated antigen-specific IgG1, IgG2a and IgE in the serum of fibrosis group mice (Figure 4.13a-c). Total serum IgE was also measured and found to be markedly elevated in fibrosis compared to control mice (Figure 4.13d). Interestingly, prophylactic ES-62 treatment of fibrosis significantly reduced ova-specific IgG1, IgG2a and IgE, but not total IgE. ES-62-mediated reduction of ova-specific IgE had previously been observed in the asthma model with prophylactic ES-62 treatment, however, reduction of antigen-specific IgG1 and IgG2a are novel actions of ES-62 in this fibrosis model. This result suggested that in the longer-term inflammation model, ES-62 was permitted to modulate a wider range of immunoglobulin isotypes. It is noteworthy that the Ig isotype most inhibited by ES-62 treatment (IgG1) is of the TH2 (inflammation-promoting) phenotype. This observation indicated that ES-62 inhibited the inflammation exhibited in the fibrosis model by, at least, modulating the antigen-specific response of the antibody production machinery.

4.2.15 OVA-induced chronic airway inflammation in ST2-deficient mice.

ST2, a protein with homology to the IL-1 receptor family, has been found to be stably and selectively expressed on the surface of CD4+ TH2 cells and not on Th1 cells. Thus, it has been suggested that ST2 expression/signalling is important for the function of TH2 cells. It is well documented that chronic airway inflammation is facilitated by a TH2 immune response. Therefore, if ST2 is a marker of TH2 cells and is required for normal TH2 cell function, perhaps long-term TH2 airway inflammation cannot be induced in a system that lacks ST2. To test this theory, the chronic airway inflammation protocol (figure 2.4) was administered in ST2 'knock out' (ST2KO) mice, which were deficient in ST2. ST2 research is currently very controversial and there is a large body of conflicting evidence as to the importance, function and action of ST2. Following one line of evidence, soluble ST2 protein has been shown to have similar effects to ES-62 on innate immune cells, by inhibiting macrophage pro-inflammatory cytokine production in response to LPS [342]. To determine any association between the effects of ES-62 and ST2, the chronic airway

inflammation model in ST2KO mice was treated prophylactically with ES-62 (in a similar manner to that used in wild-type mice).

4.2.16 Comparison of the splenocyte response in wild-type and ST2-deficient models of chronic airway inflammation

To establish the role, if any, of ST2 in the development of the chronic airway inflammation model, the OVA-induced chronic airway inflammation induction protocol was administered in ST2KO mice. Comparison of splenocyte responses in the wild-type and ST2KO models revealed an interesting difference. Whilst treated identically *ex vivo*, the cytokine responses of splenocytes from ST2KO model mice were consistently greater than those of splenocytes from wild-type mice. However, the *pattern* of cytokine production by splenocytes from both models was maintained. As observed in the wild-type model, constitutive *ex vivo* IL-12 production was displayed by splenocytes from the ST2KO model (Figure 4.12). Similarly, antigen-specific IL-4, IL-5 and IL-10 were produced by splenocytes from both models (Figure 4.12a-c and f-h) in response to re-stimulation with OVA *in vitro*. Collectively, these findings indicated that knock-out of ST2 did not prevent development of antigen-specific TH2 cells in this extended model of airway inflammation. Although ST2 deficiency appeared to induce more potent splenocyte cytokine responses, the overall antigen-specific response pattern exhibited by this cell type had not been altered.

Furthermore, ES-62-mediated enhancement of splenocyte cytokine production, exhibited in the wild-type model, was maintained in the ST2KO model, indicating that this action of ES-62 was ST2-independent. More specifically, ES-62 continued to elevate splenocyte antigen-specific IL-5, IL-4 and IL-10 and spontaneous IL-12 production, whilst not modulating TNFα secretion, in the ST2KO model (Figure 4.12). In summary, knocking out ST2 appeared to induce a more strongly polarised antigen-specific TH2 response by splenocytes, but did not modulate ES-62 action on this particular cell type.

4.2.17 Lymph node cell responses in chronic airway inflammation: the role of ST2.

Unlike the effects of the chronic airway inflammation model in wild-type mice, the extended ovalbumin administration protocol did not prove fatal in ST2KO mice. This difference in the survival of the mice might indicate that inflammation and pathology was less severe in ST2KO mice, however this proposal is somewhat at odds with the aforementioned splenocyte responses. Nevertheless, survival of sufficient numbers of ST2KO model mice in both treatment groups permitted analysis of lymph node cell responses in this model. Consistent with the splenocyte responses in the wild-type model, there was spontaneous *ex vivo* IL-12 production in ST2KO DLN (Figure 4.14d), PLN

(Figure 4.14h) and spleen cells (Figure 4.12e). Moreover, in response to re-stimulation with OVA *in vitro*, antigen-specific IL-4, IL-5, IL-10 and TNFα production was exhibited by ST2KO DLN cells (Figure 4.14a-c and e) and splenocytes (Figure 4.12a-d). However, TH1-promoting TNFα production was low, suggesting that in the ST2KO mice the cytokine response was still of a TH2 phenotype. PLN cells from the ST2KO model mice secreted only modest levels of IL-5 and IL-10 in response to OVA re-stimulation *in vitro*, indicating that few antigen-specific cells were present in the lymph nodes distal to the anatomical site of induced inflammation and that the antigen-specific response was predominantly conducted by local lymph node cells and splenocytes. Such an absent/reduced antigen-specific response of PLN cells had previously been observed in the asthma model.

4.2.18 The effects of ES-62 treatment on antigen-specific lymph node cell responses in the OVA-induced chronic airway inflammation model in ST2-deficient mice.

Prophylactic treatment of ST2KO chronic airway inflammation model mice with ES-62 further increased antigen-specific IL-4 (Figure 4.14a), yet decreased antigen-specific IL-5 (Figure 4.14b) and did not significantly modulate antigen-specific IL-10 (Figure 4.14c) production by DLN cells. Furthermore, OVA-specific DLN cell TNFα production, whilst relatively low in value, was reduced by prior treatment with ES-62 *in vivo* (Figure 4.14e). This pattern of ES-62 action on the antigen-specific response of DLN cells was different from the pattern displayed by splenocytes from this model. Although the action of ES-62 on antigen-specific IL-4 was maintained, the effects of ES-62 on antigen-specific IL-10 and IL-5 were absent and reversed respectively. Nevertheless, promotion of DLN cell antigen-specific IL-4 production was the most potent of ES-62 mediated effects in this model, suggesting that enhancement of TH2 cytokine production remained the primary function of ES-62 in these cells.

As mentioned above, antigen-specific PLN cell production of IL-5 and IL-10 (Figure 4.14f,g) was apparent but in very low concentrations (less than 30pg/ml) around the limit of detection. Interestingly, prior ES-62 treatment *in vivo* abrogated antigen-specific IL-5 production by PLN cells from the ST2KO fibrosis model. Although not significant, ES-62 also reduced the mean concentration of IL-10 produced by PLN cells in response to OVA. It was interesting that, as with the short-term asthma model, the effects of ES-62 were opposite in DLN and PLN cells from this model, i.e. promotion and reduction of antigen-specific TH2 cytokine production respectively.

Consistent with observations in the wild-type model splenocytes, the response of LN cells from the ST2KO mice to Con A treatment *in vitro* predominantly reflected production of TH1 cytokines, IL-12 and TNFα.

4.2.19 Comparison of wild-type and ST2-deficient chronic airway inflammation models: immunoglobulin production

Comparison of serum Ig profiles between wild-type and ST2KO models revealed interesting differences in the levels of antigen-specific IgE and IgG generated (Figure 4.13). Despite the relatively lower levels of antigen-specific TH2-like cytokine (IL-4 and IL-5) production *ex vivo*, serum OVA-specific IgE was greater in wild-type fibrosis mice, when compared with serum from ST2KO fibrosis mice, indicating that ST2KO mice were less able to induce an antigen-specific antibody response of TH2 signature isotype, IgE. Interestingly, serum levels of total IgE were similar in wild-type and ST2KO fibrosis mice, thus, ST2 must be necessary for induction of antigen-specific IgE, but not total IgE levels in this model. Furthermore, in the wild-type fibrosis mice, serum OVA-specific IgG2a levels were significantly less than that detected in serum from ST2KO fibrosis mice (Figure 4.13b). Given that IgG2a is a TH1-type IgG subclass, this finding indicated that a more potent TH1-mediated immune response had been activated in ST2KO mice. Thus, ST2 must also be necessary for reducing production of TH1-promoting serum IgG2a in this model. Serum levels of OVA-specific IgG1 were similar in wild-type and ST2KO fibrosis model mice, indicating that the production of this TH2-type subclass of antigen-specific IgG in this model was not dependent upon ST2 expression (Figure 4.13a).

4.2.20 ES-62 treatment of chronic airway inflammation in ST2-deficient mice does not modulate antigen-specific immunoglobulin production

To clarify the action of ES-62 in the ST2KO fibrosis model, serum levels of antigen-specific IgE and IgG and total IgE from ST2-deficient mice in each treatment group were examined (Figure 4.13). The results for analysis of serum from control mice in the wild-type fibrosis model were added to the graphs for comparison.

It was clear from comparison with control wild-type samples that serum Ig was secreted in increased amounts in ST2KO relative to wild-type mice that had undergone the extended OVA administration protocol (Figure 4.13). In contrast to the findings of serum analysis in the wild-type model, prophylactic treatment of fibrosis in ST2KO mice with ES-62 did not significantly modulate serum OVA-specific IgG1, IgG2a, IgE or total IgE (Figure 4.13a-d), suggesting that ST2 expression may be necessary for ES-62-mediated modulation of antibody production or Ig isotype.

Analysis of the effects of ES-62 on serum Ig between wild-type and ST2KO models also revealed interesting information about the roles of ES-62 and ST2 in this model. As mentioned previously, ES-62 inhibited production of OVA -specific IgE in the wild-type fibrosis mice, but not in the ST2KO fibrosis mice (Figure 4.13c). This difference could indicate that ST2 is required for ES-62 mediated inhibition of antigen-specific IgE in this model, however, as mentioned above, ST2KO fibrosis mice were unable to induce a large antigen-specific IgE response. Thus it is also possible that ES-62's inhibitory action on antigen-specific IgE is simply not apparent in ST2KO fibrosis mice because the levels are minimal anyway. It is not clear from this analysis which theory is correct. However, as the OVA-specific IgG1 response in wild-type and ST2KO mice were equivalent, it was apparent that ES-62 mediated inhibition of OVA-specific IgG1 in wild-type fibrosis mice was prevented in ST2KO mice (Figure 4.13a). This result was unambiguous, indicating that whilst ST2 was not necessary for development of the antigen-specific IgG1 response in this model, it was required for the antigen-specific inhibitory effect of ES-62 on this TH2-type IgG subclass. Clearly, in this model ES-62 was unable to mediate its normal immunomodulatory action on serum antibody levels in mice that did not express ST2. As described above, the OVA-specific IgG2a response was enhanced in ST2-deficient fibrosis mice compared with their wild-type counterparts. Nevertheless, ES-62 treatment of wild-type or ST2-deficient fibrosis mice did not induce significant modulation of the level of OVA-specific IgG2a in serum from either type of mouse in this model. Thus, despite the enhancing effect of ST2-deficiency on the antigen-specific IgG2a response, it was clear that ES-62 did not target production of this TH1-type IgG subclass in this model.

In summary, in this model ST2 was required for developing of an antigen-specific IgE response, preventing development of an antigen-specific IgG2a response and the inhibitory effect of ES-62 on antigen-specific Ig of TH2 phenotype. In terms of serum antibody profile, ST2KO mice appeared to develop a mixed IgG1/IgG2a (TH2/TH1) immune response to the OVA administration protocol, but appeared incapable of mounting an IgE response. Thus, as IgE is a TH2-type antibody isotype, it appeared that the antibody response in the ST2KO mice was polarised away from TH2 phenotype.

4.2.21 ES-62 treatment of OVA-induced airway inflammation modulates co-stimulatory molecule expression and cytokine production of bone marrow-derived cells

Following investigation of varied models of TH2-mediated inflammation and the inhibitory effects of ES-62 on such inflammation, it was of interest to determine the mechanisms employed by ES-62 to communicate with the immune system and intervene with the inflammation development machinery. Previous work published by this laboratory has demonstrated that ES-62 mediates profound and significant effects on cells in the first line

of defence or innate immune system, for example, antigen processing cells (APC) such as macrophages and dendritic cells [189-191]. Furthermore, evidence from this laboratory indicates that, via modulation of dendritic cell phenotype and cytokine production, ES-62 is permitted to indirectly modulate TH cell phenotype and hence, the nature of the ensuing immune response [189].

To determine whether APC modulation was a viable method by which ES-62 induces its inhibitory action in the short-term airway inflammation model, bone marrow-derived dendritic cells were cultured from the bone marrow of the prophylactic treatment model mice. The phenotype of these DC was assessed in unstimulated and LPS-matured conditions by analysing cell surface marker expression and cytokine production. Bone marrow-derived DC cultured from control mice that had been treated with ES-62 *in vivo* displayed a moderately matured phenotype, when compared with DC from control mice that had not been treated with ES-62. More specifically, ES-62 treatment induced upregulated cell surface expression of antigen-presentation and co-stimulatory molecules, MHC II, CD40 and CD86 (and marginal upregulation of CD80 expression) on cells subsequently cultured from bone marrow progenitors. This maturation effect was independent of OVA treatment *in vivo* and therefore only observed in DC derived from mice that had been treated with ES-62. (Figure 4.15, panel A). Indeed, DC derived from the 'Asthma' (i.e. OVA alone) treatment group were essentially phenotypically identical to those derived from the control treatment group mice. Interestingly, this upregulatory effect of prior ES-62 treatment *in vivo* on resultant bone marrow-derived DC is the reverse action to that induced by continuous ES-62 exposure *in vivo* in naïve mice [191]. In more detail, ES-62 administered continuously *in vivo* for 14 days, via the use of osmotic pumps, conferred an immature phenotype on the bone marrow DC subsequently derived from such mice. Nevertheless, the cell surface molecules that were upregulated on DC from ES-62 treated asthma model mice are important for DC mediated T cell priming. In particular, CD86 is an important co-stimulatory signal, required for successful priming of naïve T cells by antigen-presenting DC, leading to differentiation of antigen-specific T helper (TH) cells [349, 350].

In vitro stimulation of asthma model bone marrow DC with LPS induced upregulation of all cell surface molecules analysed, on DC from all four *in vivo* treatment groups (Figure 4.15, panel B). Nevertheless, a distinct ES-62-specific pattern was maintained following LPS treatment. Thus, DC from mice treated with ES-62 *in vivo* displayed a slightly inhibited LPS-induced upregulation of MHCII, CD40, CD54 and CD80. DC from the Asthma+ES-62 treatment group mice consistently expressed the lowest levels of all five surface markers in response to LPS treatment. These findings indicated that in ES-62-treated asthma model mice, the normal response of bone marrow derived DC to potent

pro-inflammatory stimuli (such as LPS) was dampened. Furthermore, such an inhibited upregulation response to LPS treatment *in vitro* was characteristic of DC derived from mice that had previously been exposed to ES-62 (administered via osmotic pumps) *in vivo* [191].

To obtain a more complete picture of the phenotype of DC derived from asthma model mice, the culture supernatants of the bone marrow-derived DC described above were analysed for cytokine content. Bone marrow-derived DC from mice in each treatment group produced detectable levels of IL-12 and TNFα. The patterns of IL-12 and TNFα production reflected the maturation status, as indicated by cell surface expression of these bone marrow-derived DC. More specifically, DC cultured from asthma model mice that had been treated with ES-62 *in vivo* displayed enhanced production of IL-12, when compared with DC from mice that had not been treated with ES-62 (Figure 4.16, panel A). Low-level induction of IL-12, following ES-62 treatment of bone marrow-derived cells has previously been demonstrated *in vitro*, but not *in vivo* [190, 191]. Interestingly, the TNFα production by DC from each treatment group was very low overall (15-20 pg/ml) and did not vary between treatment groups. In summary, ES-62 treatment *in vivo* appeared to modulate bone marrow progenitor cells in order that they differentiate into DC with slightly enhanced TH1-promoting cytokine production and expression of surface markers.

Following analysis of spontaneous IL-12 and TNFα production patterns of DC derived from this model, the DC cytokine production profile in response to LPS was determined. LPS stimulation induced markedly increased production of IL-12 and TNFα by the DC derived from the asthma model mice. Interestingly, the pattern of spontaneous IL-12 production by DC derived from the different *in vivo* treatment groups was maintained (at higher concentrations) in response to LPS (Figure 4.16, panel B). More specifically, DC from mice treated with ES-62 *in vivo* secreted more LPS-induced IL-12 than DC from Control and Asthma mice. Similarly, although the concentration of DC TNFα was increased by LPS stimulation, the level of production was not significantly different between DC from each of the *in vivo* treatment groups. Therefore, whilst the pattern of DC surface expression was altered by LPS treatment, the pattern of IL-12 secretion was not. Furthermore, the pattern of cytokine production by DC derived from ES-62 treated mice in this inflammation model was uncharacteristic of the previously determined effects of ES-62 treatment *in vivo* [191]. More specifically, prior treatment of naïve mice with ES-62 *in vivo* induces differentiation of bone marrow DC that display an inhibited TH1-promoting cytokine production response to LPS, similar to the effects of ES-62 pre-treatment on bone marrow-derived DC *in vitro* [191]. Nevertheless, in summary, ES-62 treatment of asthma model mice *in vivo* induced modulatory effects on the phenotype and function of DC derived from bone marrow of mice in this model of TH2-mediated inflammation.

4.2.22 OVA-induced chronic airway inflammation promotes development of a modulated bone marrow-derived cell phenotype, which is prevented by ES-62 treatment

As described above, prophylactic treatment of the Ovalbumin-induced asthma model with ES-62 modulated (subsequently induced) DC function and phenotype. Following demonstration of ES-62-mediated modulation of antigen-specific responses and serum antibodies in the longer-term chronic airway inflammation model, analysis was extended to determine the effects of ES-62 treatment on bone marrow-derived DC from this fibrosis model. Thus, bone marrow-derived DC from each treatment group in the fibrosis model were cultured *in vitro* for 7 days from femoral bone marrow progenitor cells and stimulated with LPS. Following culture, DC were stained for expression of cell surface molecules normally upregulated by activated DC *in vivo*. The results of this analysis are presented in Figure 4.17. Close examination revealed that DC derived from fibrosis mice displayed reduced expression of cell surface antigen-presentation and co-stimulatory molecules, when compared with DC from the control treatment group (Figure 4.17, panel A). More specifically, in fibrosis DC, a population with low expression levels of MHCII, CD40, CD54, CD80 and CD86 was apparent, that was not observed in DC from the other treatment groups. When fibrosis was treated with ES-62 *in vivo*, the low-level expressing populations were lost and the histogram of expression (for each surface marker) shifted to the right. Thus, it appeared that ES-62 treatment restored the BMDC phenotype towards that observed in DC from the control treatment group mice. It was interesting to note that the 'upregulatory' action of ES-62 on DC from this model had also been (modestly) observed in DC from the shorter-term airway inflammation model (section 4.2.21).

The response to LPS, by DC from the fibrosis model was mediated by upregulation of expression of all five surface molecules analysed. This shift was exhibited in DC from all treatment groups (at least in terms of loss of the low-level expressing populations) in response to LPS (Figure 4.17 panel B). The pattern of expression profiles displayed between untreated DC from different treatment groups was maintained in response to LPS. More specifically, DC derived from mice in the fibrosis treatment group continued to express lower overall levels of MHCII, CD40, CD54, CD80 and CD86 than DC from those in the control treatment group. Similarly, DC derived from mice in the fibrosis + ES-62 treatment group displayed slightly increased expression levels of the same cell surface markers, compared to those derived from the fibrosis treatment group. Therefore, although LPS induced upregulation of all the DC surface markers analysed *in vitro*, the patterns of surface expression induced by the fibrosis induction protocol and ES-62 treatment *in vivo* were not modulated.

In parallel with analysis of DC surface markers, the cytokine production profiles of DC derived from the fibrosis model were determined. DC derived from this model spontaneously produced detectable levels of the TH1-promoting cytokines, IL-12 and TNFα and anti-inflammatory cytokine, IL-10 (Figure 4.18, panel A). However, compared to IL-12 production, the TNFα production by the DC in this assay was modest. The OVA administration protocol induced increased constitutive production of IL-12 and slightly (but not significantly) elevated TNFα by DC. The elevated IL-12 production was prevented by ES-62 treatment *in vivo*, indicating that ES-62 treatment *in vivo* had rescued the modulatory effect of the fibrosis induction protocol on bone marrow-derived cell pro-inflammatory cytokine production. The restorative effect of ES-62 in this model had also previously been observed in terms of DC surface expression pattern. Interestingly, DC cultured from control mice spontaneously produced relatively high concentrations of IL-10 (over 700pg/ml), this was inhibited in DC derived from fibrosis treatment group mice, irrespective of ES-62 treatment *in vivo* (Figure 4.18, panel A).

Production of IL-12 and TNFα by DC derived from all the treatment groups was markedly increased upon treatment with LPS (Figure 4.18, panel B). Furthermore, the pattern of production of IL-12 and TNFα in response to LPS was uniform between the DC from different *in vivo* treatment groups. In more detail, DC cultured from control mice in this model displayed marked production of IL-12 and TNFα in response to LPS. However, the TNFα and IL-12 responses to LPS were significantly less in DC derived from the fibrosis treatment group. Interestingly, ES-62 treatment of fibrosis *in vivo* appeared to induce partial rescue of the inflammatory cytokine response to LPS, by permitting LPS-induced production of IL-12 and TNFα to a concentration not significantly different from that produced by DC derived from control mice. Therefore, similar to the pattern of IL-12 production by these cells under control conditions, *in vivo* ES-62 treatment prior to the *in vitro* differentiation of bone marrow-derived DC appeared to rescue the modulatory effect of the fibrosis induction protocol on DC cytokine production. Additionally, LPS treatment induced production of IL-10 by DC from each treatment group, however, there were no significant differences in the levels of IL-10 produced by different treatment groups.

Collectively, the analysis of bone marrow-derived DC cultured from the chronic airway inflammation model indicated that both, the fibrosis induction protocol, and ES-62 treatment, modulated the phenotype of bone marrow progenitor cells. This resulted in differentiation of DC with a modulated phenotype and function. Moreover, the modulation of DC phenotype or function induced by the fibrosis induction protocol was, at least partially, corrected by simultaneous ES-62 treatment. Furthermore, the combined fibrosis induction protocol and ES-62 treatment *in vivo* induced differentiation of bone marrow-derived DC that displayed a somewhat matured phenotype (evidenced by upregulation of

cell surface markers) and that were potentially biased towards promoting a TH1immune response (evidenced by enhanced production of TH1-promoting cytokine, IL-12).

4.2.23 ES-62 mediated restoration of bone marrow-derived cell phenotype in OVA-induced chronic airway inflammation is further promoted in ST2-deficient mice.

It had previously been determined that knock-out of ST2 prevented development of OVA-specific IgE antibody responses and mediation of some ES-62 actions. Therefore, it was important to determine the action of the OVA-immunisation protocol and the effect of ES-62 treatment on bone marrow-derived DC from the ST2-deficient fibrosis model. Bone marrow-derived DC from ST2KO fibrosis model mice were cultured as described above. DC phenotype and function was again assessed by analysing cell surface expression and cytokine production.

Analysis by flow cytometry uncovered interesting changes in the cell surface expression patterns of DC derived from the ST2KO mice, however firstly, it must be clarified that the overall cell surface expression differences observed between DC derived from different *in vivo* treatment groups were modest. Firstly, a comparison of DC derived from wild-type and ST2KO mice in the fibrosis treatment groups was made. Close inspection of the respective cell surface expression profiles (Figure 4.19) revealed that the DC derived from ST2-deficient fibrosis model mice exhibited a somewhat matured phenotype compared with the DC derived from wild-type fibrosis model mice. In more detail, a population of DC expressing low levels of MHCII, CD40, CD54 and CD86 was apparent in DC cultures from wild-type fibrosis model mice. The number of cells in this low-expressing population was however less in DC derived from ST2-deficient fibrosis treatment group mice. Thus, it appeared that knock-out of ST2 prevented differentiation of DC with an immature phenotype in response to the OVA immunisation protocol, as had been induced in the wild-type model (described above).

Interestingly, ES-62 treatment of ST2-deficient fibrosis model mice *in vivo* appeared to 'correct' this effect of ST2 knock-out. More specifically, the population of low-level expressing cells identified in DC cultures from wild-type fibrosis model mice was also apparent in DC cultures from ES-62-treated ST2KO fibrosis model mice. (Figure 4.19, panel A). Thus, the DC surface expression profiles of MHCII, CD40, CD54, CD80 and CD86 on DC derived from 'wildtype fibrosis' and 'ST2KO fibrosis+ES-62' mice were highly similar.

This down-regulatory effect of ES-62 on surface expression of DC from the ST2KO fibrosis model was particularly interesting because it was the reverse of its action on DC from the wild-type fibrosis model.

The response of ST2KO fibrosis model DC to LPS stimulation *in vitro* was also analysed and compared with that of wild-type fibrosis model DC. In agreement with previous experiments, LPS treatment induced some upregulation CD40, CD54, CD80 and CD86 analysed on DC from the three experimental groups in this model (Figure 4.19, panel B). Interestingly, the expression profiles of MHCII, CD40, CD54 and CD86 on DC derived from wild-type and ST2-deficient fibrosis model mice became more alike upon LPS treatment, mainly comprising loss of the low-level expressing population of cells in DC cultures from wild-type fibrosis mice, observed under control conditions. Similarly, surface expression levels of MHCII, CD40, CD80 and CD86 on DC derived from 'ST2KO fibrosis' and 'ST2KO fibrosis+ES-62' mice were highly similar. Thus, LPS treatment *in vitro* appeared to have a more pronounced upregulatory effect on DC derived from wild-type fibrosis mice and ST2-deficient fibrosis mice that had been exposed to ES-62, than on DC derived from ST2-deficint fibrosis mice that had not been treated with ES-62. However, it must be noted that the majority of responses to LPS were slight. These combined results highlighted two differences in this ST2KO model. Absence of the ST2 gene appeared to prevent the normal action of the fibrosis induction protocol on resultant DC phenotype. However, interestingly, ES-62 treatment of ST2-deficient fibrosis model mice appeared to 'rescue' the effect of ST2-deficiency, in terms of the modulation of DC phenotype. More specifically, in the fibrosis model in ST2KO mice, DC derived from mice treated with ES-62 were less mature than DC from mice not treated with ES-62. This was in direct contrast to the findings of the wild-type experiment, where ES-62 treatment induced production of more mature DC. To investigate this more fully, the supernatants from these cultures were analysed for cytokine content.

Culture supernatants from DC derived from the ST2KO fibrosis model were analysed for IL-12, TNFα and IL-10 and the results compared with those from the wild-type fibrosis model mice (Figure 4.20). Similar to DC derived from the wild-type model, DC derived from ST2KO mice in from both treatment groups spontaneously produced low concentrations of TNFα (approximately 80pg/ml), higher quantities of IL-12, and negligible IL-10 (Figure 4.20, panel A). Interestingly, spontaneous production of TNFα by DC derived from ST2KO fibrosis model mice was significantly greater than that released by DC derived from wild-type fibrosis model mice. Furthermore, similar to its action in the wild-type fibrosis model, ES-62 treatment of the ST2KO fibrosis model mice *in vivo* did not modulate this level of spontaneous TNFα production by resultant DC. In contrast, spontaneous IL-12 production levels by DC derived from ES-62-treated ST2KO fibrosis model mice were significantly enhanced from the levels produced by DC derived from fibrosis model mice of either strain. Thus, consistent with the findings of the DC surface

expression analysis in this model, the IL-12-induction effect of ES-62 was opposite to the observed inhibitory effect of ES-62 on IL-12 production by DC from the wild-type model.

LPS stimulation of DC *in vitro* induced marked increases in production of IL-12, TNFα and IL-10 by ST2KO fibrosis model DC, which were not significantly modulated by ES-62 treatment *in vivo* (Figure 4.20, panel B). In the wild-type model, the modulatory action of ES-62 was still apparent in response to LPS. Therefore, this difference indicated that ST2 might be required for ES-62-mediated enhancement of the TH1-promoting cytokine production response of DC to LPS.

In summary, modulatory action of prior ES-62 treatment on phenotype and function of DC subsequently derived from fibrosis model mice was apparent in the presence and absence of ST2 expression. However, the specific actions of ES-62 on DC derived from ST2KO mice were altered, when compared with those displayed in DC from wild-type mice. That is, the effect of ES-62 on surface expression and spontaneous IL-12 production was reversed in DC derived from ST2-deficient fibrosis model mice. This difference may form part of the explanation for redundancy of ES-62 action in the ST2KO fibrosis model.

4.2.24 Investigation of the phenotype of bone marrow macrophages derived from asthma model mice

Macrophages are important cells of the innate immune response involved in facilitation and maintenance of inflammation. Via recruitment and activation of inflammatory cells and destruction of pathogens or tissue debris at the site of inflammation, macrophages form an integral component of established TH1 and TH2 immune responses.

To investigate the effects of *in vivo* exposure on macrophages and their progenitors, macrophages derived from the bone marrow of asthma mice and asthma + ES-62 mice were cultured in vitro and their cytokine production was analysed. Interestingly, it was apparent that the bone marrow-derived macrophages from the different *in vivo* treatment groups spontaneously produced differential levels of cytokines. In more detail, macrophages from mice that had received the ova-induced inflammation protocol *in vivo* produced significantly enhanced levels pro-inflammatory cytokines, TNFα and IL-12, when compared with macrophages from control treatment group mice (Figure 4.21), indicating that macrophages cultured from bone marrow of ova-treated mice exhibited an activated phenotype. Low levels of IL-10 were also constitutively produced, with macrophages from the asthma group mice producing the greatest amount. Interestingly ES-62 treatment of asthma model mice *in vivo* inhibited the spontaneous production of all three cytokines by the bone marrow derived macrophages subsequently derived from this

model. In summary, ES-62 mediated impedance of inflammation in this model was associated with inhibition of spontaneous cytokine production by bone marrow-derived macrophages.

4.3 Discussion

The results presented in chapter 3 illustrated the substantial inhibitory effects of ES-62 on TH1-mediated inflammation *in vivo*, using a model of rheumatoid arthritis. In this chapter, evidence demonstrating a novel inhibitory action of ES-62 in inflammation generated by a TH2-type immune response has been put forward. Furthermore, the anti-inflammatory potential of ES-62 in this type of inflammation was made clinically relevant by demonstrating the persistence of the inhibitory action when administered after the onset of antigen-induced inflammation.

4.3.1 ES-62 inhibits TH2 mediated airway inflammation

To reiterate, ES-62 significantly inhibited OVA-induced eosinophilia and peribronchial inflammation in a short-term model of asthma–like pulmonary inflammation. Consistent with asthma in humans, the inflammation generated in this OVA-induced model was found to be of TH2 phenotype, identified by analysis of the airway cytokine profile. Furthermore, ES-62 appeared to specifically target the TH2-facilitated, allergen-induced inflammation, because the IL-4 concentration detected in the airways was inhibited by ES-62 treatment. Satisfied that ES-62 inhibited cardinal features of airway inflammation (eosinophilia and TH2 cytokines), investigation of this model proceeded to examine the immune response at various locations within the body.

The antigen-specific TH2 cytokine production observed by cells from draining lymph nodes and spleen was not apparent in peripheral LN cells, indicating the local nature of the immune response to the OVA sensitisation and challenge protocol. In contrast, the effects of ES-62 treatment were evident within and outwith the local inflammation-exhibiting areas. More specifically, ES-62 inhibited antigen-specific TH2 cytokine production by lymphocytes near the site of inflammation, whilst modestly elevating the proliferation of lymphocytes in peripheral lymph nodes, distant from the site of inflammation. The OVA administration protocol stimulated DLN cell and splenocyte proliferation and this effect was not further modulated by ES-62 treatment, indicating that the differences observed in *ex vivo* cytokine production by these cells could be considered a selective effect of ES-62 rather than non-specific suppression. Indeed, it appeared that ES-62 induced inhibition of inflammation in this model by modulating the antigen-specific response of splenocytes and DLN cells. Furthermore, the effects of ES-62 were dependent upon the location of the cells within the body and, moreover, their proximity to (and, hence, influence on) the site of inflammation.

With the aim of analysing the systemic effects of this model of TH2-mediated inflammation, serum samples and sections of lymph nodes were analysed for IgE content. Antigen-specific IgE has an important role in facilitating TH2-type allergic inflammation

[351] and, via the bloodstream, the allergy-inducing action of IgE is communicated around the body. ES-62 significantly inhibited ova-specific IgE concentration in the serum and appeared to reduce the level of IgE expressed in the lymph node follicles. This indicated that secretion and expression of the signature TH2 Ig isotype, was targeted by ES-62 action and may underlie some of the anti-inflammatory effects observed. Indeed, anti-IgE therapy has been proposed for treatment of allergic disease [336, 352], supported by the findings that reduction of IgE leads to decreased mast cell degranulation, inhibition of release of inflammatory mediators and abrogation of the inflammatory cascade [351, 353]. Relating to this, it was interesting to note that administration of ES-62 after the onset of inflammation did not modulate serum IgE. This indicated that ES-62 was able to inhibit the development of the IgE response (exhibited with prophylactic treatment), but not reduce established production levels of IgE. Moreover, this difference in the action of ES-62 between treatment protocols, suggested that ES-62 might act differently when administered before and after the onset of inflammation in this model of TH2-like inflammation. Although serum antigen-specific IgG1 and IgG2a responses were exhibited in the asthma model mice, ES-62 treatment did not significantly modulate the production of either. It is well known that IgG1 and IgG2a promote TH2 and TH1 immune responses respectively. Therefore, it is clear that ES-62 does not exert its anti-inflammatory effects in this model via disruption of IgG production or polarisation of the IgG response.

To begin to dissect the mechanisms of anti-inflammatory action of ES-62 in this model, bone marrow-derived DC were analysed. DC have been shown to expand in the lung after antigen challenge in this type of inflammation model [354] and are paramount in initiation of adaptive immune responses due to their ability to efficiently process and present antigen to naïve T cells [355]. Indeed, it has been demonstrated that depletion of $CD11c^+$ DC from a model of airway inflammation prevented development of asthma-like pathology [356]. It is well established that DC facilitate their communication with T cells by altering cell surface expression (of e.g. co-stimulatory molecules) and secreting cytokines. Indeed, it was recently demonstrated that modulation of surface expression on $CD11c^+$ antigen-presenting cells induces inhibition of allergic airway inflammation in mice [357]. Furthermore, multiple previously published studies conducted in this laboratory have highlighted profound immunomodulatory action of ES-62 on cytokine production and cell surface expression by DC [189-191]. Thus, in line with previous research, it was hypothesised that ES-62 may be inducing anti-inflammatory action in this model via modulation of this cell type. Examination of DC cultured from the short-term airway inflammation model revealed significant effects of ES-62 on DC phenotype. More specifically, DC were observed to be somewhat activated by ES-62 to produce TH1 cytokines (such as IL-12) and appeared more mature in phenotype, exhibiting increased expression of cell surface markers, such as CD40, required for priming T cells. It has been

suggested that CD40 may have a protective function in asthma development, because CD40-deficient mice exhibited enhanced responses in a model of airway inflammation [358], therefore ES-62 mediated enhancement of CD40 expression on DC may contribute to the anti-inflammatory effects observed in this model.

It has also previously been demonstrated that ES-62 treatment of DC *in vitro* (in the absence of any other immunomodulatory influence) promotes development of a TH2-type immune response [189]. This was associated with an immature phenotype of the DC, evidenced by low-level expression of cell surface markers and low-level production of TH1-promoting cytokines. Thus, by contrast, it might be hypothesised that a mature phenotype and enhanced production of TH1-promoting cytokines, as displayed by the DC derived from ES-62 treated mice in the asthma model, could promote development of a TH1-mediated immune reponse and polarise responses away from a TH2-phenotype. Therefore it might be proposed that the difference in the action of ES-62 in the asthma model may have been in response to the ongoing OVA-specific TH2-mediated immune response in these mice. However, it must be remembered that ES-62 induced these effects in control treatment group mice also, suggesting that it was most likely not in response to the effects of the existing TH2-type immune response.

Evidently, ES-62 treatment of mice in this model significantly altered the phenotype of bone marrow progenitor cells *in vivo*, resulting in modulation of the function of the cells that differentiated from these precursors. It may be possible to postulate that the increased production of IL-12 by bone marrow DC, exposed to ES-62 *in vivo*, led to inhibition of pulmonary inflammation, based on previously published research. Thus, IL-12 has been reported to suppress allergen-induced eosinophil infiltration of the lungs [359] and relieve airway constriction. Furthermore, DC production of IL-12, induced by bacteria, or by employing adenovirus to alter DC IL-12 expression [360], alleviates TH2-facilitated eosinophilic inflammation and airway hyper-reactivity in murine models of asthma. Based on these studies, the DC-activating action of ES-62 observed in the current investigation would appear to be sufficient for inhibition of pulmonary inflammation. However, this postulation is contradicted by the findings of Kuipers et al [361], who demonstrated that LPS-induced DC-facilitated inhibition of pulmonary inflammation was independent of IL-12 production by the DC.

Whilst ES-62 treatment of mice resulted in production of activated DC that produce increased levels of IL-12, the cell surface expression upregulation response of these cells to potent immunogenic agent, LPS, was inhibited. Furthermore, this inhibitory action was most pronounced in DC derived from ES-62 treated mice that had received OVA immunisation and challenge. This indicates that these cells might exhibit an inhibited

response to inflammatory stimuli, *in vivo*, for example at the site of inflammation. Indeed, it has been suggested that LPS is required for TH2 sensitisation in mouse models of asthma [362]. Furthermore, this inhibitory action of ES-62 on the response to LPS is characteristic of its previously determined actions on bone marrow-derived cells *in vitro* [190], however it must be noted that in these *in vivo* experiments, ES-62 was administered subcutaneously, therefore not directly to the cells, thus the last application was 7 days prior to the *in vitro* LPS stimulation. This provides more support for the theory that ES-62 modulates bone marrow progenitor cells *in vivo* and that this modulation is maintained as the cells differentiate.

Nevertheless, considering the previously demonstrated anti-inflammatory effects of ES-62 on DC phenotype *in vitro* and *in vivo* [191] it was unexpected that ES-62 treatment of the asthma model would induce development of DC that exhibited an enhanced cytokine production response to LPS *in vitro*. However, it might be proposed that increased production of TH1-promoting cytokine, IL-12 by DC in response to inflammatory stimuli is an effect that would help to induce a TH1-mediated immune response, and hence, polarise the immune response away from a TH2-phenotype. Thus, on a background of TH2-mediated inflammation, this TH1-promoting action of ES-62 might be anti-inflammatory.

The therapeutic effect of ES-62 treatment, after the onset of inflammation indicated that the modulatory effects of this parasite product were most likely not confined to manipulation of initiation of the immune response. It was more probable that ES-62 action also inhibited ongoing and established inflammatory effector mechanisms. Therefore, bone-marrow derived macrophages, effector cells that are commonly located in inflammatory sites and are the predominant immune effector cell resident in the alveolar spaces [363], were also analysed.

Clearly, the OVA administration protocol applied in this model induced modulatory effects on bone marrow-derived macrophage function, demonstrated by heightened production of inflammatory cytokines. This pattern of increased cytokine production indicated that these macrophages were pre-activated. Furthermore, the inhibitory effect on cytokine production by macrophages derived from ES-62 treated mice was reflected in ES-62-mediated modulation of airway inflammation in this model. Indeed, TNFα production by macrophages has been attributed to promotion of inflammation exhibited in asthma. Asthma patients often exhibit increased levels of TNFα in broncho-alveolar lavage fluid [364] and furthermore, increased airway TNFα levels have been associated with increased airway hyper-reactivity in murine models of asthma [365]. It has been demonstrated that the association between TNFα levels and airway inflammation arise

because TNFα induces production of chemokines and pro-inflammatory mediators, which induce upregulated expression of adhesion molecules (e.g. ICAM-1 and VCAM-1) on the surface of epithelial cells, that in turn, leads to recruitment of leukocytes to the airways [366]. Thus, the TNFα-reducing effect of ES-62 on macrophages may be anti-inflammatory in this model of airway inflammation. However, the overall reduction of cytokine production by macrophages derived from ES-62 treated mice in this model indicates that these cells are most likely, less activated. Nevertheless, as macrophages form an important component of inflammation induction in this model, a reduced activation status might also induce anti-inflammatory effects.

It was interesting that this action of ES-62 on macrophage cytokine production was opposite to the observed effects on bone marrow derived DC, indicating that ES-62 action on bone marrow progenitor cells in this model does not necessarily induce identical effects on the cell types into which they differentiate. However, it is well established that as part of an immune response, DC and macrophages fulfil different roles. For example, DC are a fundamental cell type for initiation of an immune response, by priming antigen-specific T cells, whilst macrophages are well-established innate cell type, with important roles in mediation of inflammation at the inflammatory site. In other words, whilst the roles of DC and macrophages within the immune system can overlap, DC are generally considered to be involved in immune response initiation, whilst macrophages are considered effector cells. Thus, it is perhaps logical that ES-62 mediates different actions on these cells. Such different actions may represent the respective effects of ES-62 on the initiation and effector mechanisms of this model, which have been depicted in Figure 4.22 and Figure 4.23.

4.3.2 The airway inflammation model does not exhibit structural changes associated with human asthma

It is well documented that the reversible inflammation exhibited in human asthma is accompanied by irreversible and progressive pathological changes in the lungs such as airway fibrosis, hyperplasia of smooth muscle and goblet cells and narrowing of the airways. However, it remains a matter of debate whether the irreversible changes in the airways induce the periods of reversible acute airway hyper-reactivity and inflammation or vice versa [367]. Nevertheless, it is generally accepted that the structural changes exhibited do exacerbate the effects of an acute attack of inflammation in asthma.

Using myography, tracheal smooth muscle contractility in the asthma model was compared with that of control mice. It was found that the short-term model of airway inflammation did not exhibit obvious associated changes in such smooth muscle

contractility. It has, however, recently been identified, using a murine model, that hyper-responsiveness of airway smooth muscle in asthma is exhibited by bronchial, but not tracheal smooth muscle [368]. Therefore, because tracheal smooth muscle was analysed in this model, this may have been the reason for this negative outcome. The myography apparatus used was not designed for analysis of airways smaller than murine trachea, however future investigations might prove more informative by examining smaller airways from the pulmonary tree in this model.

Nevertheless, this finding indicated that smooth muscle hyperplasia or hypertrophy was most likely not exhibited in the trachea of these mice. Furthermore, it can be inferred that inflammation in this model developed in the absence of prior airway smooth muscle pathology. However, it might be postulated that if the inflammation were allowed to continue in this model, prolonged exposure to inflammation may induce smooth muscle pathology, as exhibited in human asthma [84]. Prophylactic ES-62 treatment did not modulate the contractility of airway smooth muscle in this model either, indicating that the effects of ES-62 on pulmonary inflammation were exclusively immunological.

4.3.3 Differences in immunology between short-term and longer-term pulmonary inflammation models

To better represent the immunology underlying prolonged pulmonary inflammation in asthma, the short-term airway inflammation model was extended, with the aim of inducing structural remodelling of the airways. Analysis of the immunology in this model revealed many similarities when compared and contrasted with the previous model of acute pulmonary inflammation. Thus, splenocytes from both models secreted IL-5, IL-4 and IL-10 in response to OVA re-stimulation *in vitro*, highlighting the TH2 nature of the inflammation in both models. Additionally, the ovalbumin administration protocol induced secretion of antigen-specific IgG1, IgG2a, IgE and total IgE in both models. Thus, it appeared that increasing the period of administration of the OVA inflammation-induction protocol did not modulate the nature of the antigen-specific immune response that was generated. However, there were some differences observed when bone marrow-derived DC from both studies were compared. In contrast to the findings in the short-term model, bone marrow-derived DC from the chronic inflammation model exhibited reduced DC surface marker expression, yet increased spontaneous IL-12 production in response to the extended ovalbumin protocol *in vivo*. By contrast, DC from OVA-treated (fibrosis treatment group) mice in the chronic model exhibited reduced cell surface expression and TH1-promoting cytokine response to LPS *in vitro*. Thus, modulation of bone marrow-derived DC phenotype was sensitive to chronicity of OVA application in vivo. It has previously been identified that DC phenotype is pivotal in communication of the immune response throughout the circulation and hence, the secondary lymphoid organs, where

immune responses are generated. Moreover, DC generally secrete mainly TH1-promoting cytokines or anti-inflammatory cytokines such as IL-10, hence prolonged *reduction* of TH1-promoting cytokine production by these cells *in vivo* may promote development of a TH2 immune response by T cells that activated DC communicate with. Indeed, ES-62-treated DC derived from naïve mice have been shown to promote development of a TH2 immune response [189]. Furthermore, DC derived from naïve mice that were pre-treated with ES-62 *in vivo* or *in vitro* exhibit inhibited LPS-induced production of pro-inflammatory cytokines and reduced spontaneous expression levels of co-stimulatory molecules [189, 191]. Thus, DC derived from OVA-treated mice in the chronic airway inflammation model also appear to exhibit this 'DC2' phenotype. However, in this model, ES-62 action appears to change the phenotype of the DC to become mature and hence, less TH2-promoting.

4.3.4 The modulatory action of ES-62 is altered in the chronic pulmonary inflammation model.

As discussed in section 4.2.12, loss of the mice in the long-term inflammation model led to a restricted capacity for comparison of the immunology between acute and chronic airway inflammation models. Nevertheless, it was an interesting observation that the majority of mice in the fibrosis group had to be removed from the study due to the severity of the inflammation protocol, whilst the mice treated with ES-62 all maintained a level of health consistent with continuation of the study to completion. This observation indicated that ES-62 treatment reduced the severity of the inflammation induced or delayed the onset of terminal pathology in this model. Given that the immune response and action of ES-62 on splenocytes from the short term model was similar to that observed upon analysis of DLN cells, it was concluded that (in the absence of sufficient DLN cell numbers) analysis of splenocytes from the longer-term model could provide appropriate information about the local OVA-specific immune response generated in this model.

As with the acute 'Asthma' model, ES-62 modulated the immune response observed in the longer-term model of airway inflammation. Unlike its inhibitory action in the short-term model, however, ES-62 treatment induced *promotion* of splenocyte antigen-specific TH2 cytokine production in the longer-term model. This change in action suggested that the function of ES-62 in each model was different. Furthermore, it also suggested that ES-62 action on splenocyte antigen-specific responses is dependent upon chronicity of inflammation.

At first sight, these results might appear counter-intuitive as it might be postulated that promotion of TH2 cytokine production in a model of TH2-mediated inflammation would enhance severity of the inflammation, however it has recently been demonstrated that parasite-mediated inhibition of TH2-type pathology was associated with increased

production of certain TH2 cytokines [369]. More specifically, in a model of allergen-induced airway hyper-reactivity (AHR), infection with non-egg laying *Schistosoma mansoni* reduced development of AHR and it was demonstrated that the inhibitory effect was associated with elevated production of antigen-specific IL-4 and IL-10. In addition, the TH2-like cytokine, IL-10, can act as an anti-inflammatory cytokine, to counteract inflammation-promoting production of TH2 cytokines. Thus, recently it was demonstrated that $CD4^+CD25^+$ regulatory T cells function to reduce AHR in an IL-10-dependent mechanism [370]. Therefore, ES-62 mediated promotion of antigen-specific IL-4, IL-5 and IL-10 production in the long-term inflammation model does not necessarily reflect enhancement of severity, and may even indicate amelioration of disease. Indeed, the reduced mortality of ES-62 treated mice in this model would support the latter theory.

The inhibitory action of ES-62 treatment on the levels of antigen-specific serum IgE in the asthma model was maintained in the chronic model, indicating that ES-62 targets the antigen-specific IgE-mediated immune response in both models, independent of chronicity of inflammation. In the longer-term inflammation model, serum analysis uncovered additional inhibitory action of ES-62 on IgG antibody response development. More specifically, ES-62 treatment reduced OVA-specific (TH2-type) IgG1 in the chronic, but not the acute model of airway inflammation. This difference indicated that longer application of ES-62 might be required to induce a change in antigen-specific IgG secretion/metabolism or that this action of ES-62 was only induced in response to the chronic inflammation. It is of notable interest that the inhibitory effect of ES-62 on antigen-specific IgG is on the IgG1 isotype, indicating that ES-62 preferentially targets this IgG isotype despite elevating TH2 cytokines in this model. Thus, in the chronic inflammation model, ES-62 targeted production of IgE, an inflammatory TH2-isotype antibody and IgG1, the hallmark antibody isotype of TH2-mediated immune responses.

As mentioned previously, it was clear that the longer-term OVA administration protocol induced distinct effects on DC phenotype that had not been observed in the short-term acute model. These effects were partially reversed by prior ES-62 exposure *in vivo*. Thus, as mentioned above, DC derived from the chronic inflammation model spontaneously secreted elevated quantities of IL-12 and reduced IL-10 compared to control cells. This increased IL-12 production was not displayed by DC from ES-62 treated mice. Thus, in this model, ES-62 is acting in an inhibitory low level, manner on spontaneous TH1-promoting cytokine production. By contrast, although production of IL-12, TNFα and IL-10 was increased when DC were stimulated with LPS *in vitro*, the level of production was inhibited in DC from OVA-treated mice relative to that observed with DC from control treatment group mice. Under these conditions, the inhibited TH1 cytokine response (IL-12 and TNFα but not IL-10) to LPS was overcome by pre-exposure to ES-62 *in vivo*. Thus,

exposure to ES-62 appears to act to normalise the modulated spontaneous cytokine production of the DC derived from OVA treated mice and it also normalises the cytokine response of these cells to inflammatory stimuli. The discussed effects of ES-62 in the longer-term model of airway inflammation are summarised in Figure 4.24.

4.3.5 The role of ST2 in the pathogenesis of longer term pulmonary inflammation

As mentioned above (Section 4.1.3), ST2 is a gene encoding two proteins [371], which act to promote a TH2-mediated immune response [372], however, the precise nature of ST2 action or function is yet to be fully elucidated. ST2 action in an acute model of asthma has been studied by Hoshino et al [343] and found to be non-essential for initiation of allergen-induced airway inflammation. Parallel development of the long-term pulmonary inflammation model in ST2 knock-out mice revealed clues about a potential immunological role of ST2 in initiation of this type of inflammation. Firstly, the increased mortality of wild-type mice following administration of the longer-term OVA protocol, was not exhibited in ST2-deficient mice subjected to the same protocol, indicating that the pulmonary inflammation induced may not have been as severe in the ST2-deficient mice. Moreover, and consistent with the findings of Hoshino et al, no reduction of antigen-induced TH2 cytokine production was observed in the ST2-deficient chronic airway inflammation model. In fact, splenocytes from ST2-deficient mice consistently secreted *higher* concentrations of OVA-specific IL-4, IL-5, IL10 and TNFα *ex vivo* than splenocytes from wild-type mice, indicating that the antigen-specific cytokine response in was more potent in the former than in the latter.

Interestingly, serum analysis revealed that ST2-deficient mice developed significantly higher levels of antigen-specific (TH1-isotype) IgG2a and lower levels of antigen-specific IgE in this inflammation model, when compared with the wild-type model mice. The effects of ST2 knock-out on serum Ig contradicted the findings of Townsend et al, [341] who demonstrated that serum antibody isotype profiles, in a model of TH2-mediated pulmonary granuloma, were similar in their comparison of ST2-deficient and wild-type mice. In the current investigation, the observed difference in antibody response indicated that although there is evidence of both antigen specific IgG1 and IgG2a antibody responses, ST2-deficient mice appear to be mounting a less TH2-polarised response (as exhibited in the wild type mice) in response to OVA administration, evidenced by a reduced IgE response. A compensatory enhanced TH1 immune response is consistent with the findings of another study, which observed that blockade of ST2 exacerbated TH1-mediated inflammation in a model of arthritis [337]. It has been suggested that whilst ST2 is not necessary for TH2 cell differentiation, it is necessary for regulation of TH1 mediated immune responses [361], which would support the findings presented in this chapter; i.e.,

development of a more TH1-polarised serum Ig response by ST2-deficient mice in a model of TH2-mediated inflammation. Indeed, TH2-mediated cytokine production was maintained in the absence of ST2, but TH1-mediated secretion of IgG2a was not reduced as it is in the wild-type chronic inflammation model. In conclusion, it appeared that despite continued ability to generate antigen-specific TH2 cytokines and antibody responses, ST2-deficient mice seemed less able to prevent development of a TH1-type antibody response.

Continuing analysis of the immunology underlying the long-term pulmonary inflammation model, bone marrow-derived DC were cultured in vitro from each treatment group in the ST2-deficient model. Comparison of bone marrow-derived DC analyses revealed few differences between models, in terms of the role of ST2. Apart from modestly elevated TNFα production by DC derived from ST2-deficient mice, DC from OVA-treated wild-type and ST2KO mice exhibited similar cytokine production and surface expression profiles under control and LPS conditions *in vitro*. Nevertheless, although it was a small effect, the absence of ST2 expression in the fibrosis model mice appeared to prevent the development of DC with a slightly immature phenotype in response to the OVA-administration protocol. This suggests that the 'immaturing' effect of OVA immunisation on resultant DC phenotype in the chronic inflammation model may be ST2-dependent. Indeed, a DC2 phenotype (induced by ES-62 treatment of bone marrow DC *in vitro*), which normally induces priming of a TH2-type immune response, has been described as immature, evidenced by low-level expression of co-stimulatory and adhesion molecules [189]. Thus, if an 'immature' DC phenotype leads to induction of TH2-type immune responses, perhaps prevention of this immature phenotype would polarise the resultant immune response away from a TH2-phenotype, resulting in reduced TH2-type inflammation, which would support the survival-promoting effects of ST2 knock-out in this mode.

4.3.6 The relationship between ES-62 and ST2 in the long-term pulmonary inflammation model.

As mentioned in Section 4.1.5 it was proposed to determine whether ES-62-mediated action in the model of chronic airway inflammation was mediated by, or associated with, ST2 expression. Provided the ST2-dependent features of the chronic airway inflammation model (discussed above) are taken into account, comparison of the effects of ES-62 in the wild-type and ST2-deficient models will help to achieve this aim.

As mentioned previously, the survival rate of ST2-deficient fibrosis model mice was greater than that of wild-type fibrosis mice. In addition, treatment of wild-type fibrosis model mice with ES-62 also appeared to promote survival. Thus, it appears that treatment

with ES-62 *or* knock-out of ST2 have anti-inflammatory action in this model of inflammation. Hence, it might be proposed that in wild-type mice, ES-62 could act to block the action of ST2. In addition, treatment of wild-type fibrosis model mice with ES-62 resulted in promotion of antigen-specific IL-4, IL-5 and IL-10 production by splenocytes from these mice *ex vivo*, an effect also apparently induced by knock-out of ST2. Therefore, again, it appears that treatment of wild-type mice with ES-62 induced similar effects to knock-out of ST2. Furthermore, ES-62 treatment of fibrosis model mice in the wild-type model or removal of the ST2 gene both resulted in decrease of OVA-specific IgE production and differentiation of DC with enhanced surface marker expression

Nevertheless, ES-62-mediated promotion of antigen-specific cytokine production *ex vivo* was similar in splenocytes from wild-type and ST2-deficient mice, indicating that ES-62-mediated promotion of TH2 cytokine production in the longer-term inflammation model was ST2-independent. In contrast, ES-62-mediated inhibition of IgG1 TH2-type antigen-specific antibody responses was not maintained in ST2-deficient mice. More specifically, inhibition of OVA-specific IgG1and IgE production by ES-62 treatment in the wild-type model was not exhibited in the ST2-deficient model, demonstrating the role of ST2 in mediating this inhibitory action of ES-62. However, it must be considered that the OVA-specific IgE response was not fully developed in the ST2-deficient model, therefore it could be postulated that, in this case, the loss of ES-62-mediated inhibition of IgE levels simply reflected the inability to further reduce the defective production of IgE in ST2KO mice. Nevertheless, the lost ability of ES-62 to reduce antigen-specific IgG1 in ST2-deficent mice was clear.

Down-regulation of surface marker expression by DC was observed in ES-62 treated ST2-deficient fibrosis model mice. This down-modulatory action of ES-62 was opposite to that exhibited in the wild-type model (and moreover, in the acute model), thus, it might be proposed that these actions of ES-62 were normalising and acted to 'correct' the effect of OVA-treatment on DC phenotype. However, it is also possible that the upregulatory effect of ES-62 in the wild-type model was ST2-dependent. Based on the treatment groups available, it is not clear which theory is true. Furthermore, it must be re-iterated that the exhibited effects on DC phenotype were modest.

ES-62 mediated rescue of the ameliorated TH1 cytokine response to LPS by DC from the wild type model was not exhibited in ST2KO model DC, initially indicating that ST2 might be involved in this cytokine modulating action of ES-62. Indeed, recently it has been demonstrated that the cytokine production response to LPS by DC is dependent upon ST2 [373]. However in this investigation, the DC derived from the wild-type fibrosis model mice also exhibited a reduced cytokine production response to LPS, therefore the effect of

ST2-knock out on the cytokine response to LPS in DC cannot be confirmed. Nevertheless, it has also been demonstrated that ES-62 mediated modulation of LPS-induced cytokine production by DC is not transduced by ST2 [374].

In summary, ES-62 action is different in ST2-expressing and ST2-deficient models of OVA-induced airway inflammation. As it has previously been determined that ES-62 does not act via ST2 [374] it is hypothesised that these distinct actions might be induced as a result of the different immune response to the inflammation induction protocol in wild-type and ST2KO mice.

4.3.7 Conclusion

The findings presented in this chapter have highlighted novel actions of ES-62 in models of TH2-mediated inflammation. For the first time it has been demonstrated that ES-62 inhibits eosinophilic airway inflammation when administered during development of the inflammatory immune response or when used to treat established inflammation. It has previously been demonstrated that infection with Strongyloides stercoralis or treatment with an extract of A. suum inhibits development of airway inflammation in mice [375, 376]. The anti-inflammatory action of ES-62 in the short-term model of airway inflammation was associated with inhibition of antigen-specific TH2-type cytokine and antibody responses and provides support for the previous studies citing anti-inflammatory action of parasites in this type of inflammation, despite their TH2-promoting activation per se. Furthermore, it was observed that ES-62 modulated the phenotype and function of bone marrow-derived cells by treatment of bone marrow progenitors *in vivo*. Consistent with the findings of Chapter 3, it appears that rather than preventing development of an immune response, ES-62 tailors the development of immunity to result in a less inflammatory response. It is possible that this action may be mediated by disruption of the normal function of bone marrow-derived innate immune cells, which leads to disruption of the ensuing adaptive immune response as a whole.

Figure 4.1 Eosinophilia induced by ovalbumin, is suppressed by ES-62

BALB/c mice sensitised with OVA were challenged intranasally on days 14, 25, 26 and 27. ES-62 (2 μg) was administered on days –2, 12, 25 and 27 and control mice received PBS. Mice were sacrificed on day 28 and broncho-alveolar lavage (BAL) performed as described in Chapter 2. **A**, Differential counts of cells present in the BAL fluid were conducted for each mouse. The numbers of macrophages, eosinophils, lymphocytes, neutrophils and epithelial cells were determined and displayed as a percentage of the total cell count. Data are expressed as mean ± SEM (n=6 mice/group). **B**, Eosinophil count data are expressed as mean ± SEM (*n*=6 mice/group). These results are representative of at least three independent experiments. ***p<0.001 vs. Control, **p<0.01 vs Asthma (ANOVA).

C-F Intact lungs were carefully dissected from mice in each treatment group and fixed in formalin. Sections of lung tissue from Control (**C**), Asthma (**D**) and Asthma+ES-62 (**E**) mice were stained using H & E staining and viewed using a x10 objective. **F**, The peri-bronchial infiltrate was scored 1=normal, 2=mild, 3=moderate and 4=severe, by scanning 100 random bronchial walls in sufficient high power fields and data are expressed as mean ± SD (n=100). ***, p<0.001 vs Control; †, p<0.001 vs Asthma using the Mann-Whitney *U* test.

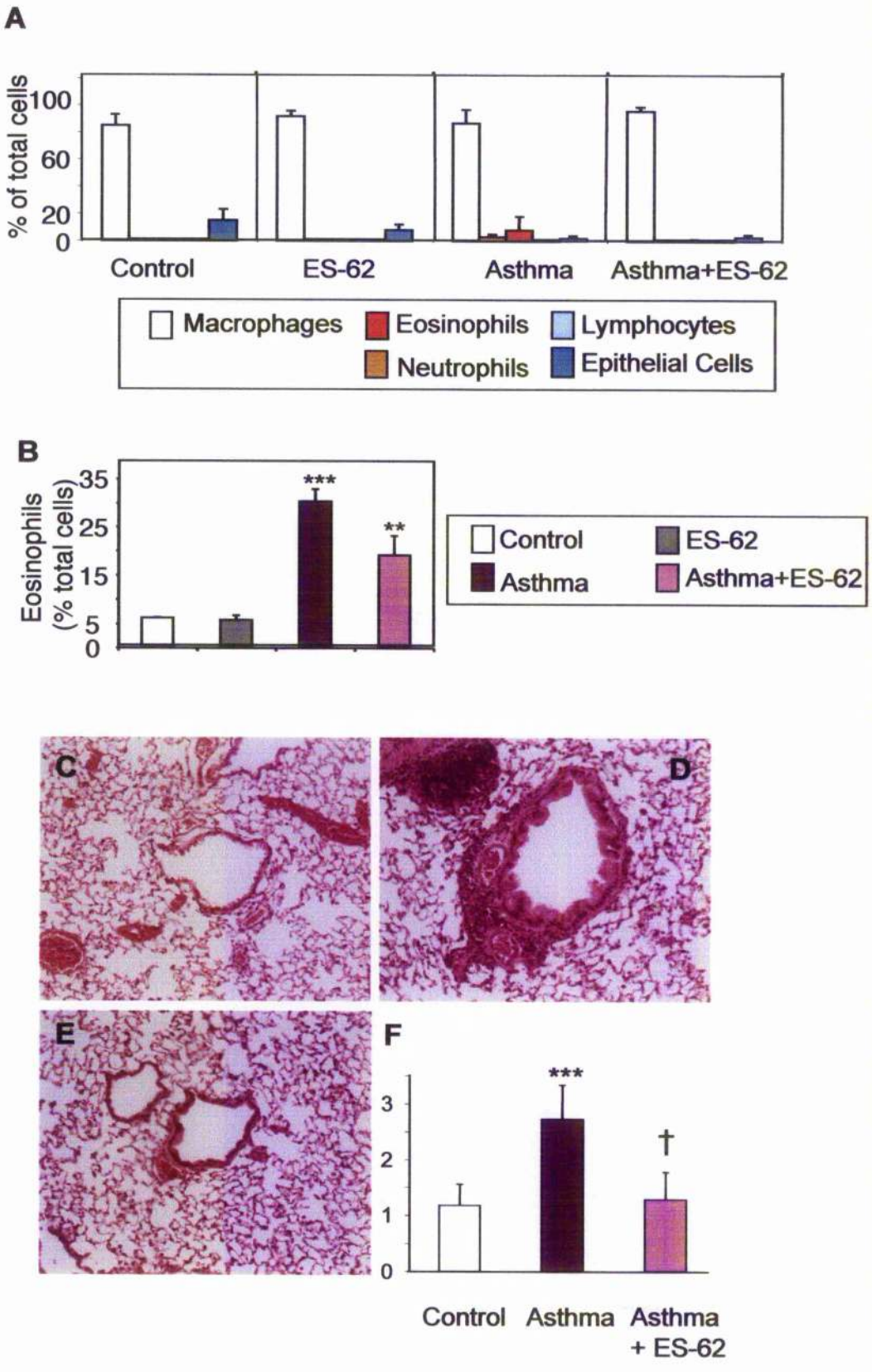

A
% of total cells
100
60
20
0
Control
ES-62
Asthma
Asthma+ES-62
Macrophages
Eosinophils
Lymphocytes
Neutrophils
Epithelial Cells
B
Eosinophils (% total cells)
35
25
15
5
0
***
**
Control
ES-62
Asthma
Asthma+ES-62
C
D
E
F
***
†
3
2
1
0
Control
Asthma
Asthma + ES-62

Figure 4.2 Ovalbumin-induced airway TH2 cytokine production inhibited by ES-62

BALB/c mice sensitised with OVA were challenged intranasally on days 14, 25, 26 and 27. ES-62 (2 μg) was administered on days –2, 12, 25 and 27 and control mice received PBS. Mice were sacrificed on day 28 and broncho-alveolar lavage (BAL) performed as described in Chapter 2. The concentration of IL-4 (A), IL-5 (B) and IFNγ (C) in the BAL fluid was analysed for each mouse by ELISA. These results are representative of at least three independent experiments. BAL fluid cytokine concentrations are depicted as individual values for each mouse, and the bar represents the treatment group mean. ***, $p<0.001$ (by ANOVA).

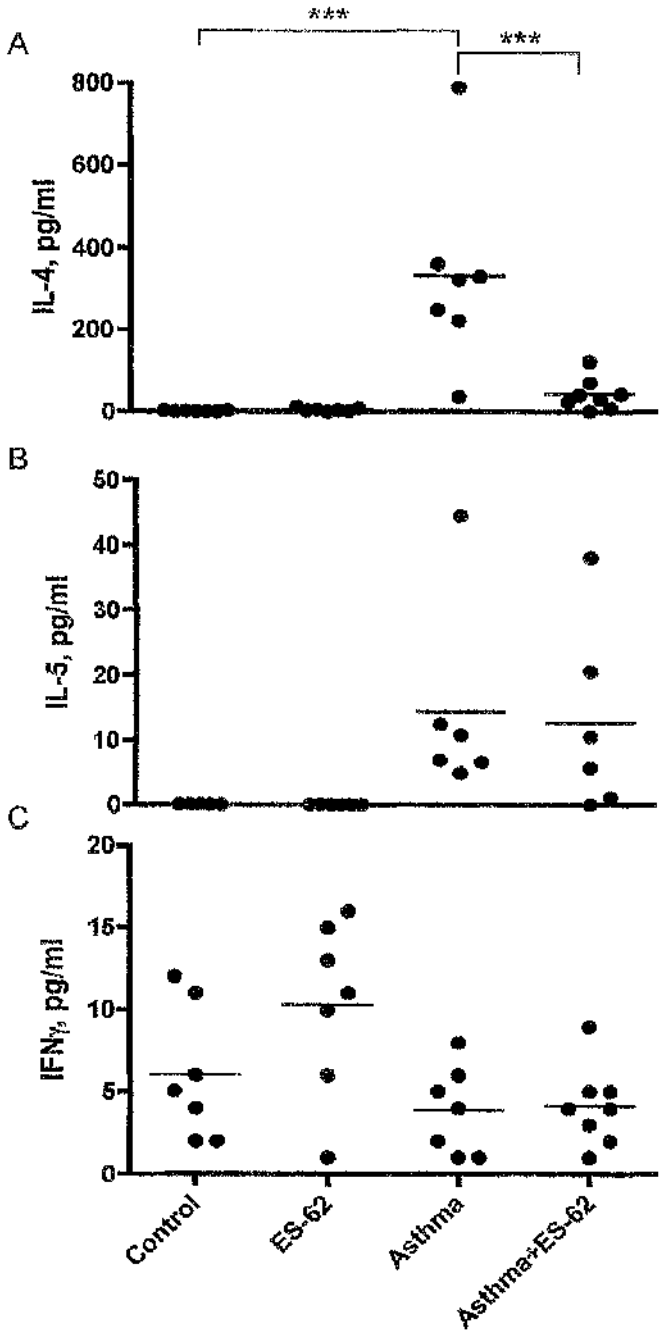

A
***
***
800
600
400
200
0
IL-4, pg/ml
B
50
40
30
20
10
0
IL-5, pg/ml
C
20
15
10
5
0
IFNγ, pg/ml
Control
ES-62
Asthma
Asthma+ES-62

Figure 4.3 ES-62 treatment *in vivo* inhibits draining lymph node cell antigen-specific TH2 cytokine production but not antigen-specific proliferation *ex vivo*

Mice were treated and sacrificed as described in the legend to Figure 4.1 and thoracic and cervical lymph nodes from each mouse removed. Lung draining lymph node cells from mice in each group (n=6/group) were pooled and cultured in triplicate with medium alone (Control) or OVA (Ova; 500 µg/ml) for 72h. T cell proliferation was assayed by uptake of [^{3}H] thymidine in the last 8 hours of culture (A). Proliferation data are expressed as mean ± SD of triplicate cultures and are representative of 3 experiments * $p<0.05$ by ANOVA, † $p<0.001$ vs Control or ES-62. Culture supernatant concentrations of IL-4 (B), IL-5 (C), IFNγ (E) and TNFα (F) were measured by Luminex. Luminex data is expressed as the mean of duplicate samples from the supernatant of pooled cells and are representative of 3 experiments. Culture supernatant concentrations of IL-10 (D) were measured in triplicate by ELISA from supernatant of pooled cells and data are expressed as mean ± SD and are representative of 3 experiments. ***, $p<0.001$ vs Asthma treatment group.

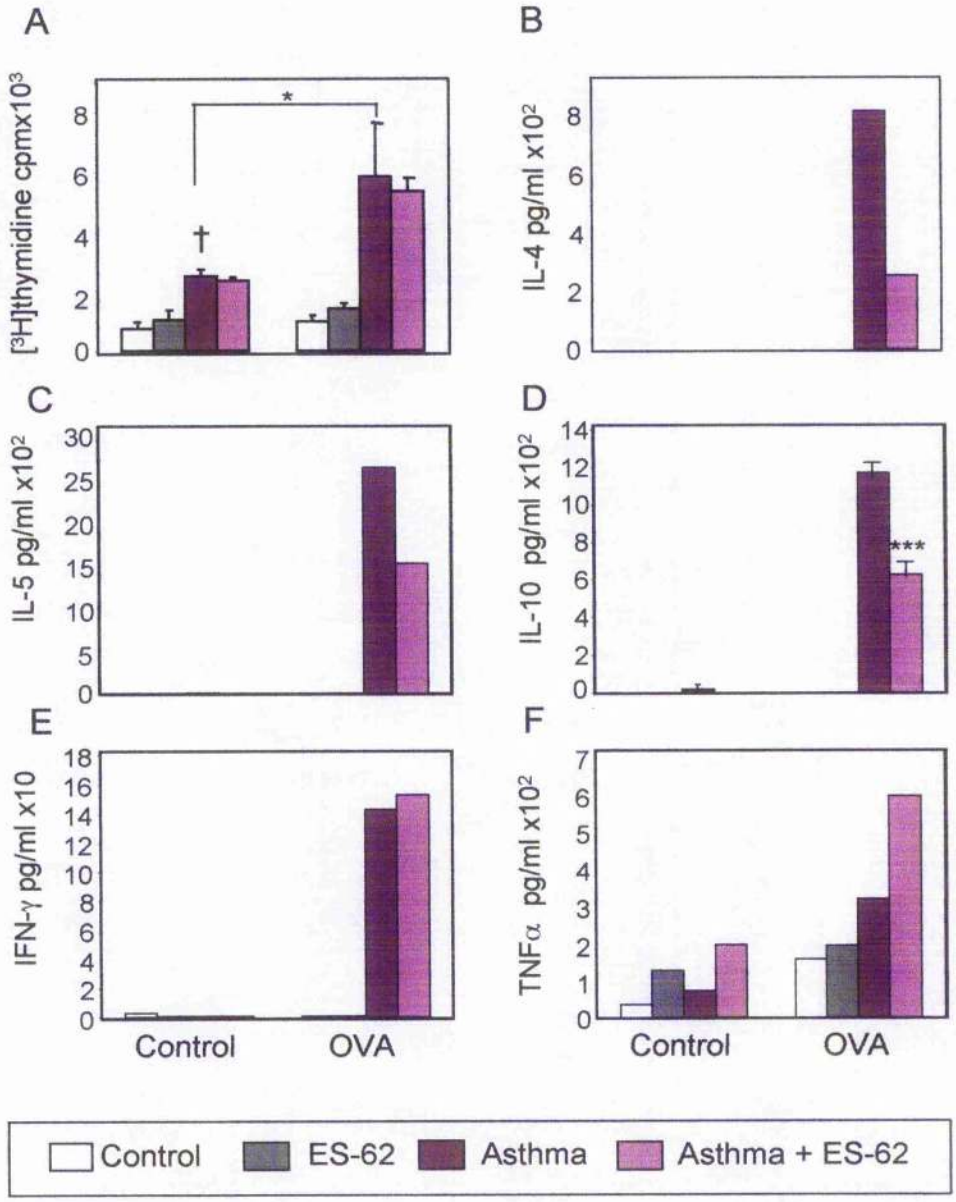

A
[3H]thymidine cpmx10³
0
2
4
6
8
*
†
B
IL-4 pg/ml x10²
0
2
4
6
8
C
IL-5 pg/ml x10²
0
5
10
15
20
25
30
D
IL-10 pg/ml x10²
0
2
4
6
8
10
12
14
***
E
IFN-γ pg/ml x10
0
2
4
6
8
10
12
14
16
18
F
TNFα pg/ml x10²
0
1
2
3
4
5
6
7
Control
OVA
Control
OVA
Control
ES-62
Asthma
Asthma + ES-62

Figure 4.4 ES-62 treatment *in vivo* does not modulate mitogen-specific TH2 cytokine production from draining lymph node cell *ex vivo*

Mice were treated and sacrificed as described in the legend to Figure 4.1 and lung draining lymph node cells from mice in each group (n=6/group) were pooled and cultured with medium alone (Control) or ConA (3 μg/ml) for 72h. T cell proliferation was assayed by uptake of [^{3}H] thymidine in the last 8 hours of culture (A). Proliferation data are expressed as mean ± SD and are representative of 3 experiments. Culture supernatant concentrations of IL-4 (B), IL-5 (C), IFNγ (E) and TNFα (F) were measured by Luminex. Luminex data is expressed as mean of duplicate samples from the supernatant of pooled cells and are representative of 3 experiments. Culture supernatant concentrations of IL-10 (D) were measured in triplicate by ELISA from supernatant of pooled cells and data are expressed as mean ± SD and are representative of 3 experiments. No statistical difference in proliferation or cytokine production was observed between cultures from Asthma and Asthma+ES-62 treatment groups.

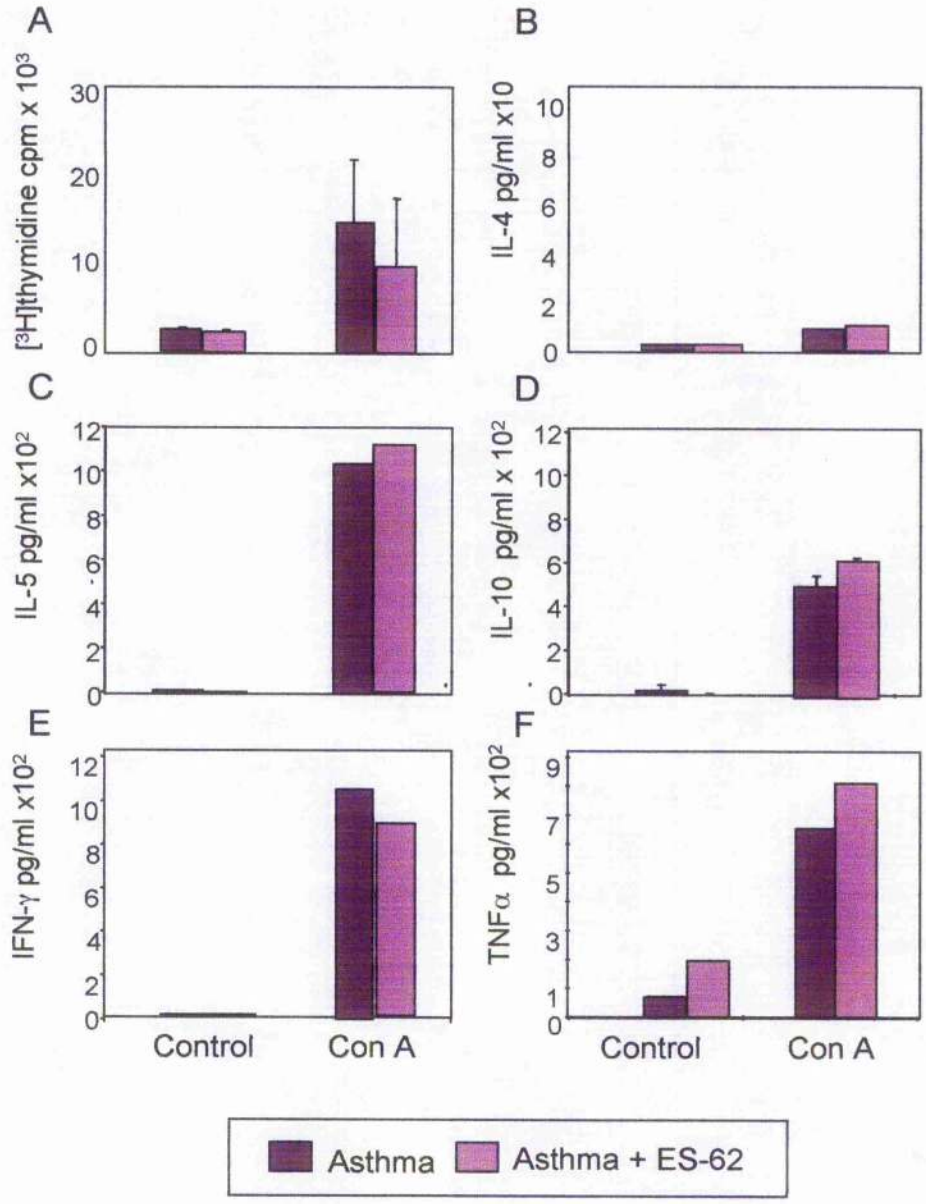

A
[3H]thymidine cpm x 10³
30
20
10
0
B
IL-4 pg/ml x10
10
8
6
4
2
0
C
IL-5 pg/ml x10²
12
10
8
6
4
2
0
D
IL-10 pg/ml x 10²
12
10
8
6
4
2
0
E
IFN-γ pg/ml x10²
12
10
8
6
4
2
0
F
TNFα pg/ml x10²
9
7
5
3
1
0
Control
Con A
Control
Con A
Asthma
Asthma + ES-62

Figure 4.5 ES-62 treatment in vivo modulates antigen-specific TH2 cytokine production by splenocytes ex vivo

Mice were treated and sacrificed as described in the legend to Figure 4.1 and spleen from each mouse was removed. Splenocytes from mice in each group (n=6/group) were pooled and cultured with medium alone (Control) or OVA (Ova; 500 µg/ml) for 72h. T cell proliferation was assayed by uptake of [^{3}H] thymidine in the last 8 hours of culture (A). Proliferation data are expressed as mean ± SD of triplicate cultures and are representative of 2 experiments. ***, $p< 0.001$ vs control conditions. Culture supernatant concentrations of IL-4 (B), IL-5 (C), IL-10 (D), and IFNγ (E) were measured by Luminex. Luminex data is expressed as mean of duplicate samples from supernatant of pooled cells and are representative of 2 experiments. Culture supernatant concentrations of TNFα (F) were measured in triplicate by ELISA from supernatant of pooled cells and data are expressed as mean ± SD and are representative of 3 experiments, **, $p< 0.01$ vs. Asthma treatment group.

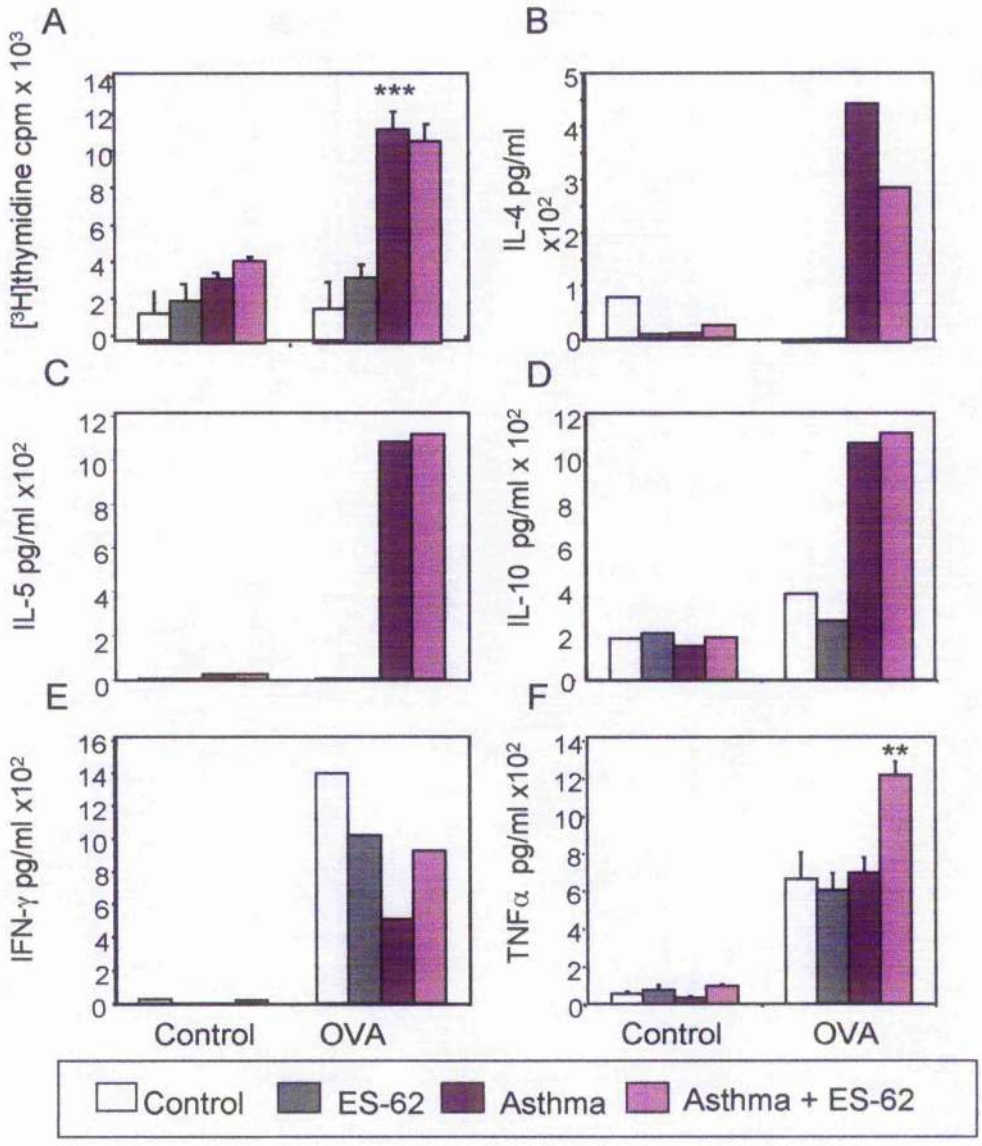

A
[3H]thymidine cpm x 10³
***
B
IL-4 pg/ml x10²
C
IL-5 pg/ml x10²
D
IL-10 pg/ml x 10²
E
IFN-γ pg/ml x10²
F
TNFα pg/ml x10²
**
Control
OVA
Control
ES-62
Asthma
Asthma + ES-62

Figure 4.6 ES-62 treatment *in vivo* does not modulate mitogen-induced TH2 cytokine production by splenocytes *ex vivo*

Mice were treated and sacrificed as described in the legend to Figure 4.1 and splenocytes from mice in each group (n=6/group) were pooled and cultured with medium alone (Control) or ConA (3 μg/ml) for 72h. T ceil proliferation was assayed by uptake of [^{3}H] thymidine in the last 8 hours of culture (A). Proliferation data are expressed as mean ± SD of triplicate cultures and are representative of 3 experiments. Culture supernatant concentrations of IL-4 (B), IL-5 (C) and IFNγ (E) were measured by Luminex. Luminex data is expressed as mean of duplicate samples from the supernatant of pooled cells and are representative of 3 experiments. Culture supernatant concentrations of IL-10 (D) and TNFα (F) were measured in triplicate by ELISA from supernatant of pooled cells and data are expressed as mean ± SD and are representative of 3 experiments. No statistical difference in proliferation or cytokine production was observed between cultures from Asthma and Asthma+ES-62 treatment groups.

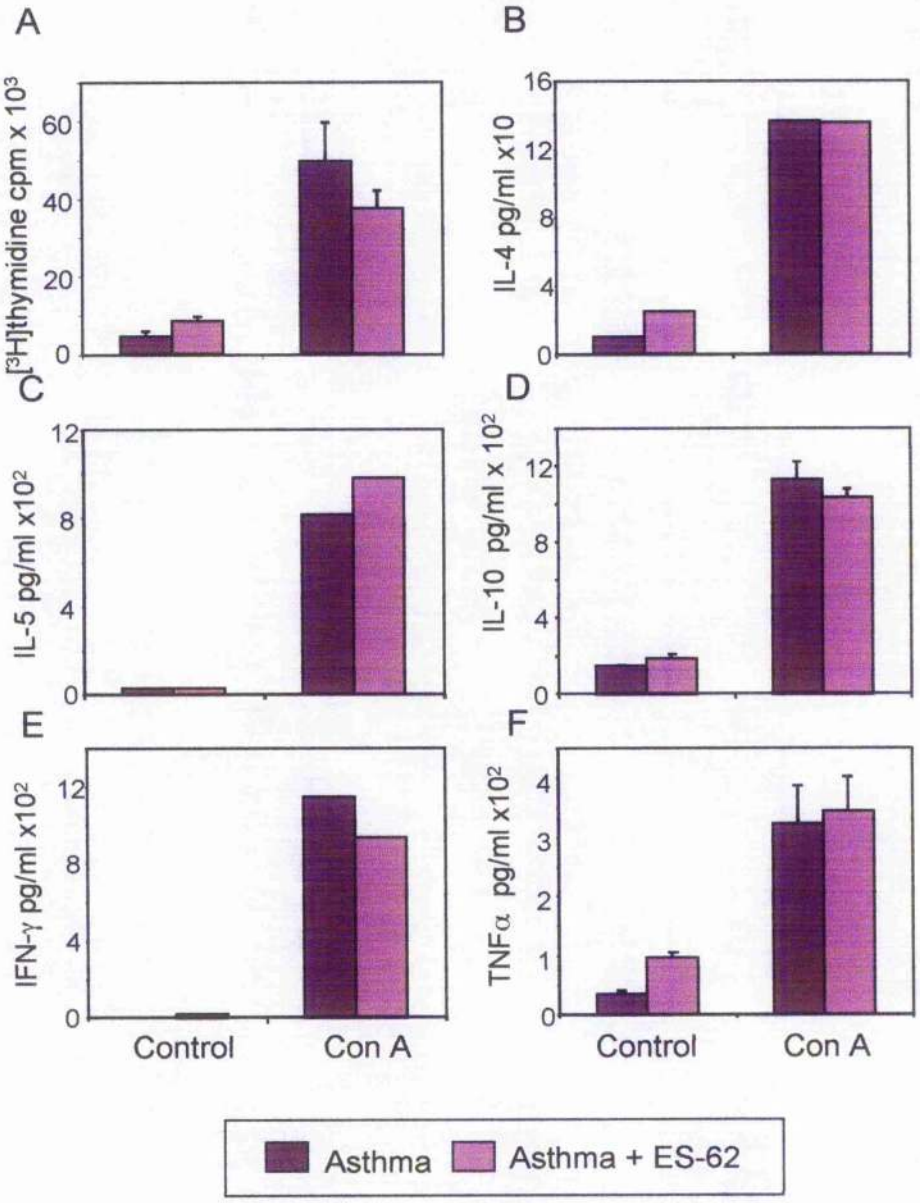

A
B
C
D
E
F
[3H]thymidine cpm x 10³
IL-4 pg/ml x10
IL-5 pg/ml x10²
IL-10 pg/ml x 10²
IFN-γ pg/ml x10²
TNFα pg/ml x10²
Control
Con A
Asthma
Asthma + ES-62

Figure 4.7 Exposure to ES-62 *in vivo* promotes peripheral lymph node cell proliferation and TH2 cytokine production *ex vivo*

BALB/c mice were treated and sacrificed as described in the legend to Figure 4.1 and non-draining lymph nodes from each mouse removed. Lymph node cells from mice in each group (n=6/group) were pooled and cultured with medium alone (Control) or OVA (Ova; 500 μg/ml) for 72h. T cell proliferation was assayed by uptake of [^{3}H] thymidine in the last 8 hours of culture (A). Proliferation data are expressed as mean ± SD of triplicate cultures and are representative of 2 experiments. ***, $p<0.001$ vs Control treatment group; ‡, $p<0.001$ vs Asthma treatment group. Culture supernatant concentrations of IL-4 (B), IL-5 (C) and IFNγ (E) were measured by Luminex. Luminex data is expressed as mean of duplicate samples from the supernatant of pooled cells and are representative of 2 experiments. IL-10 (D) and TNFα (F) were measured in triplicate by ELISA from supernatant of pooled cells and data are expressed as mean ± SD and are representative of 2 experiments. No statistical difference in cytokine production was observed between cultures from different treatment groups.

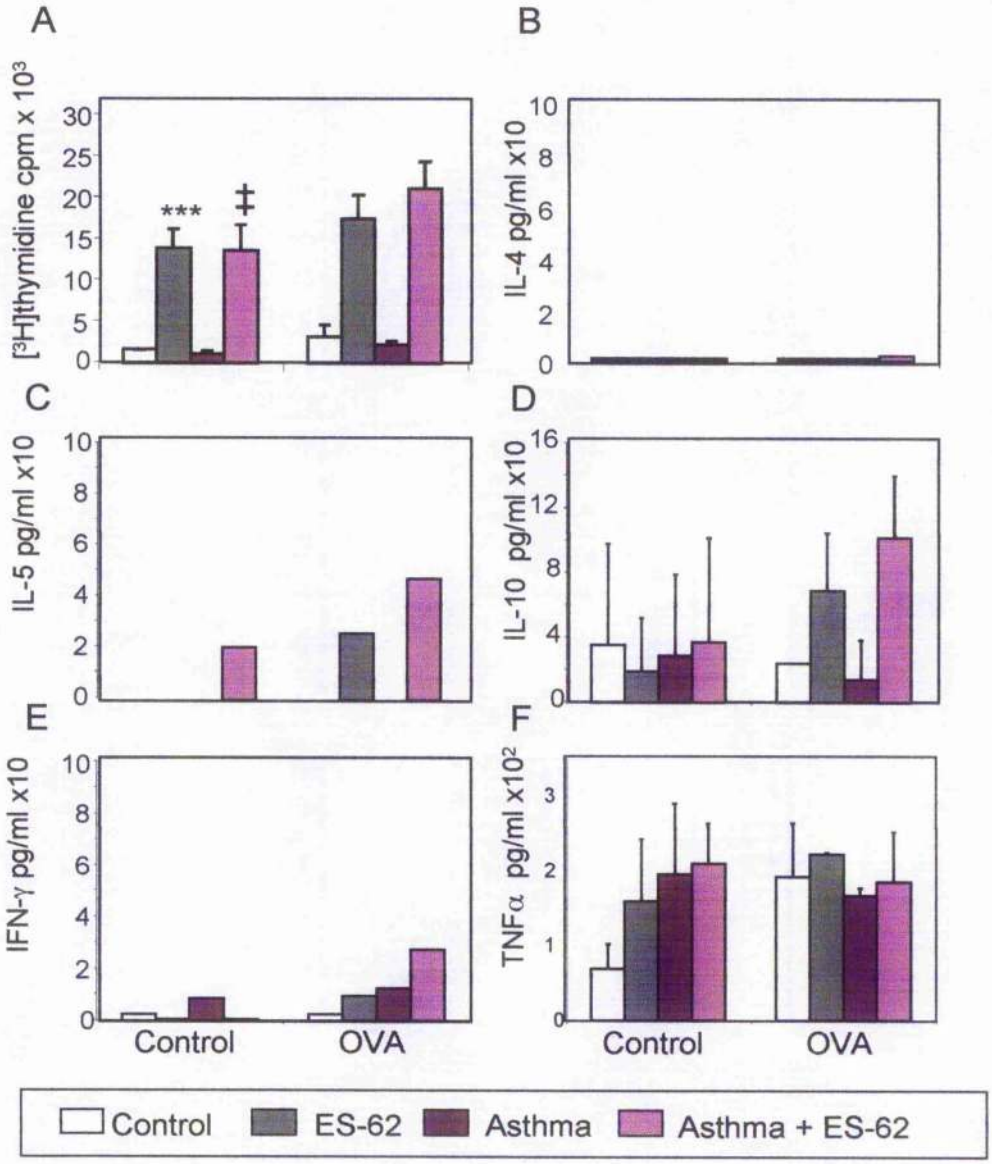
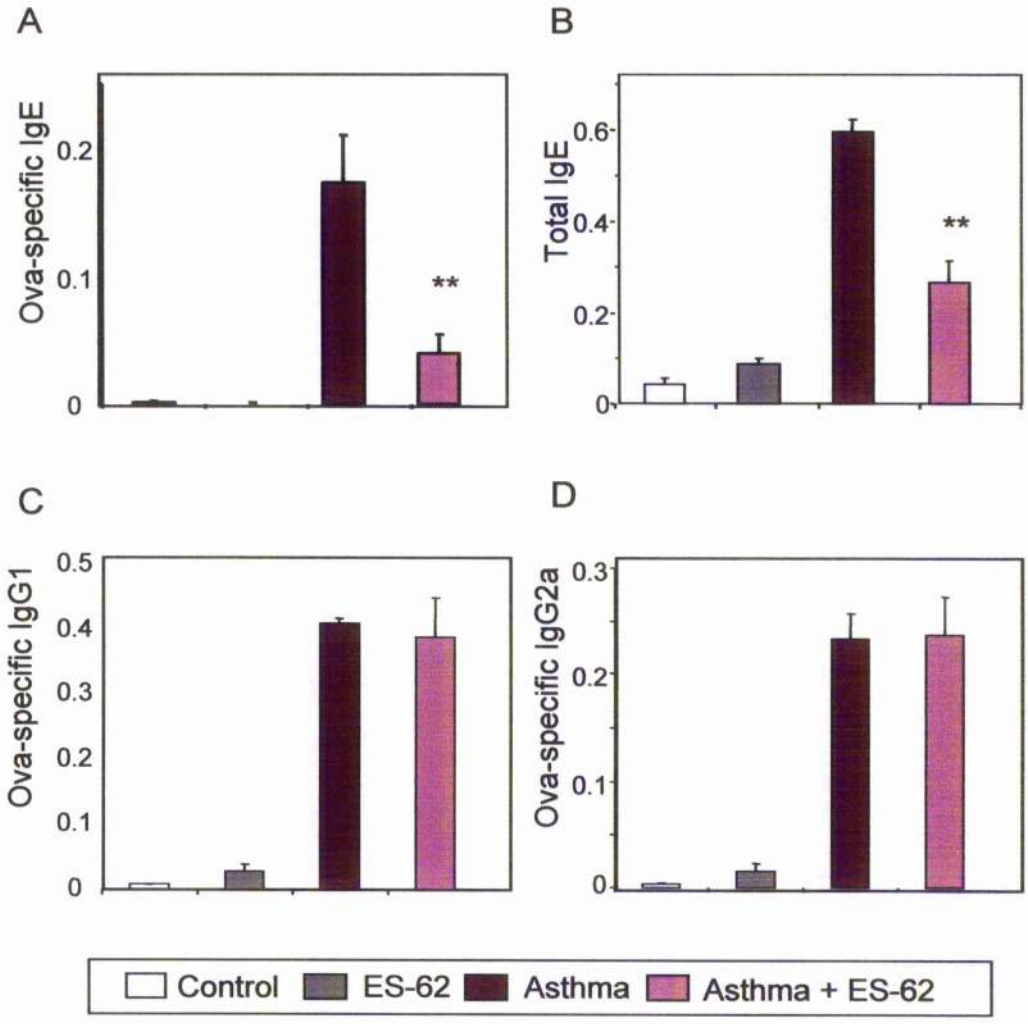

A
B
C
D
E
F
[3H]thymidine cpm x 10³
IL-4 pg/ml x10
IL-5 pg/ml x10
IL-10 pg/ml x10
IFN-γ pg/ml x10
TNFα pg/ml x10²
***
‡
Control
OVA
Control
ES-62
Asthma
Asthma + ES-62

Figure 4.8 Exposure to ES-62 *in vivo* does not modulate Ova-induced elevation of serum IgG1 and IgG2a but inhibits serum IgE

A-D, BALB/c mice were treated and sacrificed as described in the legend to Figure 4.1. Serum samples from each mouse were obtained on day 28 and diluted with 10% FCS (1/200). Diluted samples were analysed by ELISA for OVA-specific IgE (A), total IgE (B), OVA-specific IgG1 (C) and IgG2a (D). Data for each group are presented as mean ± SD (n=6 mice/group) and are representative of 3 experiments. **, $p<0.01$ vs Asthma group by Student's t-test; no significant difference was observed in serum IgG levels between Asthma and Asthma+ES-62 treatment groups.

Panel E, IgE expression in lymph node follicles is induced by ovalbumin. Intact lymph nodes from each treatment group were snap frozen in liquid nitrogen and stored at –80°C. Frozen lymph nodes were cut into 6μm sections and stained with anti-B220-FITC and anti-IgE-AF647. Sections were focused in the bright field and then viewed using red and green filters to pick up IgE and B220 signals respectively. B220 and IgE signal images were colourised according to their staining signal colour.


A
Ova-specific IgE
0.2
0.1
0
**
B
Total IgE
0.6
0.4
0.2
0
**
C
Ova-specific IgG1
0.5
0.4
0.3
0.2
0.1
0
D
Ova-specific IgG2a
0.3
0.2
0.1
0
Control
ES-62
Asthma
Asthma + ES-62

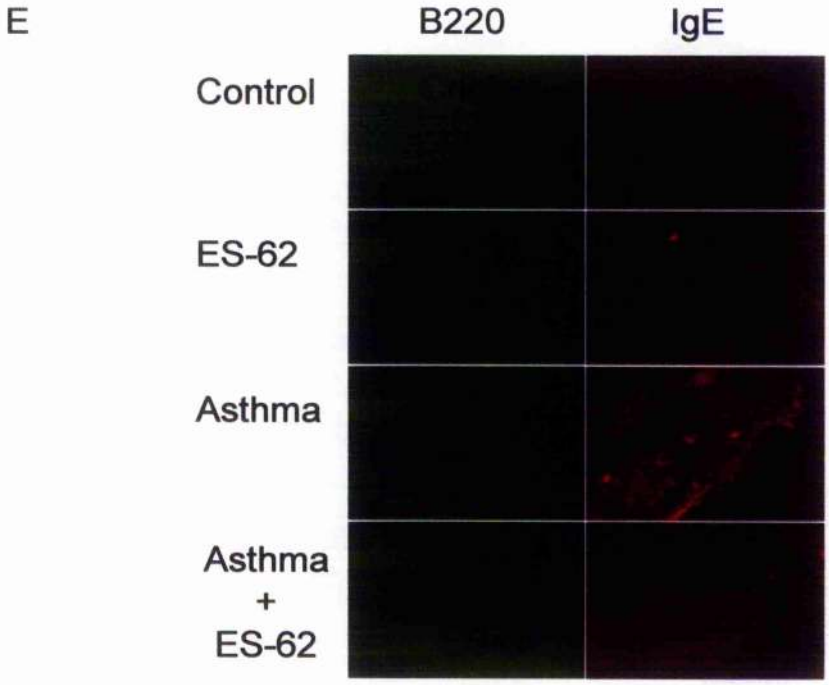

E
B220
IgE
Control
ES-62
Asthma
Asthma
+
ES-62

Figure 4.9 ES-62 inhibits Ova-induced pulmonary eosinophilia in a dose-dependent manner, when administered therapeutically

A, BALB/c mice were sensitised with OVA and challenged with OVA intranasally on days 14, 25, 26 and 27. ES-62 (0.4 µg, 2 µg or 10 µg) was administered only on days 25, 26 and 27 and control mice received PBS. Mice were sacrificed on day 28 and BAL performed as described in Chapter 2. Differential cell counts were conducted for each mouse and data for eosinophils are expressed as mean ± SD (*n*=6 mice/group). Data are from a single experiment representative of 2 experiments. *, $p< 0.05$ by ANOVA.

Panels **B** & **C**, Thoracic and cervical lymph nodes were removed from each mouse. Lung draining lymph node cells from mice in each group were pooled and cultured with medium alone (Control) or OVA (OVA; 100 µg/ml) for 72h. IL-4 (B) and IL-5 (C) released from DLN cells in response to stimulation with OVA *ex vivo* were analysed by ELISA from duplicate cultures. All data are from a single experiment representative of 2 experiments.

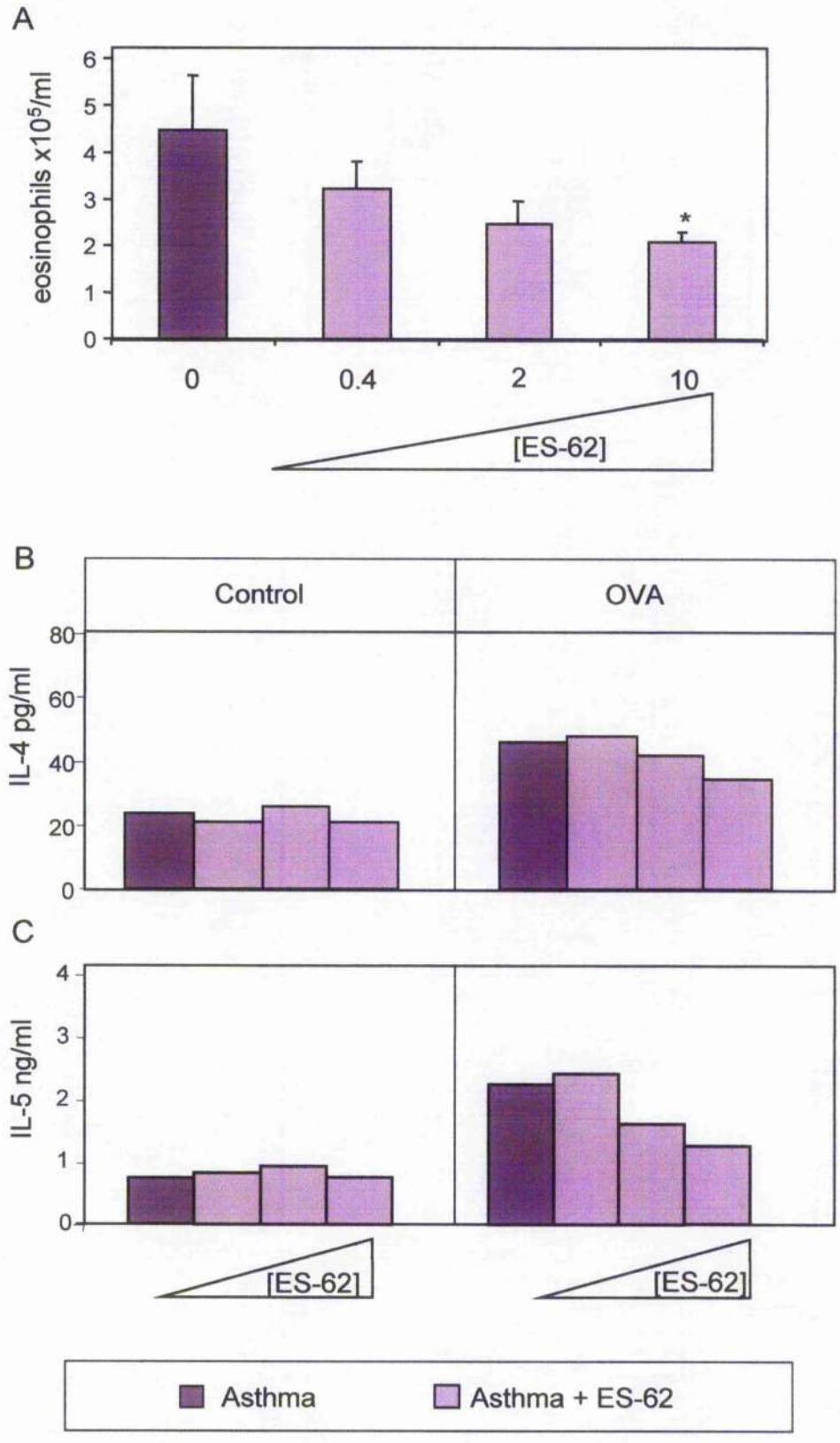

A
eosinophils x10⁵/ml
6
5
4
3
2
1
0
*
0
0.4
2
10
[ES-62]
B
Control
OVA
IL-4 pg/ml
80
60
40
20
0
C
IL-5 ng/ml
4
3
2
1
0
[ES-62]
[ES-62]
Asthma
Asthma + ES-62

Figure 4.10 Therapeutic administration of ES-62 does not significantly modulate serum ova-specific IgE, IgG or total IgE

Mice were treated and sacrificed as described in the legend to Figure 4.9. Serum samples from each mouse were obtained on day 28 and diluted with 10% FBS (in PBS; 1/200). Diluted samples were analysed by ELISA for OVA-specific IgE (A), total IgE (B), OVA-specific IgG1 (C) and IgG2a (D). Data for each group are presented as mean ± SD (n=6 mice/group) and are representative of 2 experiments. No statistical differences between serum Ig levels were observed in Asthma and Asthma+ES-62 treatment group mice.

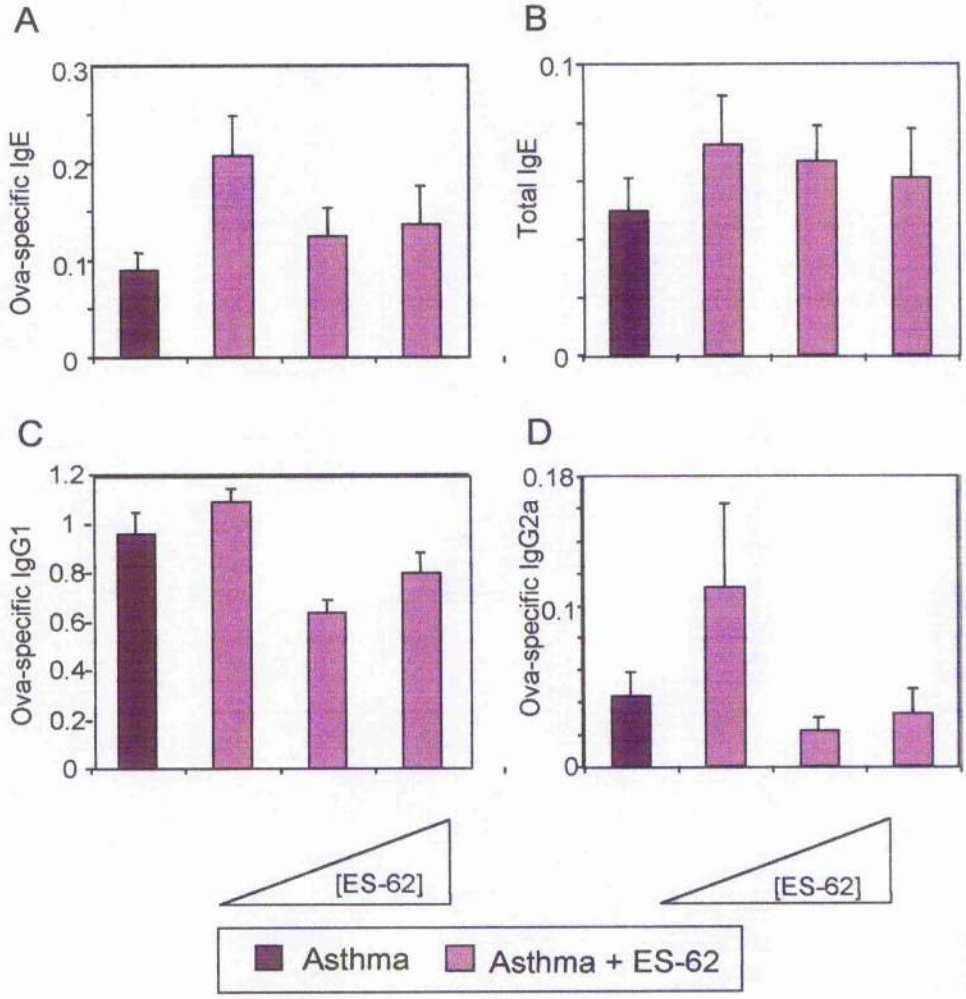

A
B
C
D
Ova-specific IgE
Total IgE
Ova-specific IgG1
Ova-specific IgG2a
0.3
0.2
0.1
0
0.1
0
1.2
1
0.8
0.6
0.4
0.2
0
0.18
0.1
0
[ES-62]
[ES-62]
Asthma
Asthma + ES-62

Figure 4.11 Ovalbumin-induced pulmonary inflammation (or ES-62 treatment of such) does not modulate airway smooth muscle contractility

BALB/c mice were treated and sacrificed as described in the legend to Figure 4.1 and trachea from each mouse carefully micro-dissected. Using a large vessel myograph, tracheal rings from each treatment group were analysed for contractile response to cumulative concentrations of methacholine (10^{-10}M to 10^{-3}M). Results are depicted as the mean ± SEM of percentage of the maximum response to KCl (n=2 at least). No statistical difference was observed between treatment groups.

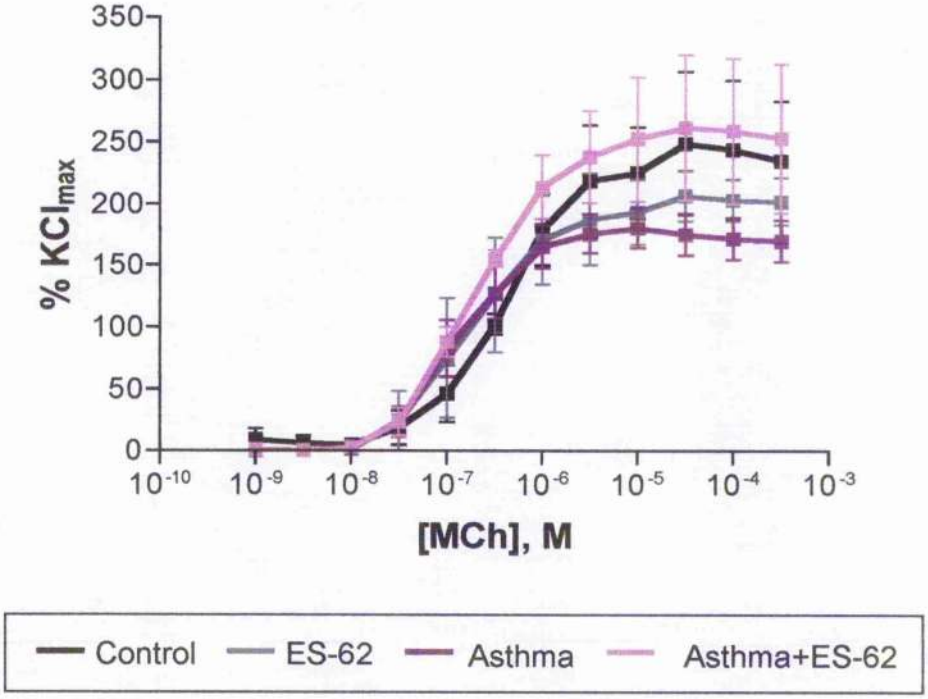

350
300
250
200
150
100
50
0
% KCl$_{max}$
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
[MCh], M
Control
ES-62
Asthma
Asthma+ES-62

Figure 4.12 The cytokine production profile of splenocytes from the chronic airway inflammation model ex vivo: wild-type vs ST2-deficient mice

BALB/c (wild-type; **A-E**) and ST2 knock-out (ST2KO; **F-J**) mice were sensitised with OVA and challenged with OVA intranasally (OVA *i.n.*) on day 14 (50μg) and once every seven days until day 56 (2μg). ES-62 treatment (2μg) was administered subcutaneously two days before and on the day of OVA administration and control mice received PBS. Mice were sacrificed on day 57 and the spleen was removed from each mouse. Splenocytes from mice in each group were pooled and cultured with medium alone (Control), OVA (500 μg/ml) or Con A (5 μg/ml) for 72h. The concentrations of IL-4 (A, F), IL-5 (B, G), IL-10 (C, H), TNFα (D, I) and IL-12 (E, J) in the culture supernatant were measured by ELISA. ELISA data are expressed as mean ± SD (n=3). ***, $p<0.001$ vs Fibrosis; ++, $p<0.01$ and +, $p<0.05$ vs wild-type control (Student's t-test). Production of all five cytokines in response to OVA treatment *in vitro* was significantly greater by splenocytes from ST2KO Fibrosis model mice than their counterparts from the wild-type model ($p<0.01$ at least).

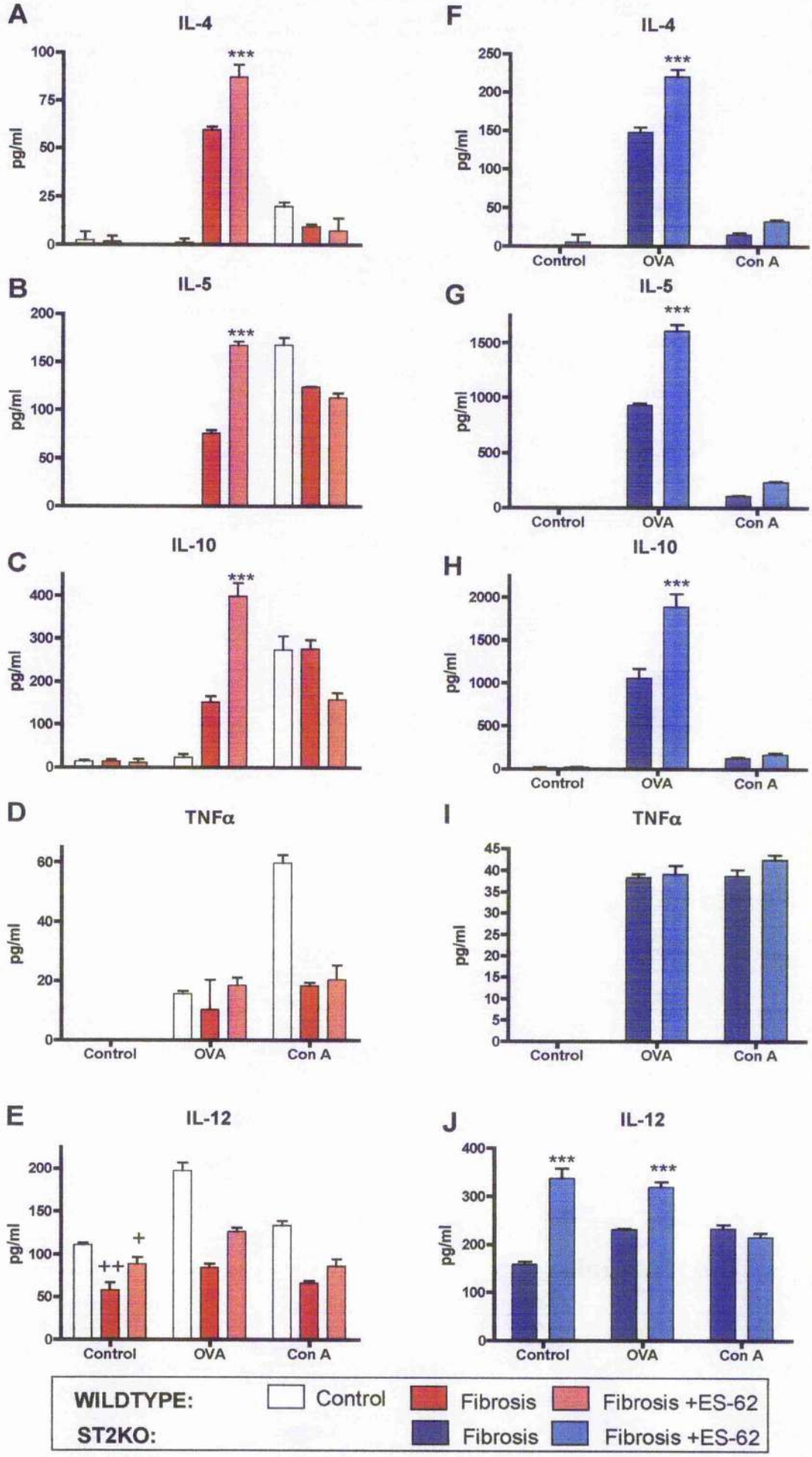

A
IL-4
pg/ml
***
B
IL-5
pg/ml
***
C
IL-10
pg/ml
***
D
TNFα
pg/ml
Control
OVA
Con A
E
IL-12
pg/ml
++
+
Control
OVA
Con A
F
IL-4
pg/ml
***
Control
OVA
Con A
G
IL-5
pg/ml
***
Control
OVA
Con A
H
IL-10
pg/ml
***
Control
OVA
Con A
I
TNFα
pg/ml
Control
OVA
Con A
J
IL-12
pg/ml
***
***
Control
OVA
Con A
WILDTYPE:
Control
Fibrosis
Fibrosis +ES-62
ST2KO:
Fibrosis
Fibrosis +ES-62

Figure 4.13 Ovalbumin-specific serum IgG and IgE levels in the wild-type chronic airway inflammation model are inhibited by ES-62 treatment and modulated in the absence of ST2

BALB/c and ST2-deficient mice were treated and sacrificed as described in the legend to Figure 4.12. Serum samples from each mouse were obtained on day 57 and titrated with 10% FBS (in PBS; 1/200). Diluted samples were analysed by ELISA for OVA-specific IgG1 (A), IgG2a (B), IgE (C) and total IgE (D). Data for each group are presented as mean ± SD (n=2 mice for WT Fibrosis group, n=6 mice for other groups). Statistical differences are as follows: OVA-IgG1: $p<0.001$,wild-type fibrosis vs wild-type fibrosis +ES-62. OVA-IgG2a: $p<0.01$ wild-type fibrosis vs wild-type fibrosis+ES-62 and $p<0.05$ wild-type fibrosis vs ST2KO Fibrosis. OVA-IgE: $p<0.05$ wild-type fibrosis vs wild-type fibrosis +ES-62. No statistical differences were observed in serum Ig levels between ST2KO Fibrosis and ST2KO Fibrosis +ES-62 mice.

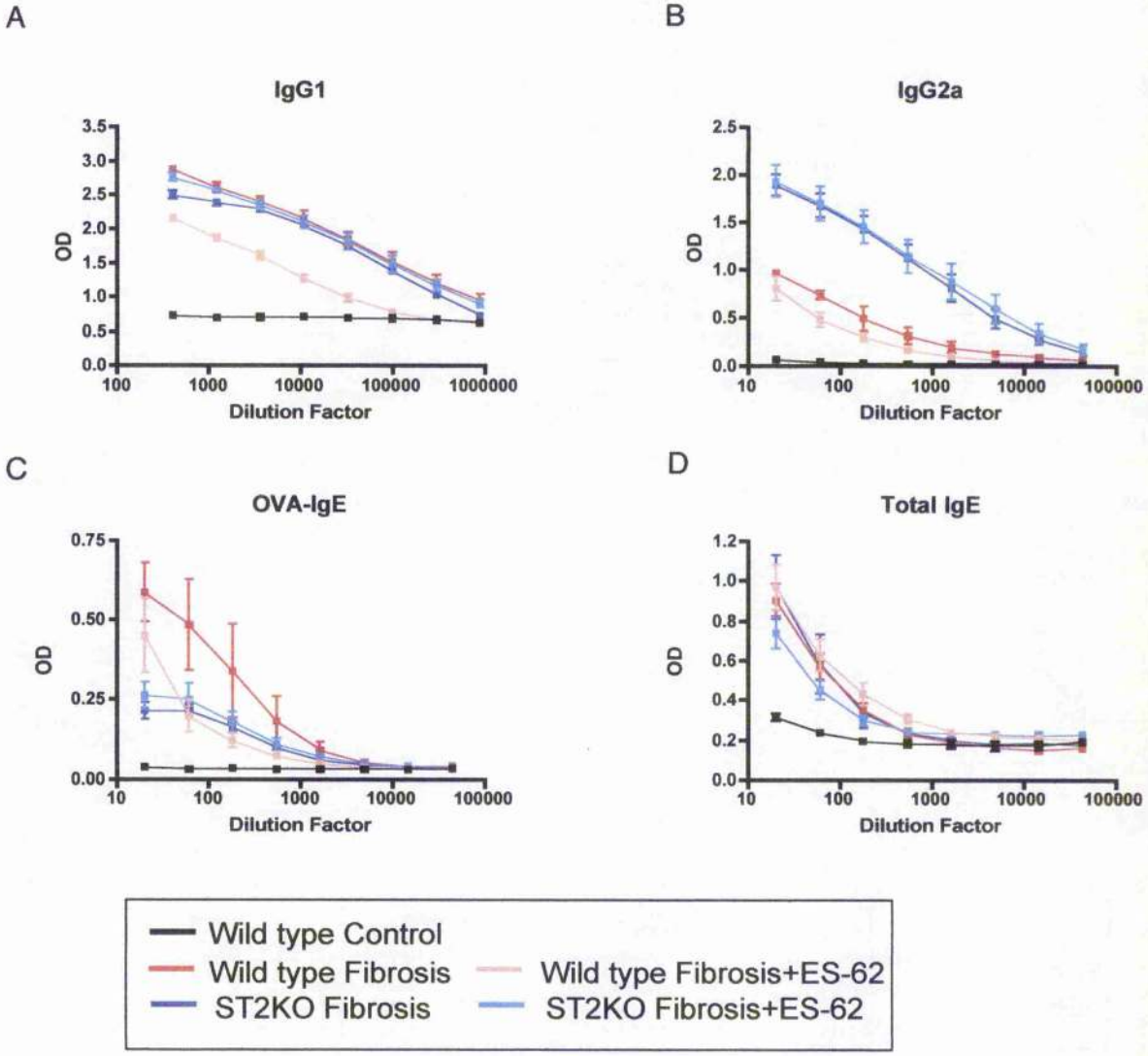

A
IgG1
OD
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
1000000
Dilution Factor
B
IgG2a
OD
2.5
2.0
1.5
1.0
0.5
0.0
10
100
1000
10000
100000
Dilution Factor
C
OVA-IgE
OD
0.75
0.50
0.25
0.00
10
100
1000
10000
100000
Dilution Factor
D
Total IgE
OD
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
10000
100000
Dilution Factor
Wild type Control
Wild type Fibrosis
ST2KO Fibrosis
Wild type Fibrosis+ES-62
ST2KO Fibrosis+ES-62

Figure 4.14 The *ex vivo* cytokine production profile of draining and peripheral lymph node cells from the chronic airway inflammation model in ST2-deficient mice

ST2-deficient mice were treated and sacrificed as described in the legend to Figure 4.12. Thoracic and cervical lung-draining lymph nodes (DLN; **A-E**) and non-draining (peripheral) lymph nodes (PLN, **F- I**) from each mouse were removed. DLN and PLN cells from mice in each group were pooled and cultured with medium alone (Control), OVA (Ova; 500 μg/ml) or Con A (5 μg/ml) for 72h. Culture supernatant concentrations of IL-4 (A), IL-5 (B, F), IL-10 (C, G), IL-12 (D, H) and TNFα (E, I) were measured by ELISA. ELISA data are expressed as mean ± SD (n=3). *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$ vs corresponding cells from fibrosis group mice.

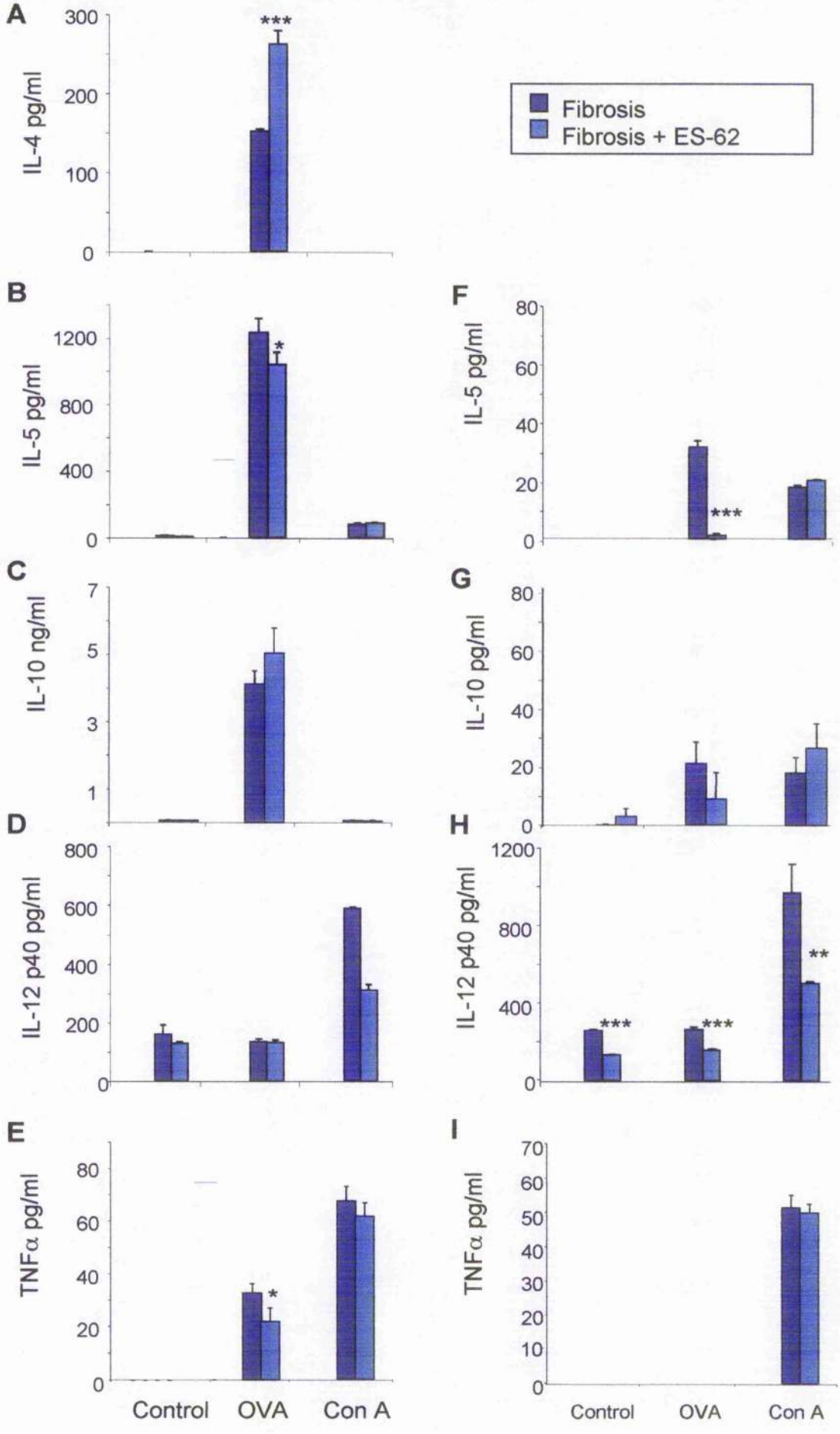

A
IL-4 pg/ml
300
200
100
0
***
Fibrosis
Fibrosis + ES-62
B
IL-5 pg/ml
1200
800
400
0
*
C
IL-10 ng/ml
7
5
3
1
D
IL-12 p40 pg/ml
800
600
400
200
0
E
TNFα pg/ml
80
60
40
20
0
*
Control
OVA
Con A
F
IL-5 pg/ml
80
60
40
20
0
***
G
IL-10 pg/ml
80
60
40
20
0
H
IL-12 p40 pg/ml
1200
800
400
0
***
***
**
I
TNFα pg/ml
70
60
50
40
30
20
10
0
Control
OVA
Con A

Figure 4.15 ES-62 treatment of the ovalbumin-induced asthma model *in vivo* modulates bone marrow-derived dendritic cell surface marker expression

BALB/c mice were treated and sacrificed as described in the legend to Figure 4.1 and femoral bone marrow from each mouse was removed. Bone marrow-derived DC were cultured *in vitro* for 7 days and stimulated with media (panel A) or LPS (1μg/ml; panel B) for 24h. Expression of MHCII, CD40, CD54, CD80 and CD86 on CD11c+ DC was analysed by flow cytometry. Data were gated on the $CD11c^+$ population and expressed as histograms depicting the expression level of the surface marker versus the number of cells, as a percentage of the maximum $CD11c^+$ cell number. Data are representative of 2 independent experiments.

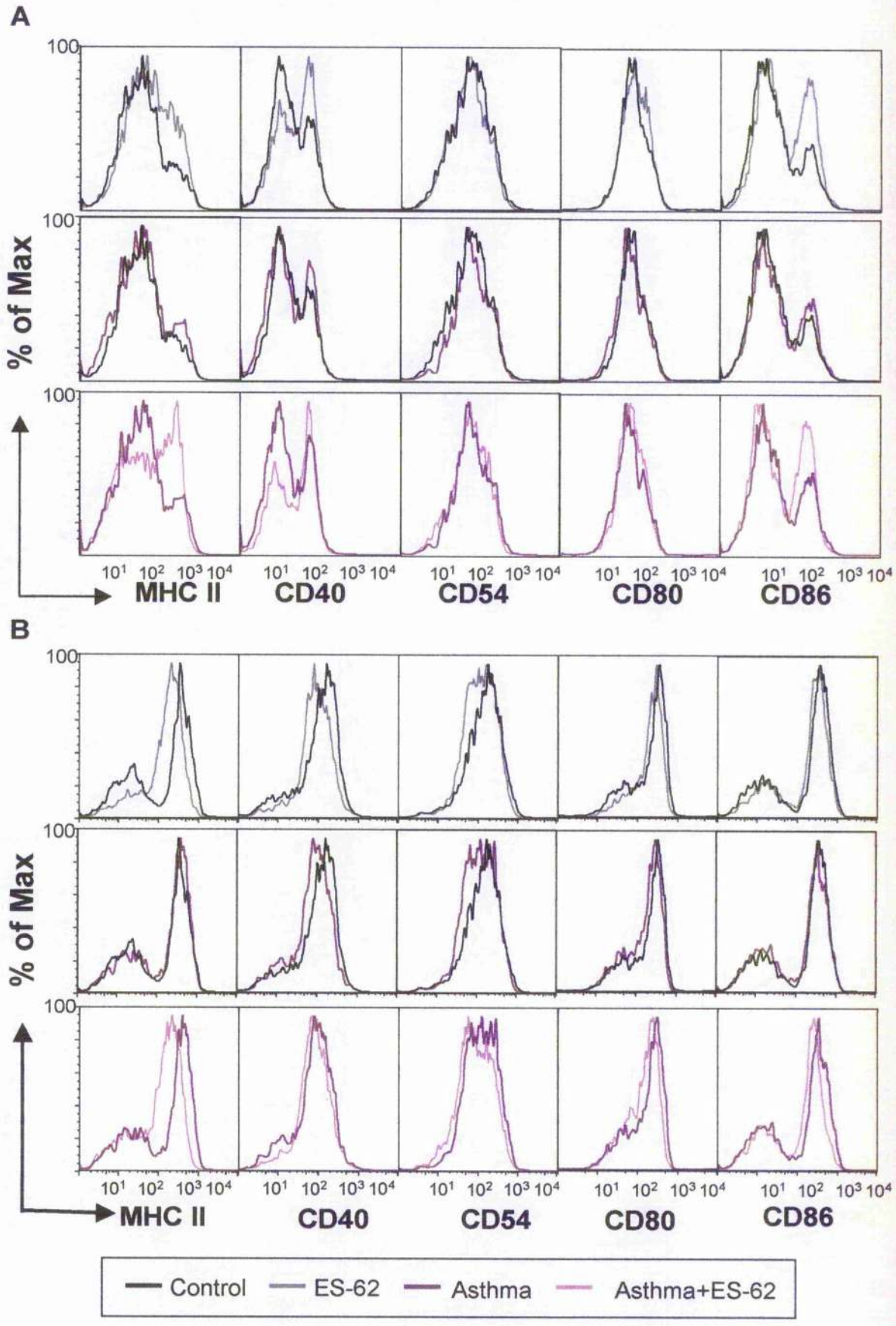

A
B
100
% of Max
10^1 10^2 10^3 10^4
MHC II
CD40
CD54
CD80
CD86
Control
ES-62
Asthma
Asthma+ES-62

Figure 4.16 ES-62 treatment of the ovalbumin-induced asthma model *in vivo* modulates bone marrow-derived dendritic cell production of TH1-promoting cytokines

BALB/c mice were treated and sacrificed as described in the legend to Figure 4.1. Femoral bone marrow from each mouse was removed and pooled by treatment group. Bone marrow-derived DC were cultured *in vitro* for 7 days and stimulated with media (panel A) or LPS (1μg/ml; panel B) for 24h. Culture supernatants were analysed for IL-12 and TNFαby ELISA. ELISA data are expressed as mean ± SD (n=3) and are representative of 2 independent experiments. ***, $p<0.001$ ES-62 vs Control and Asthma vs Asthma+ES-62; **, $p<0.01$ vs Control; *, $p<0.05$ vs Asthma.

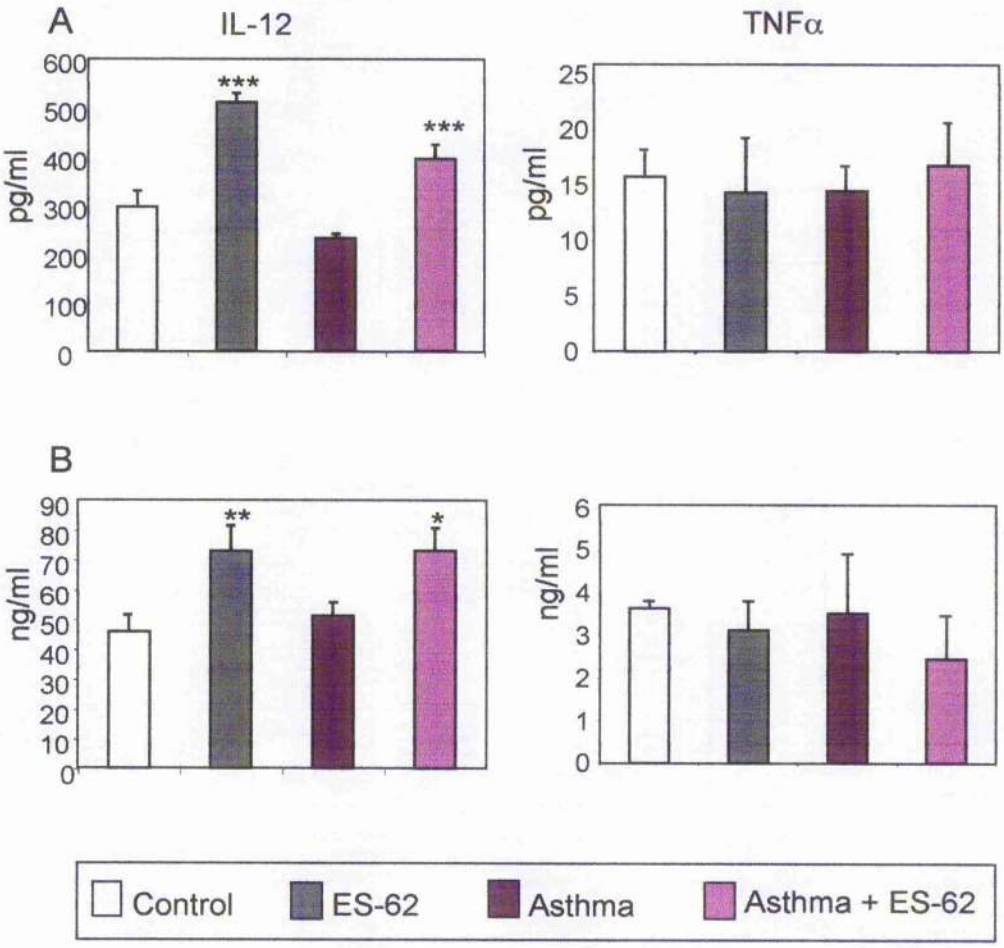

A
IL-12
TNFα
pg/ml
600
500
400
300
200
100
0
***
***
25
20
15
10
5
0
B
ng/ml
90
80
70
60
50
40
30
20
10
0
**
*
6
5
4
3
2
1
0
Control
ES-62
Asthma
Asthma + ES-62

Figure 4.17 The effects of ES-62 treatment of the ovalbumin-induced chronic airway inflammation model *in vivo* on bone marrow-derived dendritic cell surface marker expression

BALB/c mice were treated and sacrificed as described in the legend to Figure 4.12 and femoral bone marrow from each mouse was removed. Bone marrow-derived DC were cultured in vitro for 7 days and stimulated with media or LPS (1μg/ml, Panel B) for 24h. Expression of MHCII, CD40, CD54, CD80 and CD86 on CD11c+ DC was analysed by flow cytometry. Data were gated on the $CD11c^+$ population and expressed as histograms depicting the expression level of the surface marker versus the number of cells, as a percentage of the maximum $CD11c^+$ cell number. A histogram of non-stained control DC is plotted for comparison.

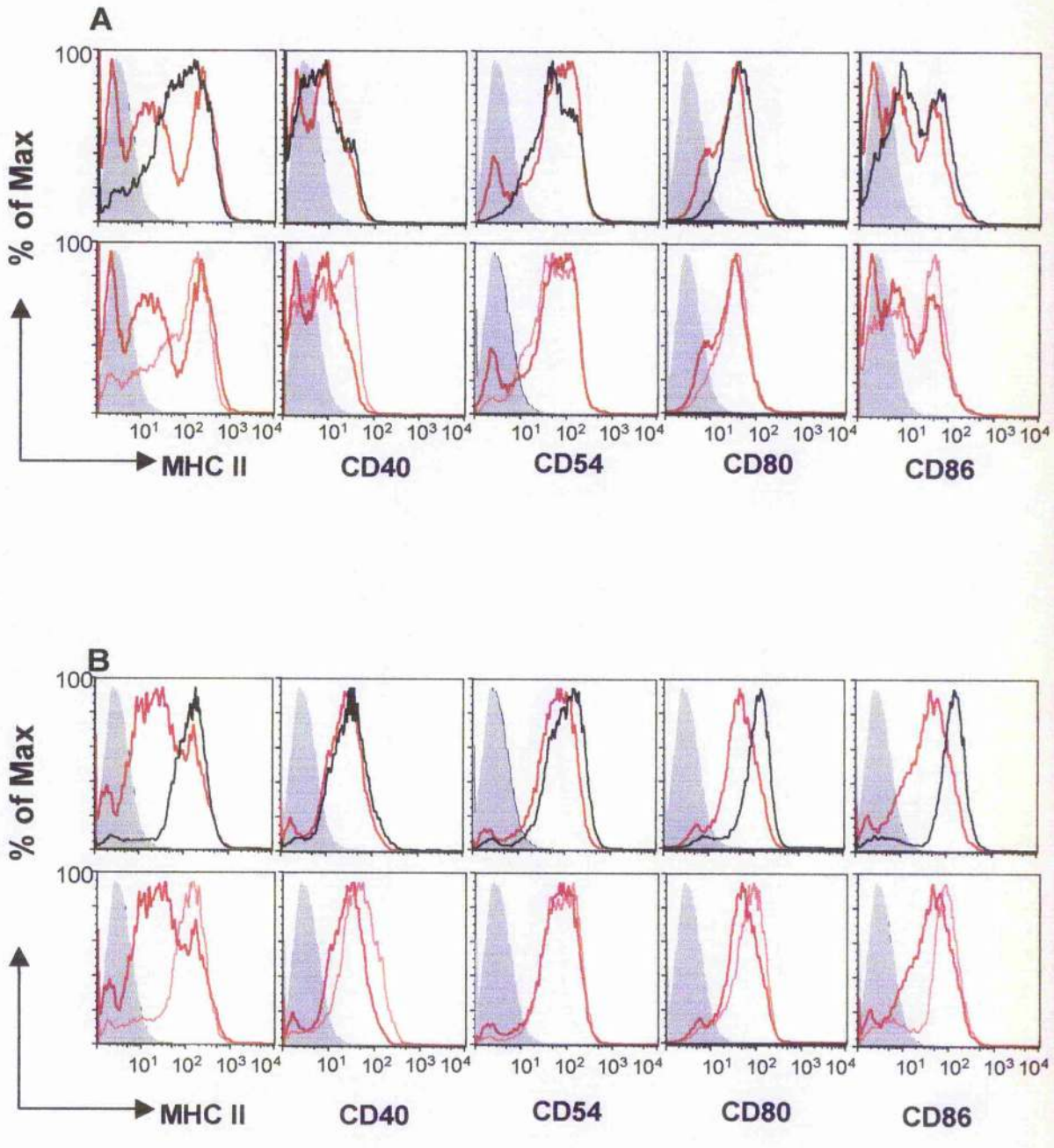

A
B
% of Max
100
MHC II
CD40
CD54
CD80
CD86
Fibrosis
Fibrosis+ES-62
Wild type (-)
Wild type (-) (no stain)

Figure 4.18 Dendritic cells derived from the chronic inflammation model display a modulated TH1-promoting cytokine production profile that is rescued by ES-62 exposure *in vivo*

BALB/c mice were treated and sacrificed as described in the legend to Figure 4.12 and femoral bone marrow from each mouse removed. Bone marrow-derived DC were cultured *in vitro* for 7 days and stimulated with media (panel A) or LPS (1μg/ml; panel B) for 24h. Culture supernatants were analysed for TNFα, IL-12 and IL-10 by ELISA in triplicate wells. ELISA data are expressed as mean ± SD (*n*=3). *, $p<0.05$ vs wild-type control; **, $p<0.01$ vs wild-type control; ***, $p<0.001$ vs wild-type control; ‡, $p<0.01$ vs wild-type fibrosis (by ANOVA).

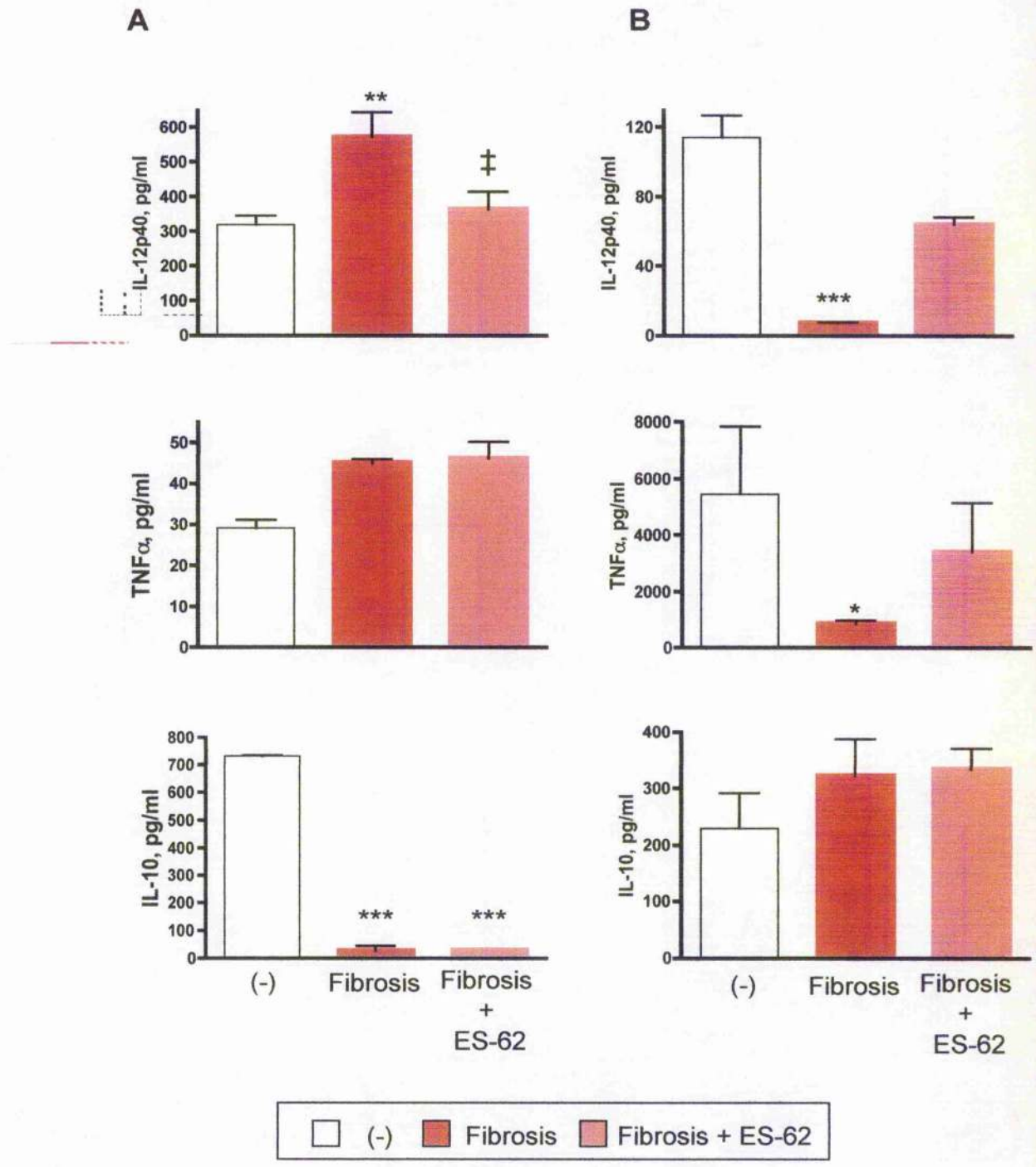

A
B
IL-12p40, pg/ml
TNFα, pg/ml
IL-10, pg/ml
**
‡
***
*
(-)
Fibrosis
Fibrosis + ES-62

Figure 4.19 The effect of ES-62 exposure *in vivo* on the surface expression profile of dendritic cells from ST2-deficient chronic inflammation model mice

ST2-deficient mice were treated and sacrificed as described in the legend to Figure 4.12 and femoral bone marrow from each mouse was removed. Bone marrow-derived DC were cultured in vitro for 7 days and stimulated with media (panel A) or LPS (1μg/ml; panel B) for 24h. Expression of MHCII, CD40, CD54, CD80 and CD86 on CD11c+ DC was analysed by flow cytometry. Data were gated on the $CD11c^+$ population and expressed as histograms depicting the expression level of the surface marker versus the number of cells, as a percentage of the maximum $CD11c^+$ cell number. A histogram of non-stained ST2KO DC is plotted for comparison.

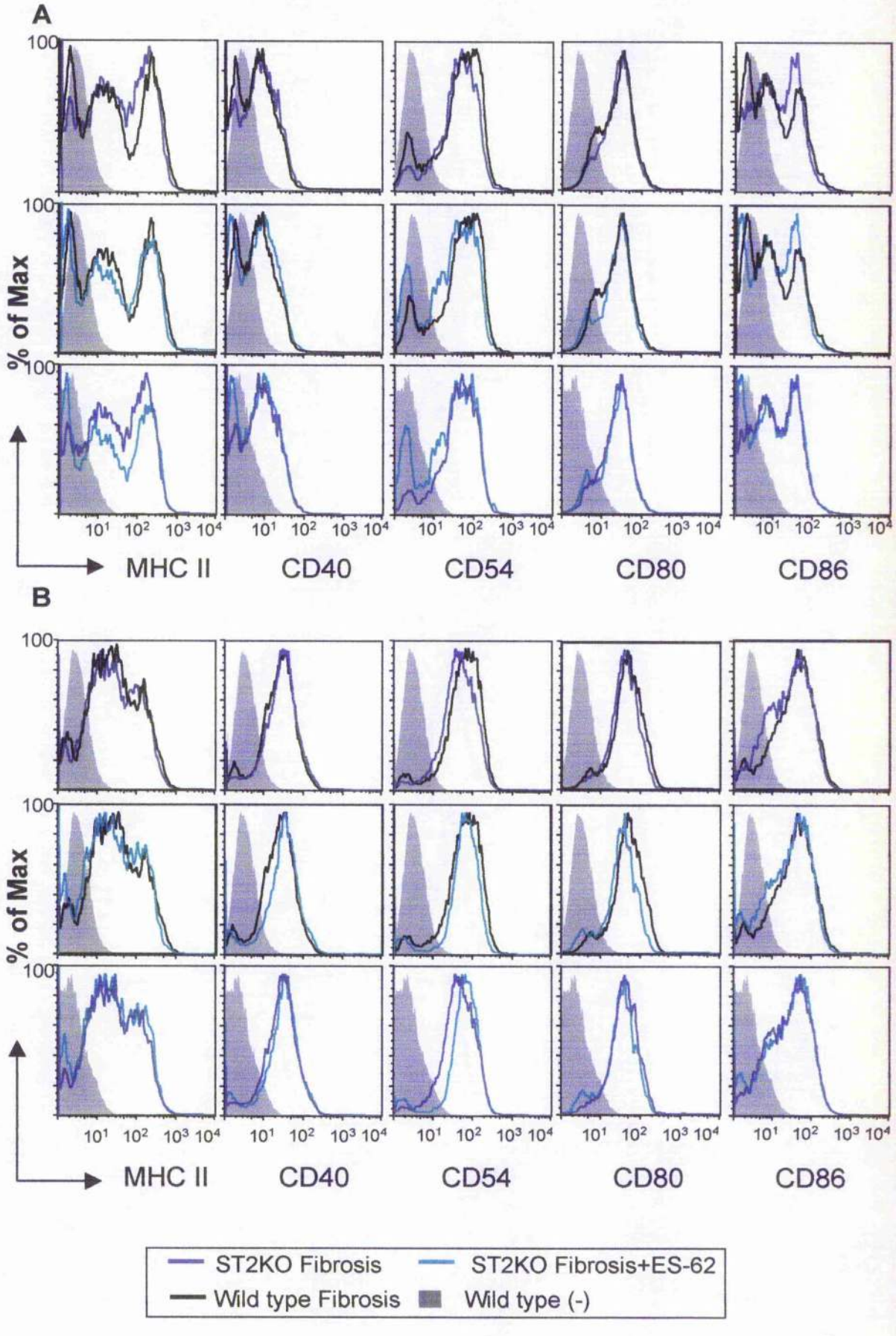

A
B
% of Max
100
MHC II
CD40
CD54
CD80
CD86
ST2KO Fibrosis
ST2KO Fibrosis+ES-62
Wild type Fibrosis
Wild type (-)

Figure 4.20 The cytokine production profile of dendritic cells derived from ST2-deficient chronic inflammation model mice

ST2-deficient mice were treated and sacrificed as described in the legend to Figure 4.12 and femoral bone marrow from each mouse removed. Bone marrow-derived DC were cultured in vitro for 7 days and stimulated with media (panel A) or LPS (1μg/ml; panel B) for 24h. Culture supernatants were analysed for TNFα, IL-12 and IL-10 by ELISA in triplicate wells. ELISA data are expressed as mean ± SD (*n*=3). **, $p<0.01$ vs ST2KO fibrosis DC; +, $p<0.05$ vs wild-type fibrosis DC, +++, $p<0.001$ vs wild-type fibrosis DC (by ANOVA).

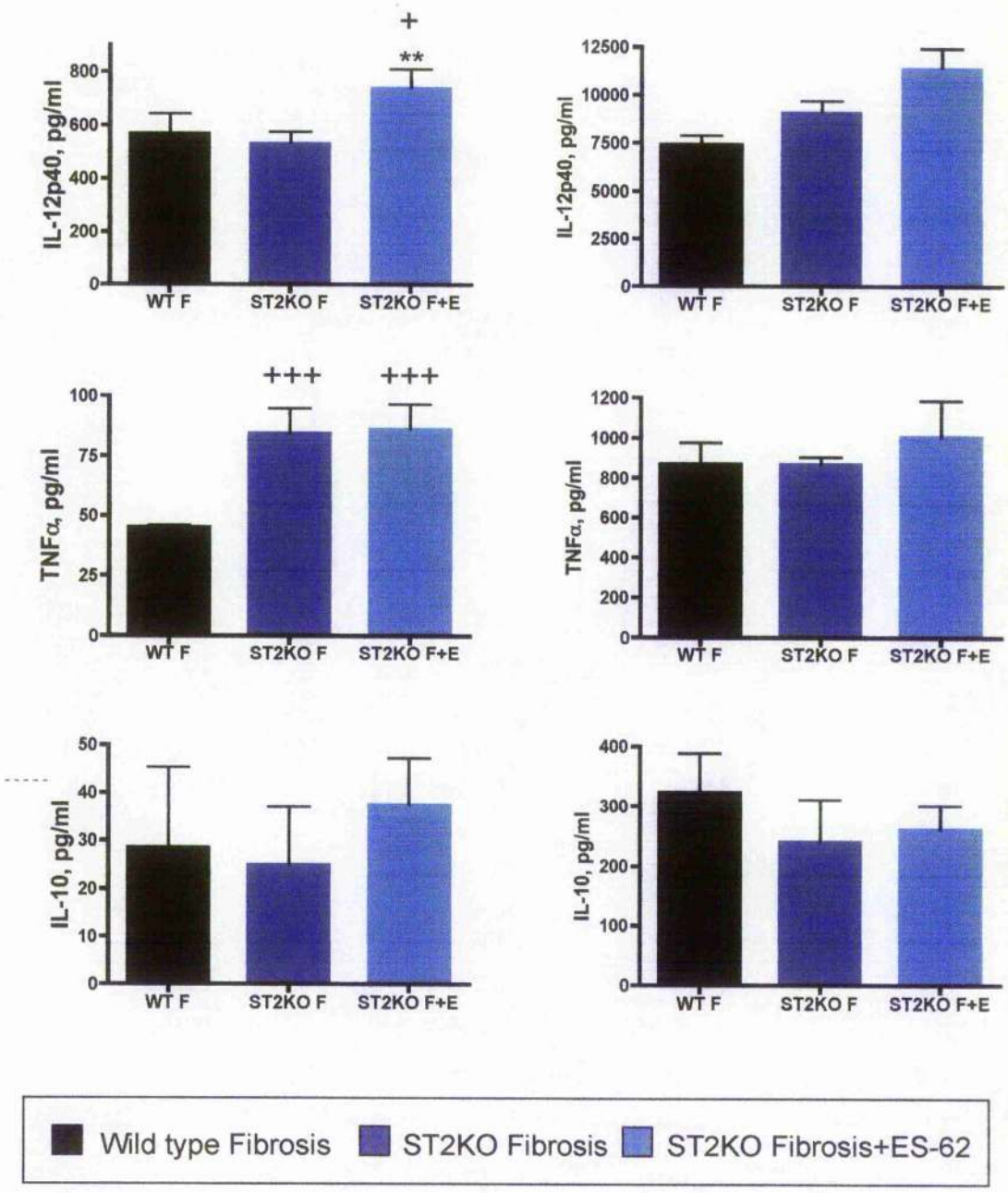

+
**
IL-12p40, pg/ml
800
600
400
200
0
WT F
ST2KO F
ST2KO F+E
12500
10000
7500
5000
2500
0
+++
+++
TNFα, pg/ml
100
75
50
25
1200
1000
800
600
400
IL-10, pg/ml
50
40
30
20
10
Wild type Fibrosis
ST2KO Fibrosis
ST2KO Fibrosis+ES-62

Figure 4.21 Spontaneous cytokine production by bone marrow macrophages derived from ovalbumin-induced airway inflammation model mice.

BALB/c mice were treated and sacrificed as described in the legend for Figure 4.1 and femoral bone marrow from each mouse was removed. Bone marrow-derived macrophages from Control, Asthma and Asthma+ES-62 treatment groups were cultured *in vitro* for 7 days before culturing in fresh media for 72h. Triplicate culture supernatants were analysed for TNFα, IL-12 and IL-10 by ELISA. Data are expressed as mean ± SD (n=3) and are the results of one experiment. *, $p<0.05$ and **, $p<0.01$ vs Asthma treatment group.

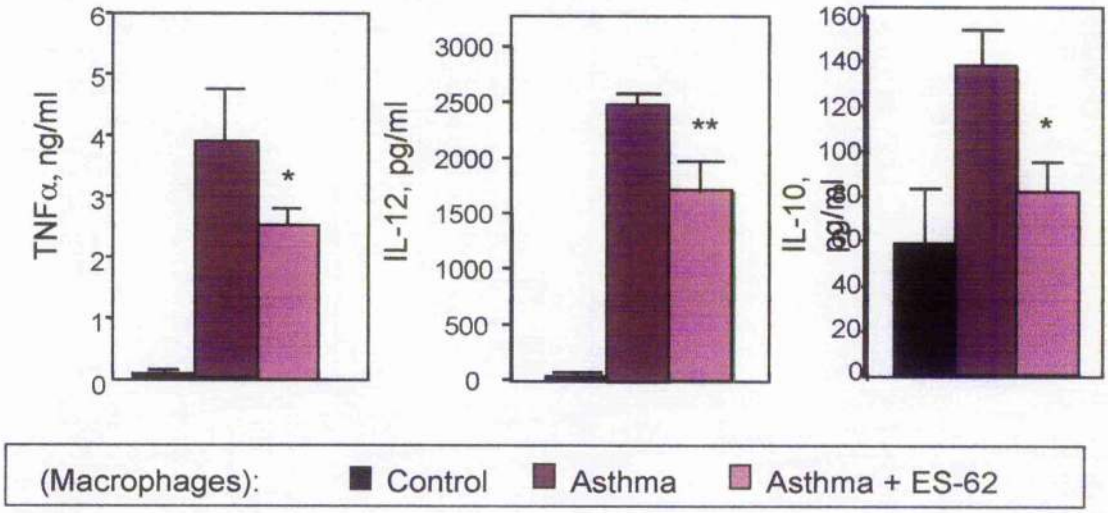

TNFα, ng/ml
6
5
4
3
2
1
0
*
IL-12, pg/ml
3000
2500
2000
1500
1000
500
0
**
IL-10, pg/ml
160
140
120
100
80
60
40
20
0
*
(Macrophages):
Control
Asthma
Asthma + ES-62

Figure 4.22 ES-62-mediated inhibition of inflammation in the short-term ovalbumin-induced asthma model: modulation of initiation mechanisms

In the short-term airway inflammation model bone marrow-derived DC are released into the periphery expressing low/moderate levels of cell surface markers and producing low levels of TH1-promoting cytokines, IL-12 and TNFα. Upon recognition of ovalbumin (OVA), these DC present antigens of OVA to naïve T cells in the secondary lymphoid organs and induce generation of an antigen-specific TH2-mediated inflammatory immune response, evidenced by production of TH2 cytokines, IL-4 and IL-5, which, in turn, induce B cell production of antigen-specific IgE, the classical inflammatory TH2-type antibody isotype. In the airways, TH2 cytokines promote recruitment of eosinophils and mast cells. Antigen-specific IgE binds to high affinity FcεRI receptors on the surface of mast cells and upon antigen-IgE complex fomation, mast cell degranulation ensues, resulting in release of pre-formed inflammatory mediators and further TH2 cytokines. All of these events contribute to development of TH2-mediated airway inflammation in this model.

Prophylactic ES-62 treatment of this model modulates the phenotype of DC that differentiate from bone marrow-progenitor cells, which results in development of DC with a more mature phenotype. This may result in development of a less potent TH2 immune response. ES-62 also acts to inhibit production of antigen-specific TH2 cytokines and IgE. Thus, recruitment of eosinophils to the airways and overall development of TH2 inflammation in this model is inhibited as a result of prophylactic ES-62 treatment.

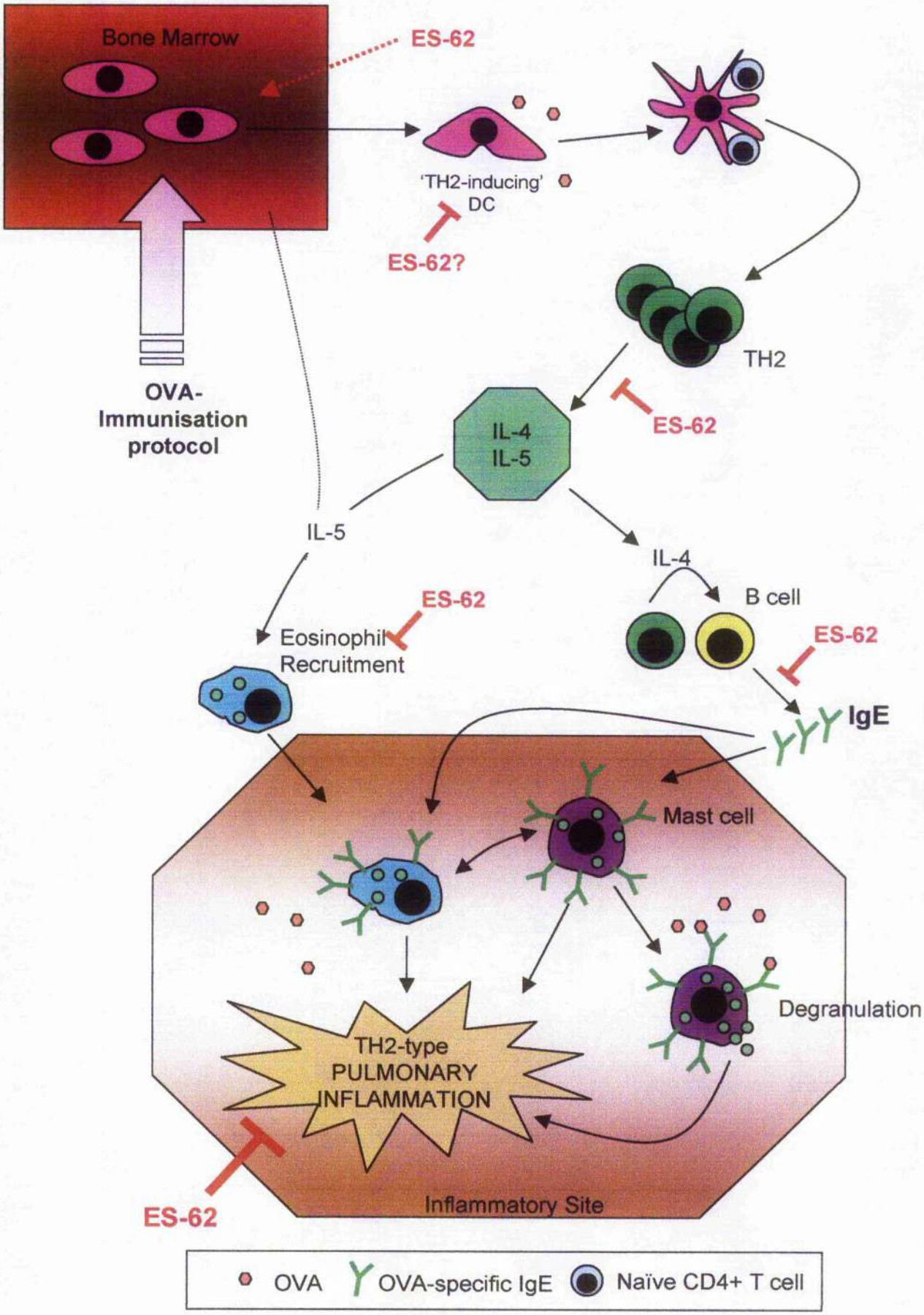

Bone Marrow
ES-62
'TH2-inducing' DC
ES-62?
TH2
OVA-
Immunisation
protocol
IL-4
IL-5
ES-62
IL-5
IL-4
B cell
ES-62
Eosinophil
Recruitment
ES-62
IgE
Mast cell
Degranulation
TH2-type
PULMONARY
INFLAMMATION
Inflammatory Site
ES-62
OVA
OVA-specific IgE
Naïve CD4+ T cell

Figure 4.23 ES-62 inhibits established TH2-mediated airway inflammation: modulation of effector mechanisms

Following establishment of a TH2-type inflammatory environment in the lungs (in the short-term model of airway inflammation) resident alveolar macrophages are activated and therefore, secrete enhanced quantities of pro-inflammatory cytokines, which act to continually recruit leukocytes to the inflammatory site. Antigen-specific TH2 cells secrete TH2-type cytokines in response to recognition of specific antigen. TH2 cytokines also help to recruit cells, especially eosinophils, to the airways. Antigen-specific IgE present in the airways binds to high-affinity FcεRI receptors on the surface of mast cells and low-affinity receptors (e.g. CD23) on other cells present. Formation of antigen-IgE complexes on the surface of the mast cell results in degranulation, inducing release of pre-formed inflammatory mediators and further TH2 cytokines. Production of inflammatory mediators and TH2 cytokines helps to maintain the inflammatory environment, which is exacerbated following inhalation of antigen (OVA). Therapeutic ES-62 treatment result in development of bone marrow-derived alveolar macrophages with a reduced activation state, which, in turn, leads to reduced production of inflammatory cytokines by these cells. In addition ES-62 treatment reduces recruitment of eosinophils to the lungs, which may be a consequence of its inhibitory action on macrophage activation status or a result of inhibition of TH2 cell cytokine production in response to antigen challenge.

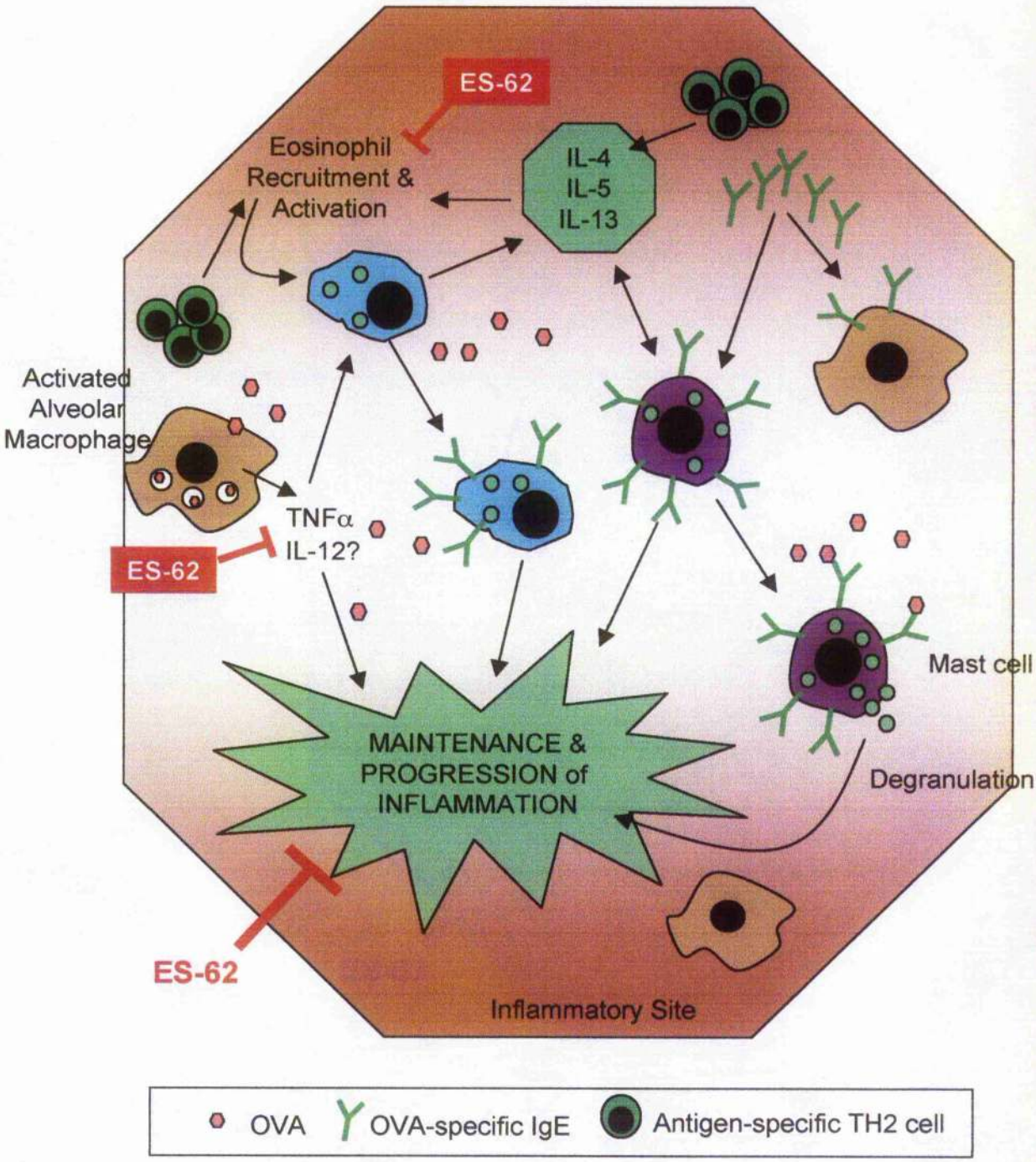
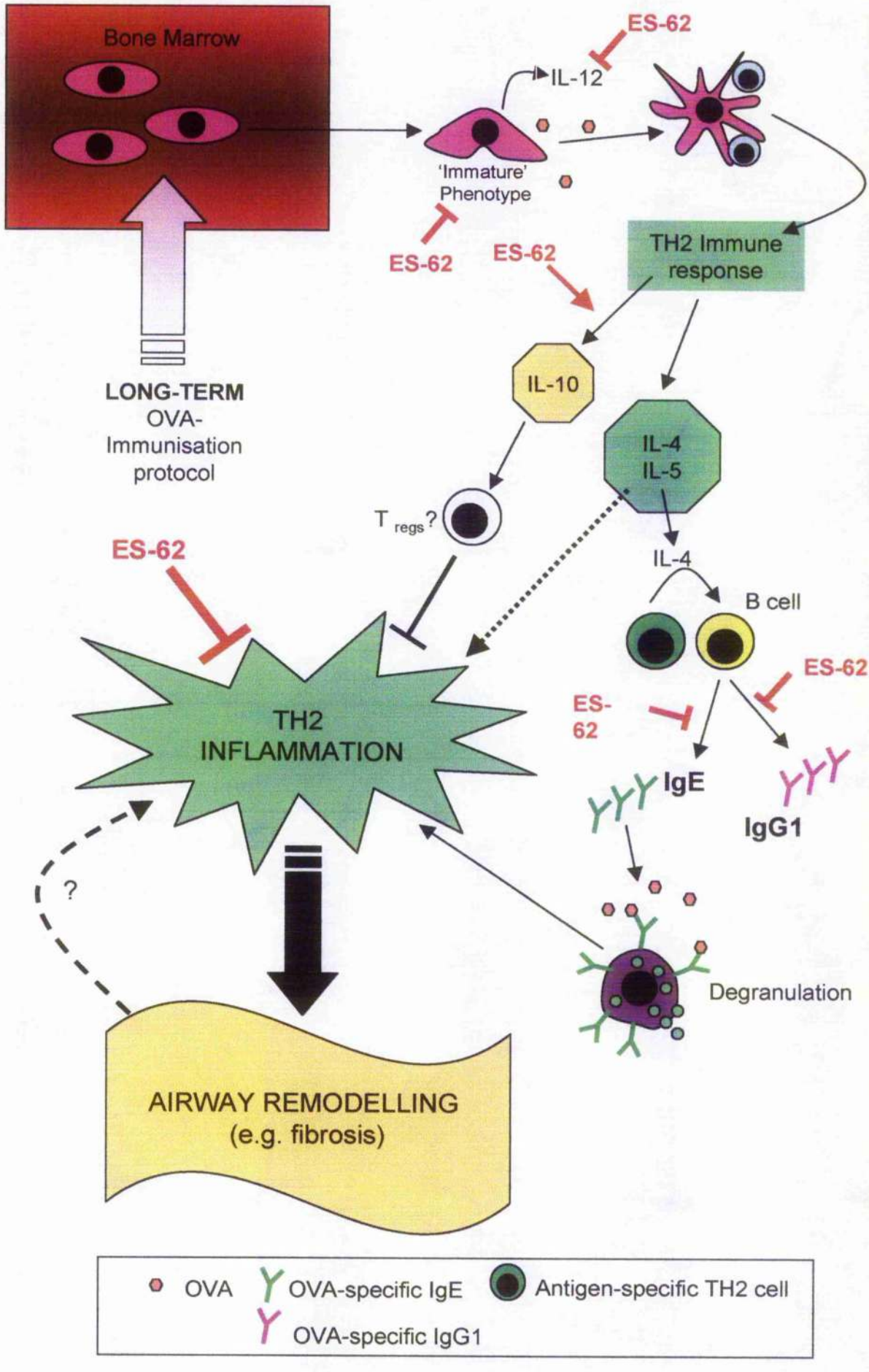

ES-62
Eosinophil
Recruitment &
Activation
IL-4
IL-5
IL-13
Activated
Alveolar
Macrophage
TNFα
IL-12?
ES-62
Mast cell
MAINTENANCE &
PROGRESSION of
INFLAMMATION
Degranulation
ES-62
Inflammatory Site
OVA
OVA-specific IgE
Antigen-specific TH2 cell

Figure 4.24 ES-62 inhibits development of severe pulmonary inflammation in a model of chronic airway inflammation

In response to the extended protocol of OVA immunisation and challenge in vivo, bone marrow-derived DC exhibit a slightly immature phenotype, evidenced by reduced expression of cell surface markers. Nevertheless, these DC also exhibit enhanced IL-12 production. Following activation of an antigen-specific TH2-type immune response, TH2 cells secrete TH2 cytokines (IL-4 and IL-5), which help to induce switching of B cell antibody production to TH2-type isotypes, IgE and IgG1. Furthermore, anti-inflammatory cytokine IL-10 is also produced in response to antigen recognition. As previously described (Figure 4.22), ligation of antigen-specific IgE bound to FcεRI receptors on the surface of mast cells in the inflammatory site, results in degranulation and release of TH2 cytokines and inflammatory mediators, adding to the inflammatory environment of the airways. Chronic airway inflammation and repeated activation of the inflammatory response to antigen challenge over an extended period of time results in development of several features of airway remodelling, for example, laying down of collagen in the airways (i.e. fibrosis). It has been suggested that the features of airway remodelling that develop as a result of chronic airway inflammation act to reduce airway integrity and further promote inflammation, thus inducing a positive feedback loop. ES-62 treatment of this model results in differentiation of bone marrow DC with a 'corrected' phenotype, evidenced by slightly enhanced expression levels of co-stimulatory molecules and inhibited spontaneous IL-12 production. ES-62 also acts to inhibit development of the antigen-specific TH2-type antibody responses, IgE and IgG1, whilst promoting production of anti-inflammatory cytokine, IL-10. Therefore it is proposed that these combined actions of ES-62 (at least) help to reduce the inflammation induced in the airways and thus, ultimately reduce the severity of the irreversible changes to airway integrity as a result of airway remodelling.


Bone Marrow
ES-62
IL-12
'Immature' Phenotype
ES-62
ES-62
TH2 Immune response
LONG-TERM OVA-Immunisation protocol
IL-10
IL-4 IL-5
T regs ?
IL-4
B cell
ES-62
ES-62
ES-62
TH2 INFLAMMATION
IgE
IgG1
?
Degranulation
AIRWAY REMODELLING (e.g. fibrosis)
OVA
OVA-specific IgE
Antigen-specific TH2 cell
OVA-specific IgG1

5 ES-62 inhibits inflammation exhibited in a model of systemic lupus erythematosus

5.1 Introduction

Systemic lupus erythematosus (SLE) is a chronic, remitting and relapsing, inflammatory disorder of unknown aetiology that is distinguished by its heterogeneous pathology in different patients. The average global prevalence of SLE is approximately 1 in 1000 [377] and it is 10-fold more prevalent in women of childbearing age than in men [21]. It is also more common in Asian and Afro-Caribbean than in European populations [378]

5.1.1 The pathology of SLE

The hallmark characteristic of SLE is the overproduction of antibodies (hypergammaglobulinemia) directed against auto-antigens (autoantibodies), including cell surface and nuclear proteins. It is understood that formation of gross autoantigen-autoantibody immune complexes (IC) initiates onset of the inflammatory pathologies exhibited in SLE [21]. These immune complexes usually form at the site of autoantigen expression, leading to organ- or cell-specific inflammation [22] and heterogeneous, SLE-associated inflammatory pathology can affect several different body systems simultaneously. In 1982 the American College of Rheumatology (ACR) proposed a set of criteria for identification and diagnosis of SLE [379, 380]. Patients exhibiting four or more of the eleven categorised manifestations of SLE (simultaneously or serially) can be confirmed as having the disorder. The eleven categories of pathology include different types of skin lesions, blood abnormalities, photosensitivity, haematological, immunological, neurological and renal disorders, arthritis and presence of antibodies specific for nuclear components (including double stranded DNA (dsDNA)). Most commonly in SLE patients, the kidneys, joints and skin are affected [381]. It is thought that SLE-associated nephritis (kidney inflammation) is initiated by ligation of pathogenic autoantibodies to antigen within the kidney glomeruli and immune complex formation, inducing proteinuria and haematuria [382, 383]. By measuring the levels of protein and blood excreted in the urine, the progression of kidney damage and therefore, disease severity, can be monitored. Non-erosive arthritis is also commonly exhibited [380]. This is most likely initiated by production of autoantibodies specific for joint proteins [21].

5.1.2 Animal models of SLE

Murine models of SLE have helped to define and clarify the underlying immunology of the inflammation exhibited in heterogeneous human SLE pathology. Human and mouse immune systems are generally similar, therefore, knowledge gained from studies using murine models has been applicable to human disease. Spontaneous onset, genetically determined murine models of SLE have been widely used, such as the NZB/W F1, BXSB and MRL/lpr models. These mouse strains have single gene mutations and

spontaneously develop autoimmune pathology similar to that exhibited in clinical SLE. Thus, pathologies developed by mice from all three strains include splenomegaly, lymphadenopathy, increased titre of IgG isotype autoantibodies and glomerulonephritis. [384, 385]. Surprisingly, whilst the genetics and pathology of each strain have been extensively characterised, the aetiology of disease in these models has not been elucidated.

NZB/W F1 mice are the hybrid progeny of a cross between New Zealand White (NZW) and New Zealand Black (NZB) mice, first proposed as a model for human SLE in 1963 [386]. Disease onset is accelerated in female NZB/W F1 mice, with symptoms arising at approximately 6 months and 50% mortality at 8.5 months old. Male NZB/W F1 mice develop disease later, with 50% mortality at 15 months of age [384].

BXSB mice were proposed as a model for SLE a decade after discovery of the NZB/W model by a single cross between a female C57BL/6 mouse and a male SB/Le mouse homozygous for linked mutant genes, satin (*sa*) and beige (*bg*) [385]. The onset of clinical symptoms is also affected by sex in this model, however, in contrast to the NZB/W F1 strain, male BXSB mice are first affected. It was identified that this was because BXSB mice carry a disease-enhancing *Yaa* gene on the Y chromosome [387], which was paternally transmitted. 50% mortality in male BXSB mice is reached at approximately 6 months and in females at 15 months.

Most recently, the MRL/lpr model of SLE was developed. By far, the most severe and extensive pathology of the three spontaneous onset models described is exhibited by MRL/lpr mice [385]. In addition to the pathologies mentioned above, MRL/lpr mice exhibit RA-like footpad inflammation and systemic vasculitis [388], which are frequently diagnosed in human SLE [389]. Lymph node hyperplasia is up to 50 times greater in MRL/lpr mice than in NZB/W F1s and, unlike the NZB/W F1 and BXSB strains, disease progression follows a similar time-course in males and females, with early onset of symptoms and 50% mortality at 5-6 months.

The genome of the MRL background strain is derived from LG (75%), AKR (12.6%), C3H (12.1%) and C57BL/6 (0.3%) murine strains [385]. A spontaneous, autosomal, recessive mutation (lymphoproliferation, *lpr*) triggered the development of MRL/lpr mice [390]. The homozygous *lpr* mutation carried by MRL/lpr mice was found to be a mutation of the *fas* gene [391], preventing expression of a functional Fas protein in this mouse. Fas is a member of the TNF receptor family and is expressed at the cell surface. Following ligation by Fas ligand (FasL), Fas initiates caspase-mediated apoptosis [392] and inflammation signal transduction [393, 394]. Apoptosis mediated by Fas is essential for elimination of

haematopoietic cells, which explains the increased levels of lymphocytes, monocytes and macrophages in MRL/*lpr* mice [395]. The mechanism by which mutations in *fas* leads to accelerated autoimmunity is unknown [21], however the strongest hypothesis suggests that the pathology exhibited is the result of autoreactive T and B cells that are resistant to apoptosis. For example, it has been suggested that upregulation of Fas antigen, normally expressed by autoreactive precursor T cells in the thymus leads to thymic selection, preventing release to the periphery [391]. Thus, Fas deficiency permits migration of autoreactive T cells to the periphery, resulting in autoimmune pathology. Fas is also involved in T cell homeostasis in the periphery. Following activation, as part of an immune response, Fas functions to switch off T cell activation, therefore, Fas deficiency might also lead to defective T cell regulation, resulting in large numbers of circulating cross-reactive T cells with low-affinity for autoantigens [396]. Whilst it is important to note there is no counterpart to the MRL/lpr mouse in humans, *fas* mutations have been identified in a small number of children with autoimmune syndromes [21, 397].

It is understood that the MRL/lpr model mimics the immunopathology and serology of human SLE better than any other animal model [395] therefore; this model was employed to examine lupus-associated inflammation in this laboratory.

5.1.3 The immunology of SLE

As mentioned previously, the aetiology of SLE is currently unknown and the heterogeneity of the exhibited pathology suggests there may be several mechanisms of pathogenesis. Hereditary studies have indicated that a pre-disposition to SLE is inherited in a polygenic manner [381]. Despite evidence supporting a genetic component in SLE, 75% of monozygotic twins are discordant for the disease [21], indicating that environmental contributions also influence onset [37, 398]. It is still not clear whether SLE is an autoimmune inflammatory disorder with many varied pathologies or whether the observed pathologies are a group of distinct inflammatory conditions. The common denominator in SLE is the production of anti-DNA autoantibodies. Irrespective of the inflammatory outcomes, almost all SLE patients exhibit high levels of autoantibodies [22]. Pathogenic autoantibodies are usually IgG isotype and their levels have been shown to correlate with disease severity [399]. Apoptotic cells are considered the source of DNA, histones and nucleoprotein complexes that drive production of pathological autoantibodies in SLE. Thus, it has been suggested that defective clearance of cell debris following apoptosis (programmed cell death) may be fundamental to lupus pathogenesis. ([400]; [401]). In addition to anti-dsDNA antibodies, there have been several suggested biomarkers of SLE [10, 402], including products of complement activation and various cytokines.

5.1.3.1 B and T lymphocytes

Mature B cells mediate autoantibody production in SLE [403]; therefore dysregulated autoreactive B cells have been implicated in SLE pathogenesis [404]. T cells do not appear to play a direct role in tissue damage exhibited in SLE however, they are necessary for the development of autoantibody production and the progression of SLE [21]. Consistent with this, it has been demonstrated that T cell deficient SLE-prone mice failed to develop lupus-associated pathology [405] and disruption of T cell signalling abrogates disease progression in a murine model of SLE [406, 407]. Thus, T and B cells (and the interactions between these two cell types, leading to autoantibody production) have been determined essential for progression of SLE. Moreover, whilst it has been demonstrated that T cells are integral to disease progression, it remains unclear whether the inflammation exhibited in SLE is of a particular TH-phenotype. It has been suggested that TH2 type immune responses may be important in SLE disease development [21], however in animal models of SLE (see below) the influence of Th1-mediated immunity has also been highlighted [408]. Therefore, T cell-derived cytokines of TH1- *and* TH2-promoting natures have been identified as important and facilitatory in the pathogenesis of SLE. Furthermore, contradictory evidence supporting a role for TH1 cytokine, IFNγ, in SLE has been published [409-411] and TH1-promoting cytokines, IL-6 and TNFα, have been implicated as important mediators of tissue injury in SLE [402]. The TH2 cytokine, IL-4, was detected in increased levels in SLE patient serum samples, however research investigating a role for this cytokine in disease pathogenesis has been conflicting [402]. In summary, SLE is an inflammatory disease of ambiguous immunological phenotype.

5.1.3.2 Dendritic cells

Dendritic cells (DC) have been dubbed the 'sentinels' of the immune system, providing the necessary communication conduit between innate and adaptive immune responses [412]. DC are necessary for priming and activating naïve T cells and for inducing anergy and deletion of autoreactive T cells [413-415]. With such an important facilitatory role, it is not surprising that DC have also been implicated in SLE pathogenesis. DC are capable of presenting auto-antigens as 'immunogenic', overproducing inflammatory cytokines or failing to adequately induce tolerance of autoreactive T cells. Under these conditions, DC could aberrantly initiate T cell priming resulting in many of the complex pathologies exhibited in clinical SLE. For example, SLE patient sera was found to induce maturation and differentiation of monocytes into DC [416], which was demonstrated to be a result of the characteristic elevated IFNα content of the serum [417]. Similarly, mice overexpressing CD40L (which ligates the DC co-stimulatory signal molecule, CD40) exhibited autoimmune pathology and autoantibodies specific for skin and nuclear antigens, including DNA [418]. Indeed, this study demonstrated that chronic DC activation leads to an autoimmune phenotype corresponding to the antigens that reside at the site of

initial activation. Repeated transfer of DC (matured by exposure to apoptotic or necrotic cells) to immunologically normal C57/BL6 mice induced expression of anti-DNA antibodies [419], but did not induce autoimmune pathology, indicating that whilst DC might have a role to play in initiation of autoantibody production, this was not sufficient for triggering autoimmunity. In summary, maturation of DC appeared to be integral to promotion of autoimmunity in SLE. Thus, it was suggested that targeting DC maturation might be a viable method of treating SLE patients [420].

5.1.3.3 Macrophages

It has been identified that SLE patients exhibit accumulation of apoptotic cell debris in certain body tissues (e.g. germinal centres in lymph nodes) [421, 422] and it has been suggested that insufficient clearance of apoptotic cells and necrotic tissue (leading to autoantibody formation against nuclear antigens) is a result of defective macrophage function in SLE. Indeed, macrophages cultured *in vitro* from $CD34^+$ stem cells obtained from SLE patients were demonstrated to be incapable of sufficiently clearing apoptotic cells, due to defects in phagocytosis [423]. It has been suggested that apoptotic cells, which are not cleared by phagocytes such as macrophages, become 'secondary necrotic', which leads to the release of danger signals from the dying cells and provides access for the immune system to target nuclear antigens [423]. Therefore, via activation of dendritic cells, an immune response specific for nuclear components can be initiated.

In particular, development of anti-double stranded DNA (dsDNA) autoantibodies has been implicated in the pathogenesis of SLE. Anti-dsDNA and anti-Sm (a nuclear ribonucleoprotein) antibodies are unique to patients with SLE [424]. Moreover, it has been suggested that anti-DNA antibodies contribute to lupus nephritis via cross-reactivity with renal antigen (α-actinin) expressed in mesangial cells of the kidney [425]. Furthermore, it has been demonstrated that polyclonal anti-DNA antibodies from patients with SLE (involving lupus nephritis) bound mesangial cells and this binding was positively correlated with severity of disease. Other kidney-derived cells (glomerular and proximal tubular epithelial cells) were also bound by the anti-DNA IgG, however, mesangial cells exhibited the greatest anti-DNA binding activity [426]. Thus, it would appear that development of anti-DNA autoantibodies leads to initiation of an inflammatory immune response particularly at the site of kidney mesangial cells. Ultimately this would lead to nephritis as exhibited in several SLE patients and in murine models of this disease.

In addition, autoantibody production in SLE appears to be dysregulated such that vast quantities of autoantibodies are generated. This leads to formation of gross immune complexes (IC), which induce tissue inflammation by immunological means (recognition of specific antigen), but also by mechanical means due to their macromolecular size. More

specifically, formation of autoantibodies and autoantigens into large IC results in trapping (or deposition) of the autoantibodies in certain tissues [424]. Indeed, it has been suggested that SLE-prone mice exhibit impaired ability to resolve immune complexes in the kidney [427], which contains some of the smallest blood vessels in the body. Therefore, it is perhaps not surprising that the kidney becomes a common end-point for autoimmune inflammation in SLE, considering that IC of autoantibodies regularly become deposited in this organ.

5.1.4 Aims

It has previously been shown that ES-62 elicits anti-inflammatory action in a model of Th1-mediated autoimmune (CIA) inflammation (Chapter 3) and a model of TH2-mediated airway inflammation (Chapter 4). Thus, it was a core aim of this investigation to determine whether ES-62 would ameliorate inflammation, of ambiguous TH-phenotype, exhibited in a model of prototypic autoimmunity, SLE.

It was planned to determine the effects of ES-62 in the MRL/lpr model of spontaneous autoimmunity, by treating with ES-62 in a prophylactic manner and monitoring the mice for signs of autoimmune pathology, such as lupus nephritis. Following identification of perceived ES-62 mediated modulation of inflammation in this model it was planned to determine the immunological mechanisms underlying such action. For example, it has been suggested that aberrant DC function might form an integral component of SLE pathogenesis [428]. Indeed, modulatory effects of ES-62 treatment on DC phenotype *in vitro* and *in vivo*, have previously been identified in other models of inflammatory disease (Chapter 3, Chapter 4, [192]). Hence, it was also planned to determine whether ES-62 treatment would modulate the phenotype of DC isolated from SLE model mice. In summary, the individual aims of this investigation are:

1. To determine the effect of prophylactic ES-62 treatment of the inflammation exhibited in the MRL/lpr model of SLE.
2. To characterise the immunology of the MRL/lpr model of SLE and determine any effect of ES-62 treatment on this.
3. To dissect the mechanisms of any ES-62 action in the MRL/lpr model by investigating the phenotype of bone marrow-derived DC.

5.2 Results

The MRL/*lpr* mouse model of systemic lupus erythematosus (SLE) exhibits several features of human SLE and has been widely established as a working system for investigation of SLE pathogenesis [429]. As mentioned above, the pathology of human lupus involves overproduction of self-specific antibody (autoantibodies) and subsequent formation of autoantigen-autoantibody immune complexes. Lymphadenopathy, splenomegaly and nephritis leading to kidney damage are common features of severe SLE and are exhibited in the MRL/lpr mouse model. MRL/lpr mice and patients with lupus persistently excrete protein via their urine (proteinuria). Consequently, proteinuria can be used as a measure of the progression of nephritis-induced kidney damage (and correlates with development of disease) in the SLE model. Thus, it was hypothesised that the anti-inflammatory action of ES-62 evident in immune cells and other models of inflammatory disease ([192]; Chapter 3; Chapter 4), would inhibit the inflammation exhibited in this model of SLE.

5.2.1 MRL/lpr mice exhibited symptoms of progressive lupus-like disease

MRL/*lpr* mice were observed from 5 weeks old. Micturation was induced twice weekly and urine was monitored for protein (proteinuria) and blood content (haematuria) as an indicator of nephritis-induced kidney damage and, hence, disease progress. PBS or ES-62 treatment was administered (subcutaneously) twice weekly throughout the investigation. Proteinuria was detected in PBS treatment group mice at approximately 11 weeks of age (Figure 5.1a). In addition, haematuria was regularly detected in urine samples from these mice (Figure 5.1b). The progression of protein and blood loss detected in these mice indicated that kidney damage was occurring, similar to that observed in human lupus nephritis.

One day prior to sacrifice (at approximately 16 weeks of age), MRL/lpr mice were inspected for signs of arthritis, a common feature of lupus-associated inflammation. Arthritis-like footpad inflammation was observed in over 70% of the PBS-treated MRL/lpr mice, 1 day prior to sacrifice (Figure 5.1c). Therefore, in addition to nephritis, the SLE model mice exhibited symptoms of lupus-induced arthritis. At 16 weeks old, mice were euthanased and carefully dissected. Lymph nodes and spleen were removed and it was observed that these secondary lymphoid organs were markedly enlarged (Figure 5.2) compared with lymph nodes and spleens from naïve, non-MRL/lpr mice (approximately 20-fold and 10-fold, respectively).

In summary, the MRL/lpr SLE model mice exhibited several pathological features commonly exhibited in SLE patients (proteinuria, haematuria, arthritis, splenomegaly and

lymphadenopathy), therefore it was concluded that this was an appropriate model with which to determine the effect of ES-62 on SLE-associated inflammation.

5.2.2 ES-62 treatment of SLE model mice inhibits onset of lupus-associated inflammation

Prophylactic ES-62 treatment of MRL/lpr mice significantly delayed the onset and reduced the concentration of proteinuria detected (Figure 5.1a), indicating inhibition of nephritis. In addition, there was a reduced incidence of haematuria in the ES-62 treatment group (Figure 5.1b). These results suggested that ES-62 treatment of SLE model mice inhibited incidence and progression of kidney damage associated with SLE disease pathogenesis.

ES-62-treated MRL/lpr mice also exhibited significantly reduced severity and markedly reduced the incidence of arthritic inflammation compared with PBS-treated mice (Figure 5.1c, d). Indicating that ES-62 treatment inhibited lupus-associated arthritic inflammation, consistent with its anti-inflammatory action in the CIA model ([192]; Chapter 3).

Similar to the PBS treated mice, mice from the ES-62 treatment group exhibited widespread splenomegaly and lymphadenopathy. There was no difference in the size of lymph nodes or spleen from mice in either treatment group (Figure 5.2a, b). To more clearly determine any difference in lymphadenopathy, $CD3^+$ T cells from each mouse were isolated and counted. Consistent with a lack of difference in lymph node size, hyperplasia of $CD3^+$ T cells and $B220^+$ B cells were not significantly different between mice from PBS and ES-62 treatment groups (results not shown). These results indicated that ES-62-mediated inhibition of kidney damage or arthritic inflammation exhibited in this model was not facilitated by modulation of splenocyte or lymph node cell hyperplasia.

5.2.3 ES-62 treatment of SLE model mice does not significantly modulate serological factors

5.2.3.1 Serum immunoglobulin

Following confirmation of the lupus-like pathology exhibited in the MRL/lpr SLE model mice, examination of the underlying immunology of this model began. SLE is characterised by hypersecretion of autoantibodies, particularly antibodies specific for nuclear antigens that are thought to derive from apoptotic and necrotic cells [400, 401]. Serum samples obtained from MRL/lpr mice at sacrifice were analysed for immunoglobulin (Ig) and cytokine content. Hypersecreted autoantibodies in MRL/lpr mice are primarily IgG (IgG2a, IgG1) and IgM isotypes [429] and it is known that pathological autoantibodies secreted in human SLE are generally IgG isotype [381], therefore, the serum samples were titrated and the serum content of total IgG1, IgG2a and IgG3 was

determined. It was noted that serum from the SLE model mice contained strikingly elevated quantities of Ig overall, observed because the samples required markedly (up to 50-fold) more dilution than was normally used for analysis of serum Ig in other models, to be measured in the normal range. Thus, high concentrations of IgG1, IgG2a and IgG3 were detected in serum samples from PBS-treated mice (Figure 5.3a-c). *In vivo* treatment with ES-62 did not significantly modulate the average levels of the serum IgG in any of the subclasses analysed.

IgM is thought to play a role in opsonization and uptake of apoptotic and necrotic cells [21]. It has also been demonstrated that the presence of IgM anti-dsDNA antibodies correlated negatively with the development of nephritis (and the level of IgG) in SLE [430]. In light of this information, the level of total IgM in serum samples from the MRL/lpr mice was determined. Similar to the results of serum IgG analysis, IgM was readily detected in samples from PBS treated SLE model mice (Figure 5.3d) and ES-62 treatment did not significantly modulate these levels.

In summary, analysis of serum Ig indicated that there was a grossly elevated production of IgG in MRL/lpr mice, consistent with the observed hypersecretion of this pathogenic Ig isotype in human SLE. The increased serum IgG might have been responsible for the observed effector phase of this disease (nephritis) via formation of large immune complexes leading to tissue damage. ES-62 treatment did not modulate the serum content of IgG or IgM, indicating that modulation of the isotype or quantity of total serum antibody secreted was not a method employed by ES-62 for inhibition of inflammatory pathology. This finding was consistent with the fact that MRL/lpr mice from both treatment groups exhibited similar numbers of lymphocytes in lymph nodes and spleen *ex vivo*.

The presence of anti-double stranded (ds) DNA antibodies, in particular, has been demonstrated as integral to SLE pathogenesis. Indeed, the presence of anti-dsDNA antibodies fulfils one of the ACR classification criteria for diagnosis of SLE [381]. It has also been suggested that serum levels of anti-dsDNA correlate with severity of inflammatory pathology exhibited in SLE, especially nephritis [399]. Thus, the content of anti-DNA Ig in serum samples from SLE model mice was analysed. In addition the effect of ES-62 treatment of SLE model mice on the serum levels of these autoantibodies was determined. Serum samples were obtained at day 95, diluted and analysed for content of anti-single stranded (ss) DNA and anti-dsDNA. Anti-ssDNA IgG2a and anti-dsDNA IgG2a antibodies were detected in serum samples from PBS-treated and ES-62 treated MRL/lpr mice (Figure 5.3e, f). ES-62 treatment did not induce significant modulation of anti- (ss or ds) DNA IgG2a levels. This finding indicated that ES-62 did not mediate anti-inflammatory

action in this SLE model by modulating production of anti-DNA autoantibodies of this isotype.

5.2.3.2 Serum cytokines

Serum samples from human SLE patients have been shown to contain elevated TH1 and TH2 cytokines [408]. Undiluted serum samples from MRL/lpr mice were analysed for cytokine content and found to contain TH1-promoting cytokines, TNFα, IL-12 and IL-2, TH2-type cytokine, IL-13 and B cell stimulating, T cell regulating cytokine, IL-10 (Figure 5.4a-e). Serum samples were also analysed for IFNγ, IL-1, IL-4, IL-5, IL-6 and IL-17, but these cytokines were not detected. This cytokine repertoire did not indicate a particular TH-phenotype of this disease. Such ambiguous immunological phenotype is consistent with the heterogeneous nature of the exhibited inflammation in human SLE. The TH-phenotype of SLE-associated inflammation is the subject of much controversy [409]. Treatment of the MRL/lpr mice with ES-62 did not significantly modulate the average serum cytokine content or repertoire of the group (Figure 5.4), indicating that modulation of serum cytokines or cytokine levels was not a method employed by ES-62 for its anti-inflammatory action in this model.

5.2.4 ES-62 significantly inhibited mitogen-induced cytokine production by LN cell from SLE model mice *ex vivo*

It has been widely accepted that T cells play an indispensable role in the pathogenesis of SLE [21], however, the TH-phenotype of these cells has been ill-defined. To determine the phenotype of lymphocytes in the MRL/lpr mice, lymph node (LN) cells from each mouse in both SLE model treatment groups were pooled and cultured *ex vivo* for 72h. It was not possible to stimulate an antigen-specific response by the LN cells *ex vivo*, because the antigen-specificity of the lymphocytes was not known. Therefore, LN cells from both treatment groups were stimulated *ex vivo* with media or the mitogen, Con A and analysed for cytokine, chemokine and growth factor production and DNA synthesis levels. Con A is a potent mitogen and when used to treat cells *in vitro*, it normally induces gross activation of cells, which demonstrated by high DNA synthesis and cytokine production levels.

Under control conditions, LN cells exhibited negligible levels of DNA synthesis ex vivo, indicating that they were not proliferating (Figure 5.5a) and ES-62 treatment *in vivo* did not significantly modulate this rate of DNA synthesis. However, when stimulated with Con A *in vitro,* SLE model LN cell proliferation was markedly elevated (Figure 5.5a). Again, ES-62 treatment of the mice *in vivo* did not significantly modulate the level of Con A-induced DNA synthesis by the LN cells (Figure 5.5a). Thus, any modulation of LN cell cytokine

secretion induced by ES-62 could be viewed as a direct effect of ES-62 on the cytokine production machinery and not a result of changes to cell number.

LN cells isolated from PBS-treated MRL/lpr mice spontaneously secreted measurable quantities of TH1-promoting cytokines, IL-1α and IL-12 (Figure 5.5). Interestingly, LN cells isolated from ES-62 treated SLE model mice exhibited significantly reduced IL-12 production *in vitro*, indicating that ES-62 treatment *in vivo* selectively modulated lymphocyte production of inflammatory mediators.

Con A treatment of LN cells induced production of additional cytokines not detected under control culture conditions (Figure 5.5). In addition to the cytokines produced spontaneously (IL-1α and IL-12,), Con A-treated LN cells produced significant quantities of TH1-type cytokines, IL-2, TNFα, IFNγ, and IL-17 and TH2-type cytokines, IL-10 and IL-13. LN cell culture supernatants were also analysed for the presence of cytokines, IL-4, IL-5 and IL-1β, but these were not detected. The range of cytokines secreted in response to mitogen indicated that the LN cells present were of different TH-phenotypes. ES-62 treatment of SLE model mice *in vivo* induced modulation of the normal LN cell cytokine response to Con A *ex vivo*. Con A-induced LN cell production of inflammatory cytokines, IFNγ, TNFα and IL-17 was significantly inhibited by ES-62 treatment of SLE model mice *in vivo* (Figure 5.5). Furthermore, LN cell production of inflammation-promoting IL-12 in response to Con A was abrogated by prior treatment with ES-62. Interestingly, LN cell production of the TH2 cytokine, IL-13, which was induced by Con A, was also inhibited by ES-62, indicating that ES-62 did not target solely TH1-type cytokines. In summary, ES-62 induced inhibition of the mitogen-induced cytokine response of lymphocytes present in the LN, independent of Th-phenotype.

5.2.5 ES-62 inhibits mitogen-induced chemokine production by LN cell from SLE model mice *ex vivo*

Chemokines are chemotactic cytokines, which have a major function in recruitment of cells to the inflammatory site and direction of cells within the immune response, thus, supernatants of the LN cell cultures were also analysed for chemokine content. LN cells isolated from PBS-treated MRL/*lpr* mice spontaneously secreted measurable quantities of IP-10 and MIG (Figure 5.6), chemokines induced by pro-inflammatory cytokine IFNγ. Interestingly, ES-62 treatment of the MRL/lpr mice *in vivo* appeared to reduce the spontaneous production of MIG, but not IP-10 by the LN cells *ex vivo*.

In addition to the chemokines produced spontaneously (IP-10 and MIG), Con A-treated LN cells produced significant quantities of inflammatory chemokines, KC, MCP-1 and

MIP-1α. Con A-induced MIP-1α production was significantly inhibited, whilst KC production was abrogated by ES-62 treatment of SLE model mice *in vivo* (Figure 5.6c, e).

5.2.6 ES-62 inhibits spontaneous and mitogen-induced angiogenic growth factor production by SLE model LN cells *ex vivo*

LN cells isolated from PBS-treated MRL/lpr mice spontaneously secreted measurable quantities of VEGF and FGF (Figure 5.7a, b), cellular growth factors with important roles in angiogenesis. Interestingly, ES-62 treatment of MRL/lpr mice induced inhibition of spontaneous VEGF, but not FGF production by LN cells *ex vivo*, suggesting that these factors may be differentially regulated.

Consistent with the enhanced proliferative response, Con A stimulation *in vitro* promoted the production levels of VEGF however a similar increase was not observed in FGF production by these cells. In addition to VEGF and FGF, Con A-treated LN cells produced significant quantities a distinct growth factor not detected under control conditions, GM-CSF (Figure 5.7c). The inhibitory action of ES-62 on VEGF production observed under control conditions was more potently observed following stimulation of LN cells with Con A. (Figure 5.7b). Furthermore, LN cell production of inflammation-promoting GM-CSF in response to Con A was abrogated by prior treatment with ES-62 (Figure 5.7c). Thus, ES-62 clearly targets LN cell production of growth factors in this model, suggesting that these factors may be important for development of the inflammatory pathology exhibited in these mice.

5.2.7 ES-62 treatment inhibits mitogen-induced proliferation of splenocytes from SLE model mice *ex vivo*

Previously it has been demonstrated that the immune response in the lymph nodes and spleen can be distinct (Chapter 4). To help determine the nature and mechanism of the immune response exhibited in the SLE model, it was important to follow up the LN cell analysis with examination of splenocytes isolated from the MRL/lpr mice. The DNA synthesis levels of SLE model splenocytes were negligible under control *in vitro* conditions (Figure 5.8a). Analogous to its action on LN cells, ES-62 treatment of SLE model mice *in vivo* did not modulate the minimal *ex vivo* proliferation rate of splenocytes. As expected, treatment with mitogenic Con A *in vitro* induced markedly elevated levels of splenocyte proliferation (Figure 5.8a). Interestingly, and in contrast to the findings of LN cell analysis, splenocytes isolated from ES-62-treated SLE model mice exhibited a significantly reduced proliferative response to Con A (Figure 5.8a), when compared with splenocytes from PBS-treated mice. This indicated that ES-62 acted to reduce the normal proliferative response of splenocytes to a potent mitogenic stimulus, such as Con A, suggesting that this inhibitory effect may also be exhibited *in vivo*.

5.2.8 ES-62 treatment inhibits mitogen-induced cytokine production by splenocytes from SLE model mice *ex vivo*

Despite exhibiting a low rate of DNA synthesis under control conditions, SLE model splenocytes spontaneously secreted detectable quantities of the TH1-promoting cytokine, IL-12 (Figure 5.8h). IL-12 had previously been detected spontaneously secreted by LN cells from these mice (Section 5.2.4), suggesting that this particular cytokine might be important for mediation of inflammation in this model. Interestingly, given that spontaneous cytokine production was minimal by splenocytes from PBS-treated MRL/*lpr* mice, *in vivo* treatment of the SLE model mice with ES-62 stimulated spontaneous production of IL-2 by splenocytes (Figure 5.8d), but did not significantly modulate IL-12 production levels. Therefore, these findings suggested that ES-62 acted to promote development of splenocytes that spontaneously produced IL-2 and to inhibit cells that spontaneously produced IL-12, in the absence of an external stimulus.

Interestingly, the low level of spontaneous IL-12 secretion by splenocytes under control conditions was not significantly modulated by Con A treatment *in vitro* (Figure 5.8h), suggesting that the IL-12-producing cell population had not been targeted by Con A. However, significant production of TH1-type cytokines, IL-2, TNFα, IFNγ, IL-1α, IL-6 and IL-17 and TH2-type cytokines, IL-13 and IL-10 by splenocytes was induced by Con A (Figure 5.8). Culture supernatants were also analysed for the presence of cytokines, IL-4, IL-5 and IL-1β, but these were not detected under either *in vitro* condition. ES-62 treatment of SLE model mice *in vivo* appeared to inhibit Con A-induced production of IL-17 and IL-6 by splenocytes *in vitro* (Figure 5.8f, e). Thus, the pattern of IL-17 and IL-6 production (under Con A stimulation) reflected the pattern of proliferation in splenocytes from the two *in vivo* treatment groups. Nevertheless, the levels of the other Con A induced cytokines secreted by splenocytes from PBS treated mice (IL-12, IL-2, TNFα, IFNγ, IL-1α, IL-13 and IL-10) were not significantly modulated by ES-62 treatment *in vivo*, indicating that the action of ES-62 was specific for IL-6 and IL-17 in these cells.

5.2.9 ES-62 treatment inhibits mitogen-induced chemokine production by splenocytes from SLE model mice *ex vivo*

In addition to TH1-promoting cytokine, IL-12, SLE model splenocytes spontaneously produced detectable levels of inflammatory chemokines, MCP-1 and IP-10 (Figure 5.9a, d). As mentioned previously, IP-10 is induced by IFNγ, whilst MCP-1 is induced by IL-1 and TNFα and has been detected in increased levels in the serum of SLE patients [431]. Interestingly, although the spontaneous production of MCP-1 was low overall, splenocytes from ES-62 treated mice exhibited a trend of reduced production of MCP-1 (Figure 5.9d), however this difference was not statistically significant.

The increase in proliferation of splenocytes induced by Con A treatment *in vitro* was reflected in the production levels of IP-10 and MCP-1 that had been secreted spontaneously by these cells under control conditions. In addition, inflammatory chemokines, KC, MIG and MIP-1α were induced by Con A treatment of splenocytes (Figure 5.9b, c, e). In a similar pattern to that of IL-17 production, the pattern of Con A-induced MCP-1 production reflected the pattern of proliferation in splenocytes from the two *in vivo* treatment groups (Figure 5.9d) That is, ES-62 treatment of SLE model mice *in vivo* significantly inhibited MCP-1 production by splenocytes in response to Con A treatment. However, the levels of other Con A induced chemokines secreted by splenocytes from PBS treated mice (IP-10, KC, MIG and MIP-1α) were not significantly modulated by ES-62 treatment *in vivo*. It was interesting to note that ES-62 targeted production of distinct chemokines by LN cells and splenocytes in this model respectively.

5.2.10 Analysis of growth factor production by SLE model splenocytes

Ex vivo, splenocytes from the SLE model mice spontaneously secreted low, but measurable quantities of angiogenic growth factors, FGF and VEGF (Figure 5.10a, b) and furthermore, production of both of these factors was also detected in splenocytes from ES-62 treated mice. FGF and VEGF are important factors with roles in angiogenesis, a process often initiated in chronic inflammation to provide enhanced blood (and hence, cell) supply to the site(s) of inflammation. Thus, it was interesting that ES-62 did not target secretion of either of these factors by splenocytes from this model, particularly considering it had inhibited spontaneous production of VEGF by LN cells.

In accordance with its action on proliferation, Con A treatment of splenocytes *in vitro* induced enhanced production of FGF and VEGF (Figure 5.10a, b). Furthermore, and similar to the results obtained during LN analysis, production of GM-CSF, a growth factor involved in differentiation of monocytes, was detected in splenocyte culture supernatants following Con A treatment (Figure 5.10c). However, unlike the action of ES-62 observed during LN cell analysis, the levels of Con A induced FGF, VEGF or GMCSF secreted by splenocytes from PBS treated mice were not significantly modulated by ES-62 treatment *in vivo*. Overall, ES-62 mediated inhibitory action on growth factor secretion observed in LN cells from this model was not exhibited in splenocytes, indicating that this effect of ES-62 treatment was targeted specifically at growth factor-secreting LN cells, but not splenocytes.

In summary, under control conditions, splenocytes from the SLE model mice spontaneously secreted a reduced range of inflammatory chemokines and cytokines when compared with LN cells from the same mice. In response to Con A stimulation the

range of factors secreted was greatly increased to include both TH1-promoting and TH2-promoting cytokines, indicating that cells of distinct immune phenotypes were present in the spleen. Whilst ES-62 treatment *in vivo* resulted in an inhibited proliferative response of splenocytes to Con A, it appeared to have a more profound modulatory action on the *in vitro* cytokine response of LN cells, than on splenocytes in this model. This indicated that targeting the function of LN cells in preference to splenocytes might facilitate the anti-inflammatory effects of ES-62. Nevertheless, it was of particular interest that Con A-induced IL-17 production by LN cells and splenocytes was consistently inhibited by ES-62 and therefore, this finding may represent a major mechanism by which ES-62 induces inhibition of inflammation in this model.

5.2.11 ES-62 treatment significantly modulates the phenotype of bone marrow DC derived from SLE model mice

ES-62 has previously exhibited marked immunomodulatory action on bone marrow derived cells of the innate immune response, such as dendritic cells [189]. Dendritic cells (DC) facilitate communication between innate and adaptive immune responses and function as important antigen-presenting cells. In particular, it has been suggested that DC play an important role in pathogenesis of human lupus [420]. Having uncovered a potential anti-inflammatory effect of ES-62 on LN-localised lymphocytes and as previous work has suggested that ES-62 does not directly target T cell responses [186], it was hypothesised that ES-62 might target DC to mediate its observed anti-inflammatory effects in the MRL/*lpr* model of SLE. Previously, it has been demonstrated that ES-62 treatment of bone marrow *in vivo* results in differentiation of bone marrow-derived DC with a modulated phenotype, thus it was suggested that ES-62 mediated modulation of DC pre-cursors in the bone marrow was a mechanism by which the anti-inflammatory actions of ES-62 might be facilitated *in vivo*. To test this hypothesis, DC derived from pooled bone marrow samples isolated from the SLE model mice in the PBS and ES-62 treatment groups were cultured *in vitro* and then stimulated with LPS (or media) for 24h and their surface expression and cytokine production determined.

DC cultured from bone marrow of SLE model mice treated with ES-62 did not exhibit a markedly modulated phenotype to that of DC derived from PBS-treated mice (Figure 5.11, panel A). However, it was noted that DC derived from ES-62-treated SLE model mice exhibited marginal upregulation of MHCII, CD40, CD54, CD80 and CD86, compared to DC from PBS-treated SLE model mice. LPS treatment of DC *in vitro* induced uniform upregulation of all cell surface markers analysed on DC from both *in vivo* treatment groups. The modest upregulation of surface markers on DC derived from the ES-62 treatment group mice (exhibited under control conditions) was maintained when DC were treated with LPS *in vitro* (Figure 5.11, panel B). Thus, prior ES-62 treatment of SLE model

mice *in vivo* did not prevent activation of subsequently cultured bone marrow derived DC in response to a potent inflammatory stimulus, such as LPS. This action was in direct contradiction to the previously demonstrated inhibitory effects of ES-62 on this cell type, when DC were derived from naïve non-MRL/lpr model mice [191]. Moreover, it was unlikely that this understated effect would be responsible for the significant anti-inflammatory effects of ES-62 on SLE model pathology.

To more comprehensively analyse the phenotype of the DC derived from the SLE model mice, culture supernatants of the bone marrow derived DC were also analysed for cytokine content. DC cultured from bone marrow of PBS-treated SLE model mice spontaneously secreted low (almost negligible) levels of TH1-promoting inflammatory cytokines, IL-1 and TNFα (Figure 5.12). Consistent with co-stimulatory molecule upregulation, LPS stimulation induced marked increases in cytokine production by DC from both treatment groups. Thus, DC cultured from PBS-treated MRL/lpr mice secreted elevated levels of IL-1 and TNFα, whilst additionally secreting IL-12 and IL-6 in response to LPS (Figure 5.12). Furthermore, secretion of regulatory/TH2 cytokine, IL-10 was induced by LPS stimulation of DC from the SLE model mice

Interestingly, ES-62 treatment of mice *in vivo*, mediated an inhibitory effect on the bone marrow derived DC cytokine secretion response to LPS. When compared with PBS DC, DC cultured from ES-62 treated mice secreted significantly inhibited levels of all the cytokines detected (IL-1, TNFα, IL-12, IL-6 and IL-10) in response to LPS treatment. Thus, ES-62 treatment of SLE model mice resulted in differentiation of bone marrow-derived DC, which displayed an inhibited cytokine, but not cell surface expression response to LPS stimulation *in vitro*. This marked effect of ES-62 treatment *in vivo* on the cytokine production pattern of subsequently cultured DC *in vitro* is typical of the previously identified anti-inflammatory effects of ES-62 on bone marrow-derived cells [191] and highlighted an potential mechanism by which ES-62 might interfere with the normal progression of inflammatory pathways activated during pathogenesis of lupus-like disease.

5.3 Discussion

SLE is an autoimmune disease of ambiguous immunological phenotype, with a prevalence of 0.1%. Its pathologies are diverse and heterogeneous, which adds to the complexity of diagnosis of this disease in humans. As described previously in this thesis, the anti-inflammatory effects of filarial nematode excretory-secretory product, ES-62 have been demonstrated in models of TH1 and TH2-mediated inflammation, collagen-induced arthritis and ovalbumin-induced asthma respectively. To incorporate examination of ES-62 action in another inflammatory disease, MRL/lpr mice were monitored and it was confirmed that this was an appropriate model for the study of human lupus. Thus, by employing a genetically determined, spontaneous onset murine model of SLE, a novel anti-inflammatory action of ES-62 in a prototypic autoimmune disorder was uncovered.

5.3.1 ES-62 selectively inhibits progression of SLE-associated inflammatory pathology

SLE model mice displayed evidence of progressive nephritis, a common inflammatory pathology incurred in human SLE, evidenced by proteinuria and haematuria that increased in severity with age. Indeed, it has previously been demonstrated that the levels of proteinuria and haematuria correlate with severity of nephritis in murine SLE models and in SLE patients [429]. ES-62 treatment of SLE model mice significantly inhibited proteinuria and non-significantly reduced the incidence of haematuria, indicating delayed onset and reduced progression of nephritis in the ES-62 treatment group.

Consistent with a lupus-like phenotype, symptoms of inflammatory arthritis were exhibited in the majority of the SLE model mice towards the end of the experiment. This is a common component of SLE-associated pathology in humans and has previously been observed regularly in this murine model [429]. ES-62 treatment of MRL/lpr mice abrogated onset of arthritis-like symptoms and where arthritis did occur, the severity was significantly reduced. Indeed, anti-arthritic action of ES-62 has previously been demonstrated in a murine model of rheumatoid arthritis [192], thus, in the current model this observation indicated that, supplementary to amelioration of nephritis, ES-62 mediated inhibition of additional inflammatory pathology exhibited in SLE.

In addition to nephritis and arthritis, splenomegaly and lymphadenopathy were evident in SLE model mice. Interestingly, these inflammatory pathologies were not targeted by ES-62. Treatment with ES-62 did not significantly modulate the hypertrophy of lymphoid organs or hyperplasia of cells exhibited in SLE model spleen and lymph nodes. These findings illustrated that ES-62 mediated anti-inflammatory action was focussed on the pathologies that caused the greatest morbidity (and ultimately, mortality) in this disease (that is, nephritis and arthritis).

5.3.2 ES-62 mediated inhibition of SLE-associated inflammation is not induced by modulation of serum antibodies or cytokines.

Accelerated lupus-like disease, such as that exhibited in the MRL/lpr model of SLE, has been associated with TH1-mediated immune responses [408]. Thus whilst MRL/lpr mice predominantly exhibit enhanced serum immunoglobulin of IgG and IgM isotypes, TH1-type IgG2a and IgG3 subclasses are particularly nephritogenic in murine SLE [408, 432].

Consistent with hyperplasia of lymph node and spleen cells, hypersecretion of IgG antibodies (including DNA-specific) was also detected in the SLE model mice. The concentration of total monoclonal IgG circulating in the bloodstream has been shown to correlate with the degree of glomerulonephritis and necrotising vasculitis in MRL/lpr mice [429]. Furthermore, enhanced serum IgG is consistent with the antibody profile of SLE patients [21, 411, 429]. Thus, as an indicator of the onset of nephritis, an elevated titre of serum IgG and IgM antibodies was revealed in SLE model mice. ES-62 treatment of MRL/lpr mice did not significantly modulate the quantity or subclass balance of total serum IgG. In addition, ES-62 did not modulate total serum levels of IgM, suggesting that target of total serum IgM or IgG content was not necessary for modulation of synovial or renal inflammation. Furthermore, DNA-specific IgG2a was not significantly modulated by ES-62 either, confirming that the anti-inflammatory action of ES-62 did not target antigen-specific or non-specific antibody production for mediation of its anti-inflammatory effects. These findings corresponded with the absence of modulatory action of ES-62 on splenocyte and lymph node cell hyperplasia in these mice and, thus, supported the finding that modulation of B cell number was not a method employed by ES-62 for mediation of anti-inflammatory action in this model. Nevertheless, the lack of effect of ES-62 treatment on serum Ig in this model was surprising. It has been a commonly accepted theory that enhanced serum IgG induces production of IC that initiate onset of inflammatory pathologies, such as nephritis. Therefore, ES-62-mediated inhibition of nephritis, without inhibition of serum Ig appeared contradictory. Furthermore, ES-62 must therefore have employed a mechanism distinct from modulation of hypergammaglobulinemia to mediate inhibition of onset of renal inflammation and arthritis. This mechanism may be down stream of IC formation, for example, at the point of interaction of IC and target antigens. However, it must also be considered that serum was sampled at a single time-point (at the time of sacrifice). Serum factors are most likely in continuous flux, therefore it is possible that repeated analysis over a series of time-points might have reveal alternate findings.

Examination of cytokines present in serum samples obtained from SLE model mice revealed a combination of TH1 (TNFα, IL-2, IL-12), TH2 (IL-10, IL-13) promoting factors and T-regulatory cytokine (IL-10), which were not significantly modulated by ES-62

treatment. The mean concentration of IL-12 was modestly reduced in ES-62 treated mice, however this difference was not significant. Cytokines of different immune phenotypes have been identified as simultaneously elevated in SLE patients, suggesting that lupus is a complex disease that may be supported by different cytokine patterns at different time-points during the disease. Clearly, ES-62 did not elicit profound modulatory effects on the balance of serum cytokines in this model. In summary, analysis of SLE model serum revealed an elevated IgG titre and an ambiguous immunological profile, characteristics similar to serum samples of lupus patients [381]. ES-62 did not significantly alter serum levels of antibody or cytokine, indicating that, whilst these factors provided information about the immunological status of the model, they did not necessarily directly influence progression or inhibition of inflammatory pathologies, such as nephritis.

5.3.3 ES-62 mediated inhibition of SLE-associated inflammation correlated with mitogen-induced cytokine production by SLE model LN cells.

The MRL/lpr model of SLE mimics an autoimmune disease of marked inflammation. Paradoxically, LN cells isolated from the SLE model mice exhibited relatively low spontaneous activation, demonstrated by minimal levels of spontaneous proliferation and cytokine production. Interestingly, impaired responsiveness to TCR ligation is characteristic of CD4+ T lymphocytes isolated from SLE patients [433]. Furthermore, whilst the phenotype of the immune response in SLE patients is often ambiguous, in the MRL/lpr model of autoimmunity the inflammation exhibited is often mediated predominantly by a TH1 immune response [408]. This perhaps reflects the spontaneous secretion of IL-12 by the SLE model LN cells *ex vivo* as IL-12 is necessary for initiation of a TH1-mediated immune response [434]. Moreover, in the absence of stimulation *ex vivo*, SLE model LN cells spontaneously secreted a selection of inflammation promoting factors, highlighting the inflammatory nature of this disease. For example, IP-10, a TH1 chemoattractant [435] spontaneously secreted by SLE model LN cells in this investigation, has been associated with pulmonary inflammation in MRL mice [436]. In addition, IP-10 is detected in elevated concentrations in serum samples from SLE patients [431], suggesting that this inflammatory chemokine is involved in the pathogenesis of SLE.

ES-62 treatment abrogated spontaneous IL-12 production and reduced VEGF secretion by the LN cells, indicating that ES-62 had targeted its actions to reduce production of circulating factors necessary for initiation and maintenance of the exhibited TH1-type inflammation and angiogenesis respectively. As previously mentioned, IL-12 promotes TH1-type immunity and the MRL/lpr model of SLE is propagated by a TH1-mediated immune response. Furthermore, it has been demonstrated previously that IL-12 deficient MRL/lpr mice exhibited delayed onset of nephritis and intra-renal IFNγ expression [437]. Therefore, inhibitory action of ES-62 on LN cell IL-12 secretion might reflect a mechanism

by which ES-62 inhibited progression of SLE. The angiogenic chemokine, VEGF is generally elevated in serum samples from SLE patients and has been identified as overexpressed in renal tissue in patients with lupus nephritis [438]. Moreover, serum levels of VEGF have been shown to correlate with the severity of inflammation exhibited in SLE [439]. Thus, inhibition of VEGF production by LN cells as a result of ES-62 treatment also appears to be a potential mechanism of anti-inflammatory action in the SLE model.

Currently the auto-antigen or auto-antigens to which the immune response is targeted in MRL/lpr mice is unknown. Therefore, it was not possible to stimulate an antigen-specific response by the LN cells *ex vivo*. Instead, LN cells were stimulated *ex vivo* with the potent mitogen, Con A, which induces non-specific proliferation of cells. Thus, it was proposed that analysis of the cellular responses to Con A treatment would help to reveal the phenotype of the cell types present, because it would stimulate non-specific activation of cells.

As expected, DNA synthesis levels and the cytokine-producing repertoire of the SLE LN cells was markedly enhanced by treatment with Con A, compared with unstimulated cells, indicating that T cells of distinct phenotypes were present. In more detail, Con A treatment revealed the presence of TH2 cells in the LN cell culture, identified by secretion of IL-13. TH1 cells were also present, as indicated by detection of IFNγ and IL-2 in the Con A-treated culture well. Detection of regulatory cytokine, IL-10 suggested that a population of regulatory cells might be present within the SLE model lymph nodes, for example IL-10-secreting regulatory T cells. Indeed, IL-10 is an important co-factor for survival of B cells (and hence, maintenance of autoantibody production), however no correlation between IL-10 levels and SLE severity has been identified [440]. In addition, inflammatory chemokines, IP-10, MIG, MIP-1α, MCP-1 and KC were detected in Con A-treated LN cell cultures from SLE model mice. These inflammatory factors would help to sustain an inflammatory milieu *in vivo*. All of these chemokines have been detected in elevated concentrations in serum samples from SLE patients, implicating roles in SLE pathogenesis [431].

In addition to inhibition of constitutive IL-12 production, ES-62 treatment of SLE model mice appeared to dampen the production of selected Con A-induced TH1-promoting (IFNγ, IL-12, IL-17, TNFα, KC, MIP-1α, VEGF) and TH2-promoting (IL-13) factors, suggesting that although ES-62 had not significantly modulated the mitogen-induced proliferation of LN cells, the cytokine secretion function of these cells had been inhibited.

IFNγ is a definitive TH1 cytokine and as mentioned previously, the MRL/lpr model of SLE is predominantly associated with a TH1-mediated immune response. By contrast, the role of IFNγ in human SLE has been the subject of much controversy: evidence has been published to demonstrate, both, enhancement and impairment of IFNγ levels in SLE patients [410, 411]. Nevertheless, it has been demonstrated that IFNγ is essential for development of glomerulonephritis in the MRL/lpr model [441] and exacerbations of SLE-induced nephritis are mediated by TH1 cytokines such as IFNγ [442]. Therefore, ES-62-induced inhibition of IFNγ would disrupt IFNγ-mediated development of nephritis and, therefore, promotion of TH1-mediated immune responses in the MRL/lpr mouse. This represents a potential mechanism for mediation of the inhibitory action of ES-62 in this model of SLE.

Likewise, IL-17 is an inflammatory cytokine that induces release of other pro-inflammatory cytokines, mobilises and recruits neutrophils and inflammatory cells and has been demonstrated to have important inflammatory effects on rheumatoid synoviocytes [443]. Consistent with such a role in inflammation, LN cells from the SLE model were induced to secrete IL-17 after mitogen treatment. Therefore, it is possible that IL-17 could induce maintenance of the arthritis-like inflammation exhibited in the SLE model. Moreover, IL-17 induces production of TNFα [444] and GM-CSF [445], inflammatory cytokines also secreted by SLE LN cells in this investigation. Importantly TNFα has a proven integral role in pathogenesis and maintenance of inflammation in human and murine arthritis [207]. As mentioned in Chapter 3, blockade of TNFα is an established method for therapy of arthritis in humans and consistent with a link between these cytokines, inhibition of IL-17 also attenuates inflammation in murine collagen-induced arthritis [446].

IL-17 is a powerful inducer of neutrophilia and it has been identified that the neutrophilic action of IL-17 is mediated by KC [447], an inflammatory chemokine that potently recruits neutrophils and was also secreted by LN cells from the SLE model in response to Con A. Furthermore, IL-17 has been demonstrated to stimulate production of the angiogenic growth factor, such as VEGF and has been shown to co-operate with TNFα in mediation of this effect [448]. It has been identified that IL-17 induces production of TNFα also [449] and therefore, co-operation with TNFα to induce production of VEGF promotes a synergism between these two pro-inflammatory cytokines. Serum levels of VEGF, but not FGF have been shown to correlate with inflammation severity in SLE patients, suggesting that these angiogenic factors are differentially regulated [439]. Indeed, unlike VEGF, FGF is not directly induced by IL-17 and in support of this, ES-62 treatment of the SLE model mediated inhibition of VEGF, but not FGF production by LN cells. Thus, neutrophilic inflammation and angiogenesis are clearly integral components of the pathology exhibited in the MRL./lpr mice. In summary, ES-62 treatment inhibited secretion of IL-17, KC, VEGF

and TNFα by LN cells from the SLE model, consistent with a common link in their production (Figure 5.13). Therefore, the results presented in this chapter indicate that ES-62 may be targeting this group of related inflammatory mediators to facilitate its anti-inflammatory action in this model of SLE.

It has been suggested that IL-17 production is induced by IL-23, a cytokine closely related to IL-12 [450]. Furthermore, it has been suggested that IL-23 represents a key therapeutic target in autoimmune inflammation [450]. Therefore, as an extension of the current investigation, it would be interesting and informative to additionally examine *ex vivo* IL-23 production by LN and spleen cells from this model (Figure 5.13). This would determine whether ES-62 also targets the cytokine production upstream of IL-17 in the MRL/lpr mouse.

As previously mentioned, LN cell secretion of IL-17 and TNFα was inhibited by ES-62 treatment in SLE model mice and hence, it is possible that this action of ES-62 could contribute to the observed inhibited arthritis in the SLE model mice. However, the role of TNFα in SLE is not as straightforward as its action in rheumatoid arthritis pathogenesis. For example, there have been several reports of anti-TNF therapy (in RA patients) inducing a lupus-like autoimmunity, after prolonged administration [451, 452] and furthermore, it has been suggested that TNFα might have a protective role against lupus onset [453]. Moreover, it has been identified that genetically determined TNFα production levels in humans are inversely associated with onset of lupus nephritis [453]. In contrast, TNFα has been cited as pathogenic in cutaneous lupus, because it is activated by ultra-violet B light that exacerbates lupus-associated skin inflammation [454]. Ultimately, TNFα is a pro-inflammatory cytokine therefore, ES-62 mediated inhibition of TNFα in this model of inflammation is likely to be anti-inflammatory in action.

However, as mentioned previously, LN cells from the SLE model mice secreted both TH1 and TH2 cytokines. IL-13, a TH2 cytokine, which is recognised by the same receptor as the prototypic TH2 cytokine, IL-4, has been detected at increased levels in serum samples from SLE patients compared with normal individuals [455, 456], highlighting its role in rheumatic diseases including SLE. Based on these postulations, inhibition of IL-13 secretion by ES-62 would therefore induce inhibition of SLE pathogenesis.

Elucidation of the cytokine inhibiting action of ES-62 in the SLE model implicated mitogen-induced LN cell cytokine production in the progression of inflammatory pathologies, such as nephritis and arthritis, exhibited in this disease. ES-62-mediated inhibition of cytokine production correlated with ES-62 mediated inhibition of nephritis and arthritis in this model. This suppressive effect of ES-62 was not TH-specific as it targeted TH1 and TH2

LN cells. It must also be remembered that ES-62 was not present in the *in vitro* culture dish of these LN cells therefore, ES-62 treatment of the SLE model mice had (directly or indirectly) modulated the functionality of the LN cells *in vivo* and this had been maintained *ex vivo*. The sustained modulatory action of ES-62 that is maintained after ES-62 exposure has stopped has been demonstrated previously in other models of inflammatory disease, such as collagen-induced arthritis model (Chapter 3) and the ovalbumin-induced asthma model (Chapter 4). Indeed, it appears that ES-62 treatment of cells in vivo results in permanent modulation of cell function, which is usually anti-inflammatory in effect. For example, in cells from a model of TH1-mediated inflammation (CIA), lymphocytes from ES-62 treated mice produce inhibited levels of TH1-promoting cytokines following re-stimulation *ex vivo*. Conversely, in cells from a model of TH2-mediated inflammation (asthma), cells from ES-62 treated mice produce inhibited levels of TH2 cytokines when re-stimulated *ex vivo*. Therefore, it appears appropriate that in the current model of inflammation of TH1 and TH2 phenotypes, cells from ES-62 treated mice produce inhibited levels of both, TH1- and TH2-promoting cytokines *ex vivo*.

5.3.4 ES-62 mediated inhibition of mitogen-induced splenocyte proliferation ex vivo.

In a similar pattern to that observed in LN cells, splenocytes isolated from SLE model mice exhibited minimal DNA synthesis levels and secreted low levels of inflammation-promoting cytokines, including IL-12, inflammatory chemokines, IP-10 and MCP-1 and growth factors, FGF and VEGF. Interestingly, spontaneous production of MCP-1 had not been detected in untreated SLE LN cell cultures. ES-62 treatment inhibited VEGF secretion by untreated SLE splenocytes (and mildly reduced the secretion of MCP-1), but, unlike the effect of ES-62 on LN cells, IL-12 secretion was not significantly modulated. In summary, it appeared that the inhibitory action of ES-62 observed in LN cells was not as potent in splenocytes from this model.

Surprisingly, ES-62 induced production of IL-2, predominantly described as a T cell growth factor [457], by SLE model splenocytes. However, interestingly, T cells from human SLE patients and SLE mouse models exhibit impaired IL-2 production [429, 458, 459]. Furthermore, it is understood that IL-2 deficiency promotes breach of tolerance leading to production of autoantibodies in SLE [457]. It has been suggested that this is because IL-2 acts not only as a growth factor but also as a signal to promote tolerogenic responses *in vivo*, for example, development of $CD4^+CD25^+$ regulatory T cells [460]. In addition, it has been demonstrated that induction of IL-2 by expression of the human IL-2 gene ameliorates SLE disease activity in the MRL/lpr mouse [461]. Thus, ES-62-mediated promotion of IL-2 production by splenocytes in the SLE model mice, might help to prevent development of inflammation in this model.

Con A treatment of splenocytes induced elevated DNA synthesis levels accompanied by marked production of several more cytokines and chemokines. The profile of cytokines produced under these conditions was similar to that observed with Con A induced LN cell cytokine production, and included TH1- and TH2-promoting factors. Interestingly, the action of ES-62 on splenocytes from this model targeted the proliferative response of these cells to Con A, in addition to the cytokine secretion response. More specifically, ES-62 induced significant inhibition of mitogen-induced splenocyte proliferation. It has previously been demonstrated in this laboratory that ES-62 inhibits proliferation of T and B cells *in vitro*, however this anti-proliferative action was specific for proliferation triggered by ligation of the antigen receptor [185, 186]. In addition to inhibition of DNA synthesis by splenocytes from the SLE model, ES-62 inhibited production of IL-6, IL-17 and MCP-1 by splenocytes.

As discussed above (section 5.3.3), as an inducer of several inflammatory mediators, IL-17 has both direct and indirect roles in promoting development of inflammation exhibited in SLE. Therefore, ES-62-mediated inhibition of IL-17 production by LN cells and splenocytes most likely represents an efficient mechanism for anti-inflammatory action in the developing SLE model.

MCP-1 is a potent inflammatory chemoattractant for monocytes and T cells [12, 462], is upregulated in SLE and has been demonstrated to positively correlate with inflammation severity in SLE patients [463]. More specifically, it has been demonstrated that MCP-1 functions to recruit and activate inflammatory cells in nephritis in MRL/lpr mice [464]. Furthermore, anti-MCP-1 gene therapy attenuated nephritis in MRL/lpr mice [464].

The TH1-promoting pro-inflammatory cytokine, IL-6 has widespread roles in inflammation-induced tissue damage and regulation of inflammation [465]. Moreover, IL-6 is known to induce differentiation of B cells into antibody forming cells and activates T cells, therefore it is not surprising that IL-6 has been implicated in the pathogenesis of SLE. Furthermore, based on experiments conducted in murine models of SLE, it has been suggested that blockade of IL-6 may ameliorate inflammation exhibited in SLE patients [466].

Thus, ES-62-mediated inhibition of IL-17, MCP-1 and IL-6 production by splenocytes would be considered anti-inflammatory in SLE model mice. In summary, the anti-inflammatory nature of ES-62, exhibited previously in the cytokine production profile of LN cells from this model, was also apparent, albeit in a reduced range of factors, in SLE model splenocytes.

5.3.5 ES-62 modulation of DC function as a potential mechanism for inhibition of inflammation exhibited in SLE model mice.

It has recently been suggested that, as professional antigen-presenting cells, DC have a role in activating self-reactive T cells, which brings about the autoimmune inflammation exhibited in SLE [428]. Examination of the phenotype of DC derived from bone marrow of the SLE model mice indicated that ES-62 treatment of SLE model mice induced differentiation of a DC with a similar expression profile of cell surface markers to DC derived from control (PBS-treated) SLE model mice. Nevertheless previous work from this laboratory has demonstrated that whilst ES-62 treated DC appear relatively immature, they are capable of priming T cells to differentiate into an anti-inflammatory phenotype [189, 467].

Stimulation of bone marrow-derived DC from SLE model mice with potent inflammatory stimulus, LPS, induced markedly enhanced cytokine production and maturation of cells, demonstrated by uniform upregulation of cell surface marker expression. Interestingly, treatment with LPS revealed additional information about the nature of the DC derived from ES-62 treated mice. Unlike the PBS DC, the ES-62 DC exhibited a suppressed cytokine response to LPS treatment, indicating that whilst these DC appear matured (and hence, primed for direct T cell communication) by LPS treatment, they were programmed to elicit an inhibited response to an inflammatory stimulus. Dendritic cells are key organisers of the immune system therefore, modulation of DC activity as a result ES-62 exposure might permit modulation of downstream immune signalling pathways and effector cells also. In the introduction to this chapter, it was mentioned that DC are integral to the pathogenesis of SLE. ES-62 treatment of SLE model has clearly resulted in differentiation of bone marrow-derived DC that exhibit a modulated phenotype. This modulated phenotype was represented by inhibition of the cytokine production response to inflammatory stimulus. This immunomodulatory action of ES-62 has previously been demonstrated in naïve, non-MRL/lpr mice by exposing bone marrow cells to ES-62 *in vivo* (administered via osmotic pumps) and subsequently culturing DC ex vivo [191]. Thus, in summary, the combined results suggest that ES-62 was able to manipulate bone marrow cell function ensuring that when they matured, they differentiated into re-programmed DC, promoting an ameliorated inflammatory immune response. ES-62 treatment of the SLE model mice delayed the onset of morbidly severe pathology and hence, would induce a prolonged life expectancy of the MRL/lpr mice. This action of ES-62 in a parasite-infected individual would be important for longevity of the host, and therefore, of the parasite also.

In conclusion, evidence has been provided which demonstrates a novel anti-inflammatory action of ES-62 in a distinct model of autoimmune disease, SLE. ES-62 mediated action inhibits onset and reduces severity of morbidity-inducing inflammatory nephritis and

arthritis in a spontaneous onset murine model of SLE. This anti-inflammatory action appears to reflect modulation of LN cell and splenocyte cytokine production and alteration of DC function (illustrated in Figure 5.14). Nevertheless, it must be remembered, that the results presented in this chapter are preliminary (summarising the findings of two independent experiments), therefore, further investigations are required to more precisely identify the mechanisms employed by ES-62.

Figure 5.1 ES-62 inhibits proteinuria and arthritis exhibited in the MRL/lpr model of SLE

ES-62 (2μg) or PBS was administered subcutaneously to MRL/lpr (SLE model) mice twice weekly. Micturation was also induced twice weekly. Protein (A) and blood (B) content of the urine was measured using Multiistix (Bayer, UK). Incidence (C) and severity (D) of arthritic inflammation was measured in the footpad of SLE model mice 1 day before mice were euthanased. Score data are expressed as mean ± SEM (n=11 (PBS) and 12 (ES-62)) and are representative of two independent experiments. * $p<0.05$ and ** $p<0.01$ by Student's t-test.

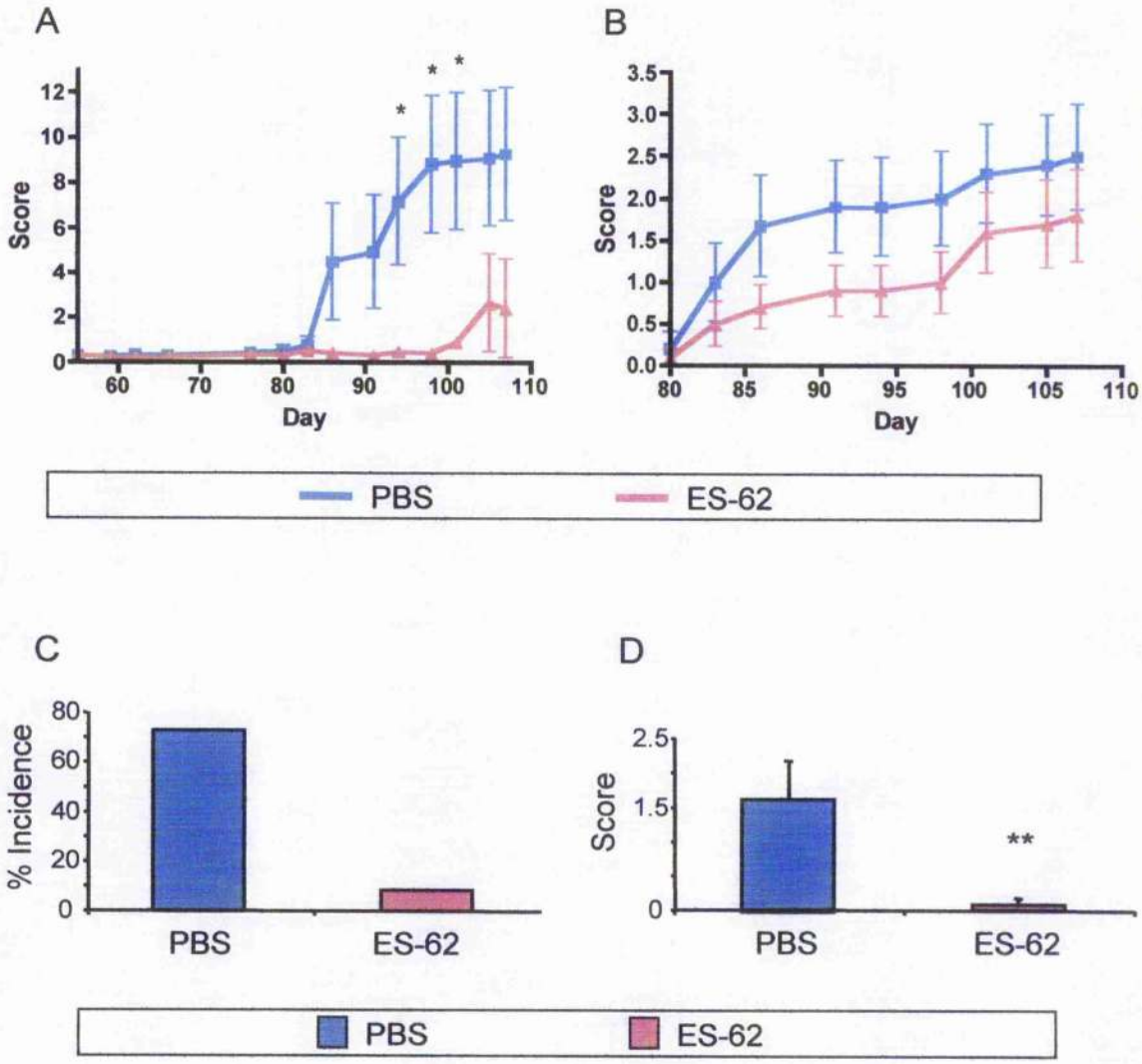
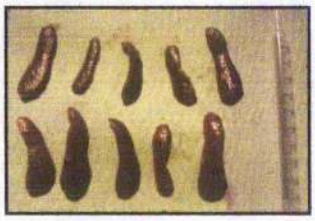

A
B
Score
Day
PBS
ES-62
C
D
% Incidence
Score
PBS
ES-62
**

Figure 5.2 ES-62 does not modulate lymphadenopathy or splenomegaly exhibited in the MRL/lpr model of SLE

ES-62 (2μg) or PBS was administered subcutaneously to MRL.lpr (SLE model) mice twice weekly. Following sacrifice, mice were dissected and photographed, to illustrate lymphadenopathy (A) and splenomegaly (B). Pictures are representative of two independent experiments.

A

B



PBS

ES-62

Figure 5.3 ES-62 does not modulate total serum IgG or IgM or DNA-specific IgG2a in SLE model mice

MRL/*lpr* mice were treated as described in the legend for figure 5.1.

A-D Serum samples from each SLE model mouse were diluted with 10% FBS (in PBS; 1/1000). Diluted samples were titrated and analysed by ELISA for total IgG1 (A), IgG2a (B), IgG3 (C) and IgM(D). Data for each group are presented as mean ± SD (*n*=11 (PBS) and 12 (ES-62) mice/group) and are representative of 2 independent experiments.

E, F Serum samples (obtained at sacrifice) from each SLE model mouse were diluted with 10% FBS (in PBS; 1/50,000). Diluted serum samples were analysed by ELISA for IgG2a specific for single stranded DNA (ssDNA; E) and IgG2a specific for double stranded DNA (dsDNA; F). Data are presented as the mean of duplicate samples for each mouse (n=9 (PBS) and 10 (ES-62)) and the bar represents the group mean. Data are representative of two independent experiments.

There were no statistical differences between serum levels of total IgG1, IgG2a, IgG3, IgM, ssDNA-specific IgG or dsDNA-specific IgG in mice from PBS or ES-62 treatment groups.

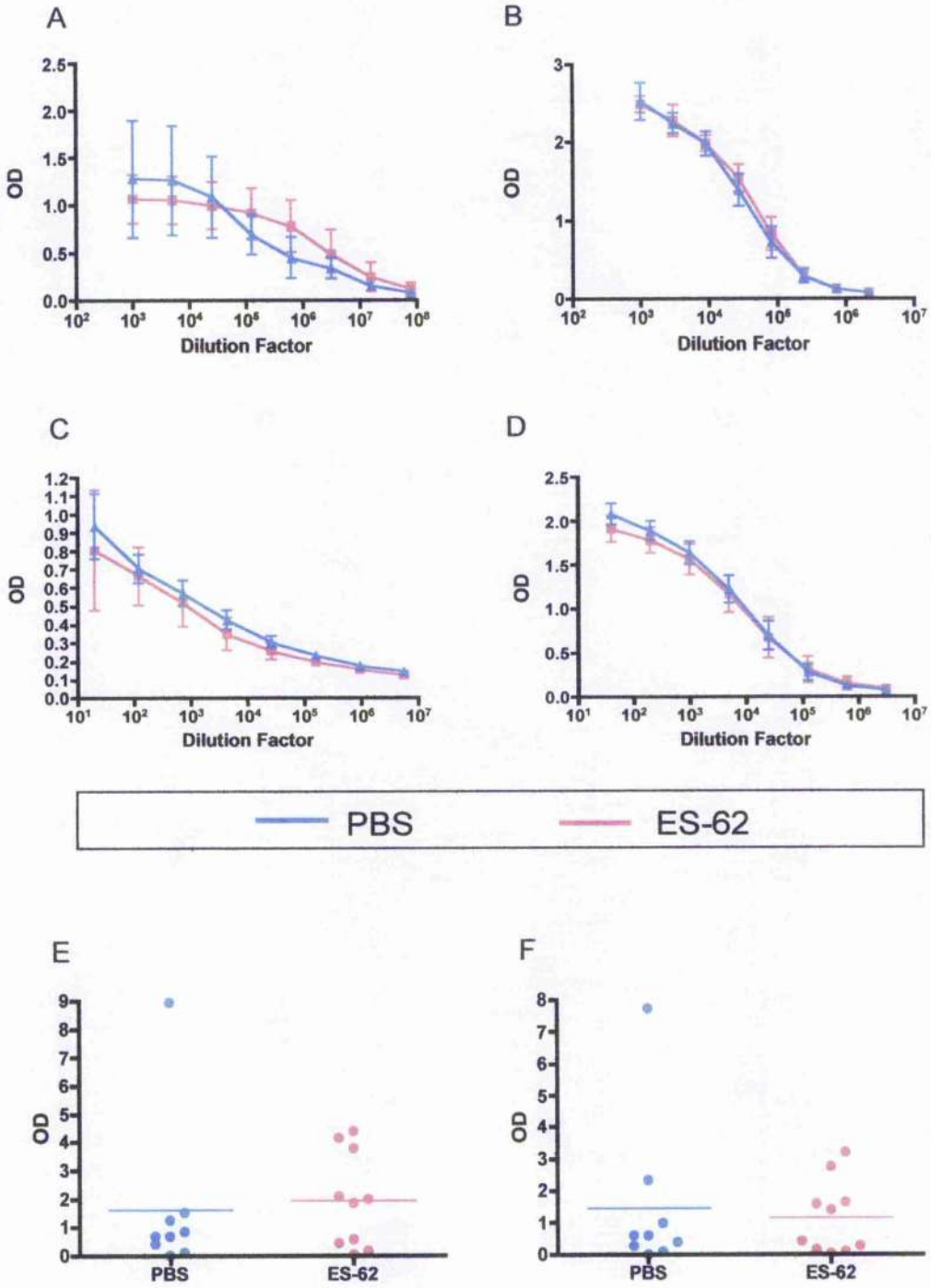

A
B
C
D
OD
Dilution Factor
PBS
ES-62
E
F
PBS
ES-62

Figure 5.4 ES-62 does not modulate SLE model serum cytokine content

SLE model mice were treated as described in the legend for figure 5.1. Serum samples from each SLE model mice were analysed in duplicate by Luminex for content of TNFα(A), IL-12 (B) IL-2 (C), IL-13 (D), and IL-10 (E). Data are expressed as the mean value of duplicate samples for each mouse (n=11 (PBS) and 12 (ES-62)) with the bar representing the treatment group mean. Data are representative of two independent experiments. There were no statistical differences observed between serum cytokine concentrations in PBS and ES-62 treatment groups.

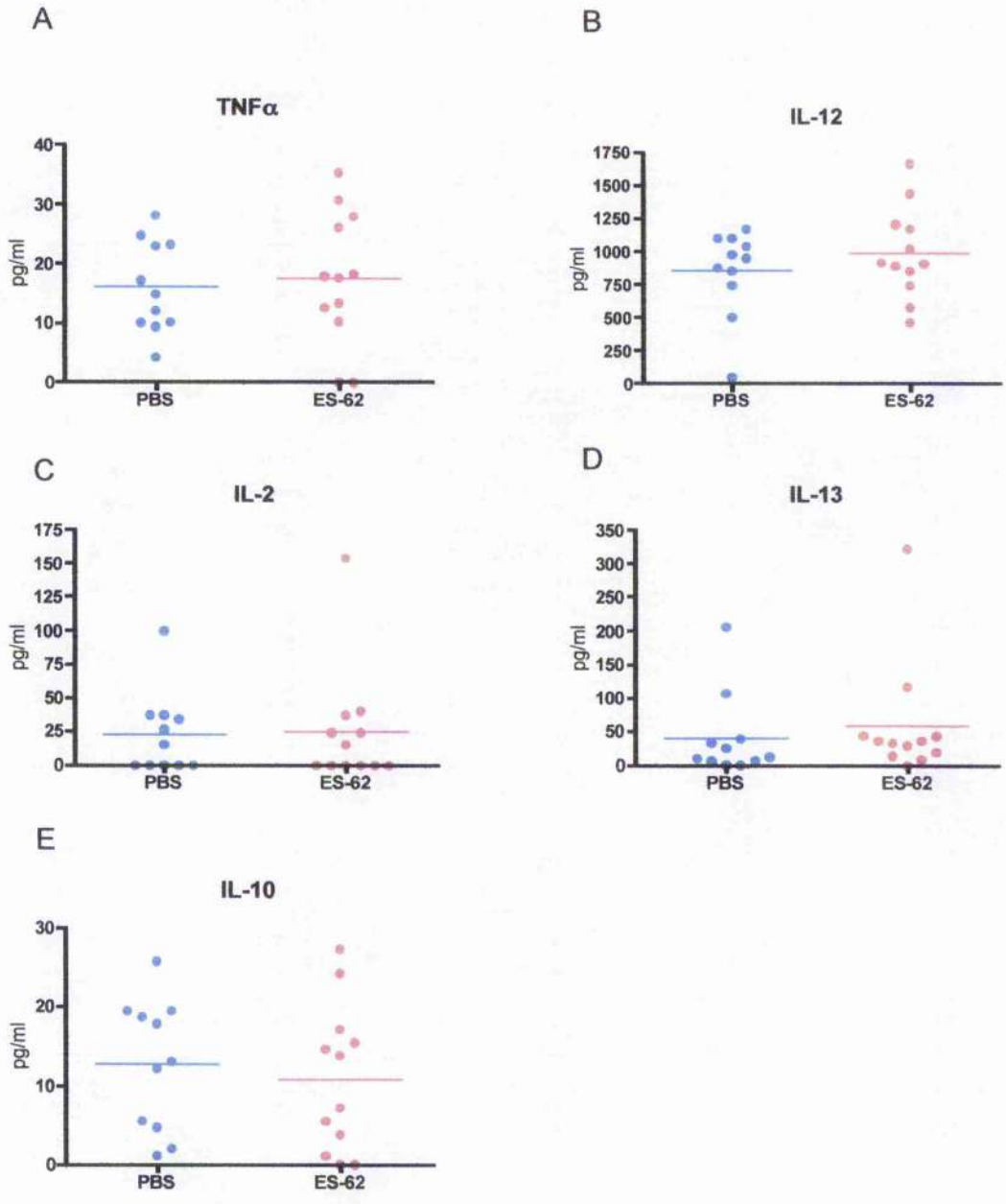

A
TNFα
pg/ml
40
30
20
10
0
PBS
ES-62
B
IL-12
pg/ml
1750
1500
1250
1000
750
500
250
0
PBS
ES-62
C
IL-2
pg/ml
175
150
125
100
75
50
25
0
PBS
ES-62
D
IL-13
pg/ml
350
300
250
200
150
100
50
0
PBS
ES-62
E
IL-10
pg/ml
30
20
10
0
PBS
ES-62

Figure 5.5 ES-62 inhibits mitogen-induced SLE model LN cell cytokine production

SLE model mice were treated as described in the legend for figure 5.1. After sacrifice and dissection, lymph node cells from mice in each treatment group were pooled and cultured in triplicate with medium alone or stimulated with concanavalin A (Con A; 5 μg/ml) for 72h. T cell proliferation (DNA Synthesis) was assayed by uptake of [^{3}H] thymidine in the last 8 hours of culture (A). Data are expressed as mean ± SD (*n*=3) and are representative of 2 experiments. Culture supernatant concentrations of IFNγ (B), IL-1α (panel C), IL-2 (D), IL-17 (E), TNFα (F), IL-12 (G), IL-13 (H) and IL-10 (I) were measured by Luminex. The absence of bars indicates a zero value (or a value so small as not to be visible) for cytokine content. Data are expressed as the mean of triplicate cultures ± SD in each treatment group and are representative of 2 independent experiments. ** $p<0.01$, *** $p<0.001$ by Student's t-test.

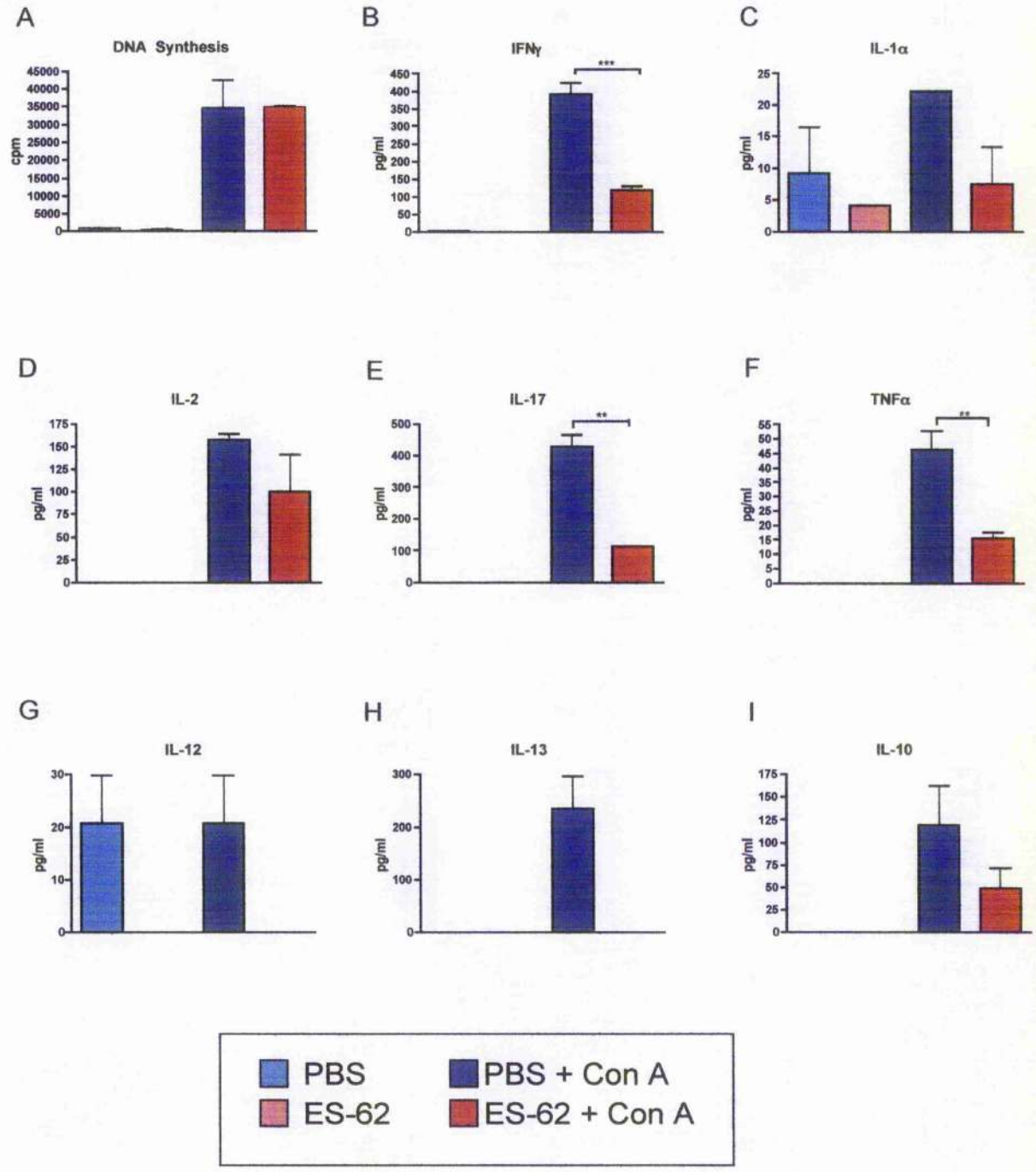

A
DNA Synthesis
cpm
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
B
IFNγ
pg/ml
***
450
400
350
300
250
200
150
100
50
0
C
IL-1α
pg/ml
25
20
15
10
5
0
D
IL-2
pg/ml
175
150
125
100
75
50
25
0
E
IL-17
pg/ml
**
500
400
300
200
100
0
F
TNFα
pg/ml
**
55
50
45
40
35
30
25
20
15
10
5
0
G
IL-12
pg/ml
30
20
10
0
H
IL-13
pg/ml
300
200
100
0
I
IL-10
pg/ml
175
150
125
100
75
50
25
0
PBS
ES-62
PBS + Con A
ES-62 + Con A

Figure 5.6 ES-62 inhibits mitogen-induced SLE model LN cell chemokine production

SLE model mice were treated as described in the legend for figure 5.1. After sacrifice and dissection, lymph node cells from mice in each treatment group were pooled and cultured in triplicate with medium alone or stimulated with concanavalin A (Con A; 5 μg/ml) for 72h. The concentrations of IP-10 (A), MIG (B), MIP-1α (C), MCP-1 (D) and KC (E) in the culture supernatants were measured by Luminex. The absence of bars or error bars indicates a zero value (or a value so small as not to be visible). Data are expressed as the mean of triplicate samples ± SD in each treatment group and are representative of two independent experiments. *, $p<0.05$ and ***, $p<0.001$ by Student's t-test.

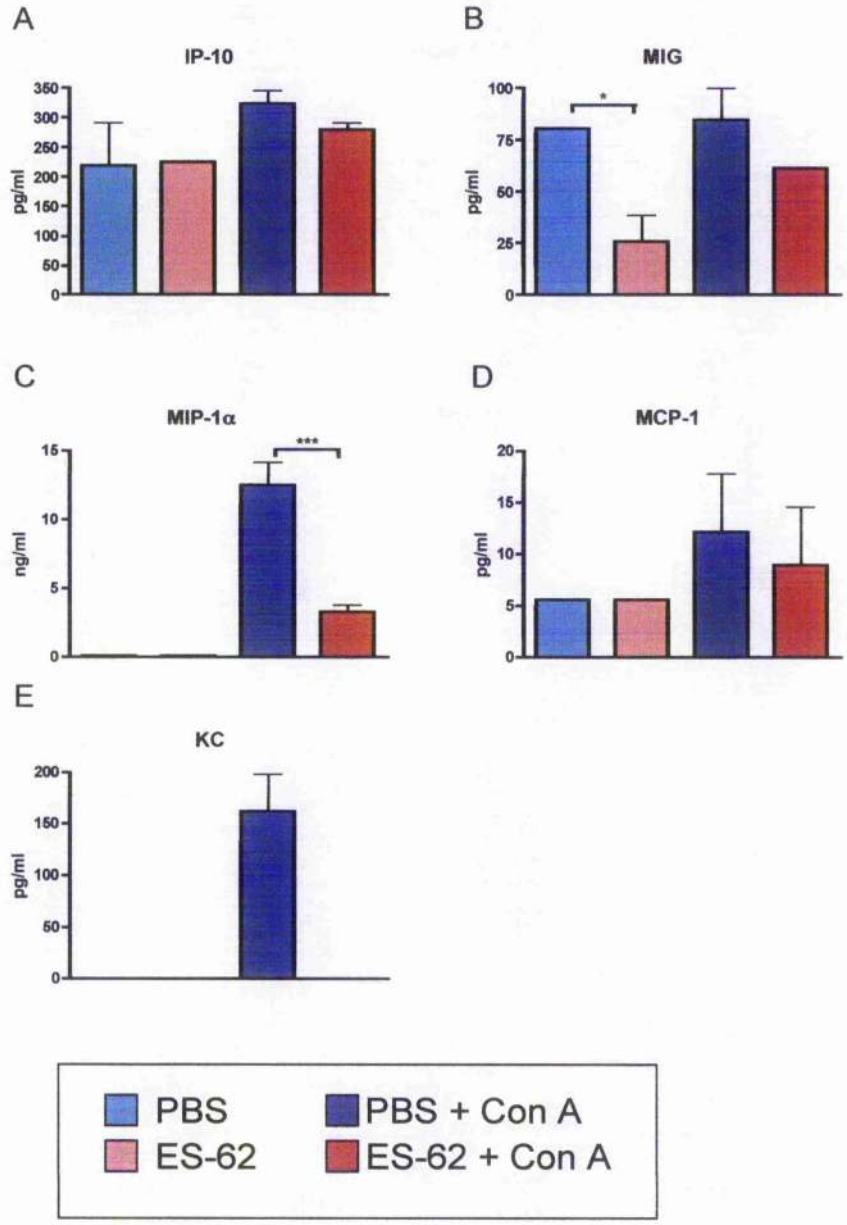
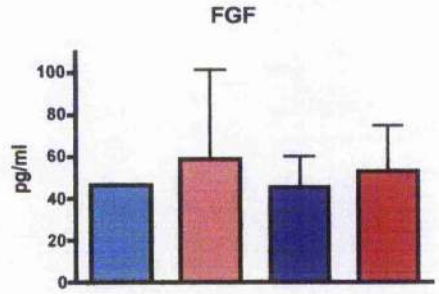

A
IP-10
pg/ml
350
300
250
200
150
100
50
0
B
MIG
pg/ml
100
75
50
25
0
*
C
MIP-1α
ng/ml
15
10
5
0
***
D
MCP-1
pg/ml
20
15
10
5
0
E
KC
pg/ml
200
150
100
50
0
PBS
ES-62
PBS + Con A
ES-62 + Con A

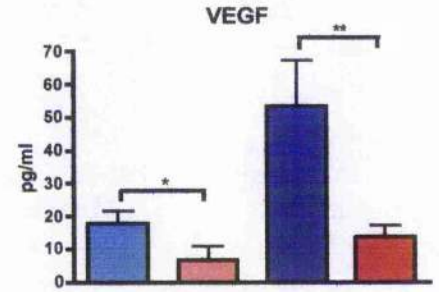
Figure 5.7 ES-62 inhibits SLE model LN cell growth factor production

SLE model mice were treated as described in the legend for figure 5.1. After sacrifice and dissection, lymph node cells from mice in each treatment group were pooled and cultured in triplicate with medium alone or stimulated with concanavalin A (Con A; 5 µg/ml) for 72h. The concentrations of FGF (A), VEGF (B) and GM-CSF (C) in the culture supernatants were measured by Luminex. The absence of bars or error bars indicates a zero value (or a value so small as not to be visible). Data are expressed as the mean of triplicate samples ± SD in each treatment group and are representative of two independent experiments. *, $p<0.05$; **, $p<0.01$ by Student's t-test.

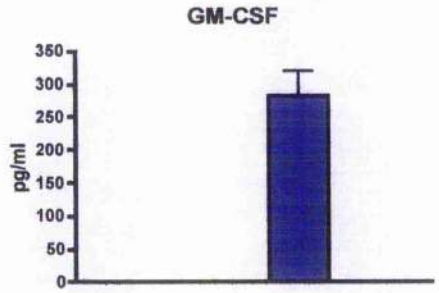
A


FGF
pg/ml
100
80
60
40
20
0

B


VEGF
pg/ml
70
60
50
40
30
20
10
0
*
**

C


GM-CSF
pg/ml
350
300
250
200
150
100
50
0

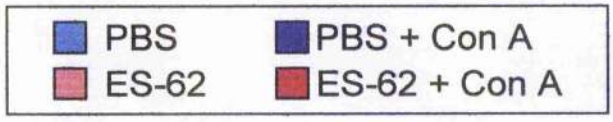

PBS
PBS + Con A
ES-62
ES-62 + Con A

Figure 5.8 ES-62 inhibits mitogen-induced SLE model splenocyte proliferation and cytokine production

SLE model mice were treated as described in the legend for figure 5.1. After sacrifice and dissection, splenocytes from mice in each treatment group were pooled and cultured in triplicate with medium alone or concanavalin A (Con A; 5 μg/ml) for 72h. T cell proliferation was assayed by uptake of [^{3}H] thymidine in the last 8 hours of culture (panel A). Data are expressed as mean ± SD (*n*=3) and are representative of 2 experiments. ** $p<0.01$ by Student's t.-test. Culture supernatant concentrations of IFNγ (B), IL-1α (C), IL-2 (D), IL-6 (E), IL-17 (F), TNFα (G), IL-12 (H), IL-13 (I) and IL-10 (J) were measured by Luminex. The absence of bars or error bars indicates a zero value (or value so small as not to be visible). Data are expressed as the mean of triplicate cultures ± SD in each treatment group and are representative of 2 independent experiments. * $p<0.05$, ** $p<0.01$ by Student's t-test.

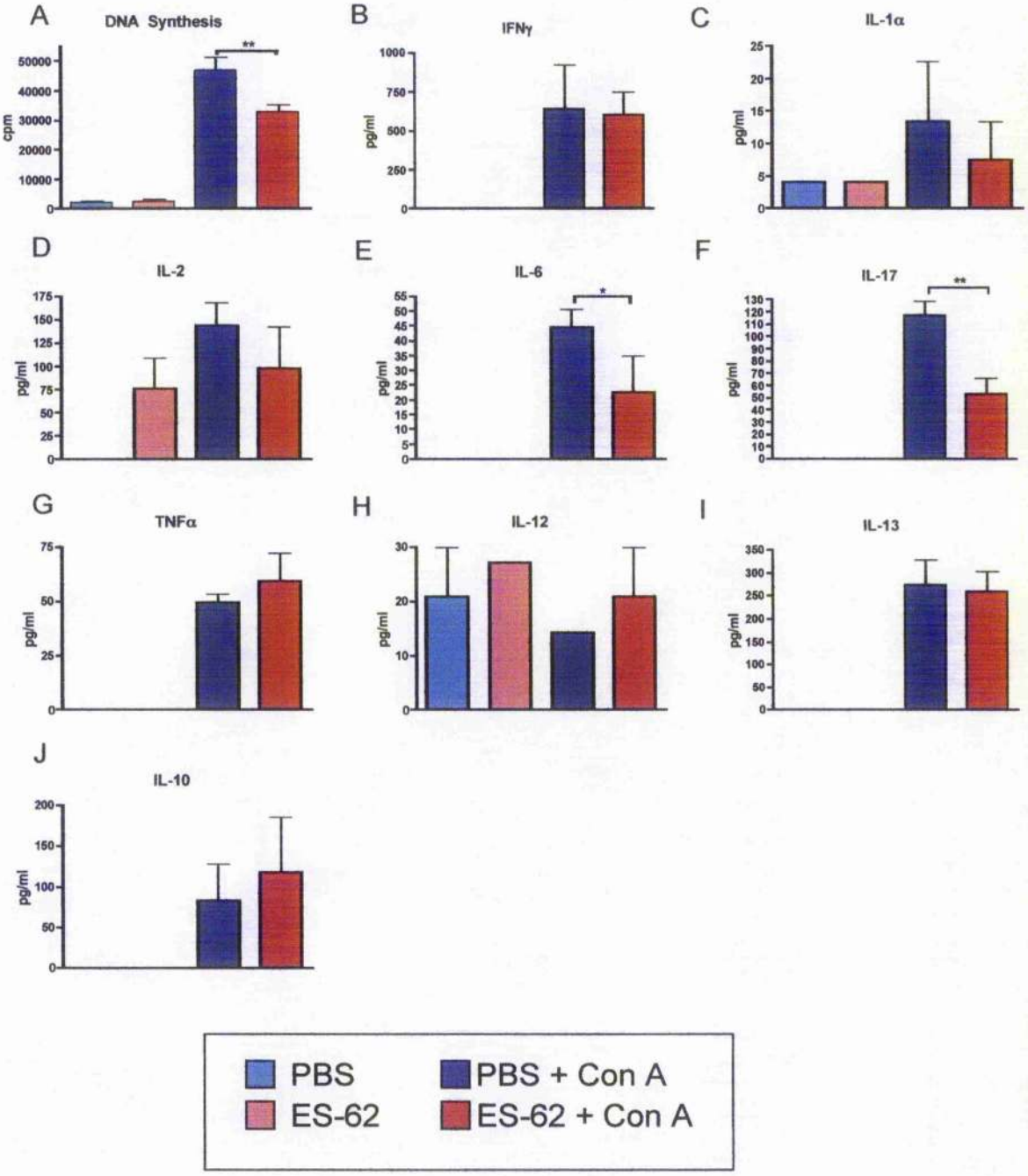

A
DNA Synthesis
cpm
**
B
IFNγ
pg/ml
C
IL-1α
pg/ml
D
IL-2
pg/ml
E
IL-6
pg/ml
*
F
IL-17
pg/ml
**
G
TNFα
pg/ml
H
IL-12
pg/ml
I
IL-13
pg/ml
J
IL-10
pg/ml
PBS
ES-62
PBS + Con A
ES-62 + Con A

Figure 5.9 ES-62 inhibits mitogen-induced SLE model splenocyte chemokine secretion

SLE model mice were treated as described in the legend for figure 5.1. After sacrifice and dissection, lymph node cells from mice in each treatment group were pooled and cultured in triplicate with medium alone or concanavalin A (Con A; 5 μg/ml) for 72h. Culture supernatant concentrations of IP-10 (A), MIG (B), MIP-1α (C), MCP-1 (D) and KC (E) were measured by Luminex. Data are expressed as mean of triplicate cultures ± SD in each treatment group and are representative of 2 independent experiments. *** $p<0.001$ by Student's t-test.

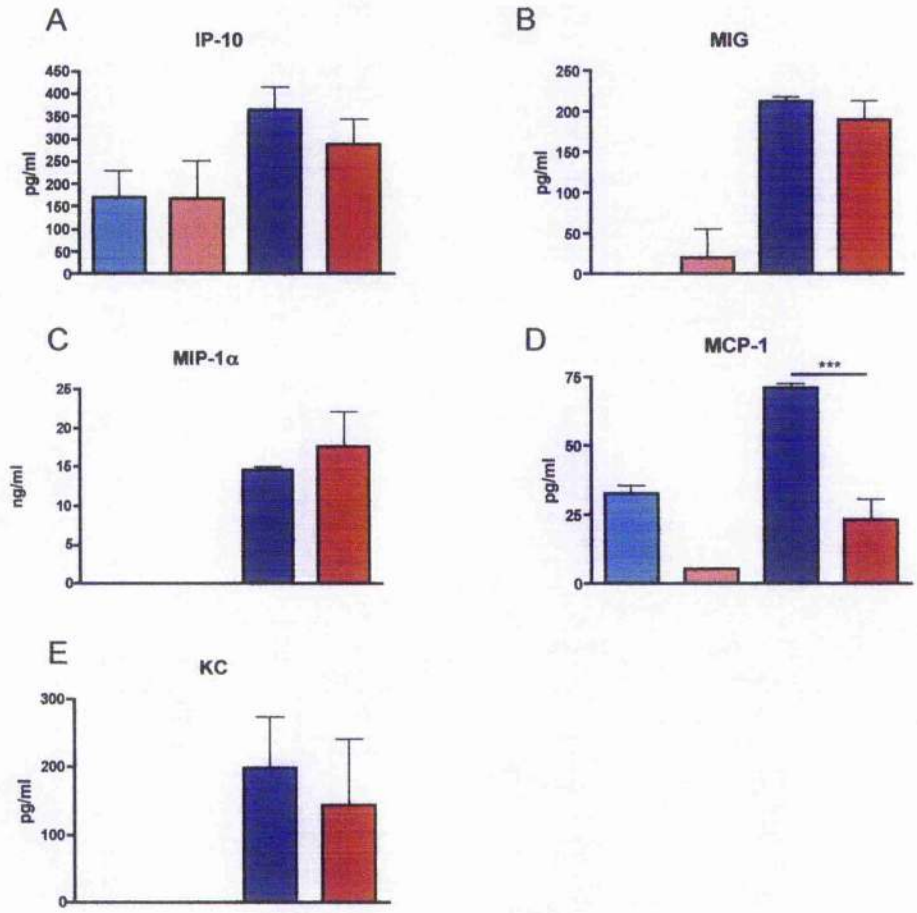

A
IP-10
pg/ml
450
400
350
300
250
200
150
100
50
0
B
MIG
pg/ml
250
200
150
100
50
0
C
MIP-1α
ng/ml
25
20
15
10
5
0
D
MCP-1
***
pg/ml
75
50
25
0
E
KC
pg/ml
300
200
100
0
PBS
ES-62
PBS + Con A
ES-62 + Con A

Figure 5.10 SLE model splenocyte growth factor production *ex vivo*

SLE model mice were treated as described in the legend for figure 5.1. After sacrifice and dissection, lymph node cells from mice in each treatment group were pooled and cultured in triplicate with medium alone or concanavalin A (Con A; 5 µg/ml) for 72h. Culture supernatant concentrations of FGF (A), VEGF (B) and GM-CSF (C) were measured by Luminex. Data are expressed as the mean of triplicate cultures ± SD in each treatment group and are representative of 2 independent experiments. No statistical differences in growth factor secretion were observed between treatment groups.

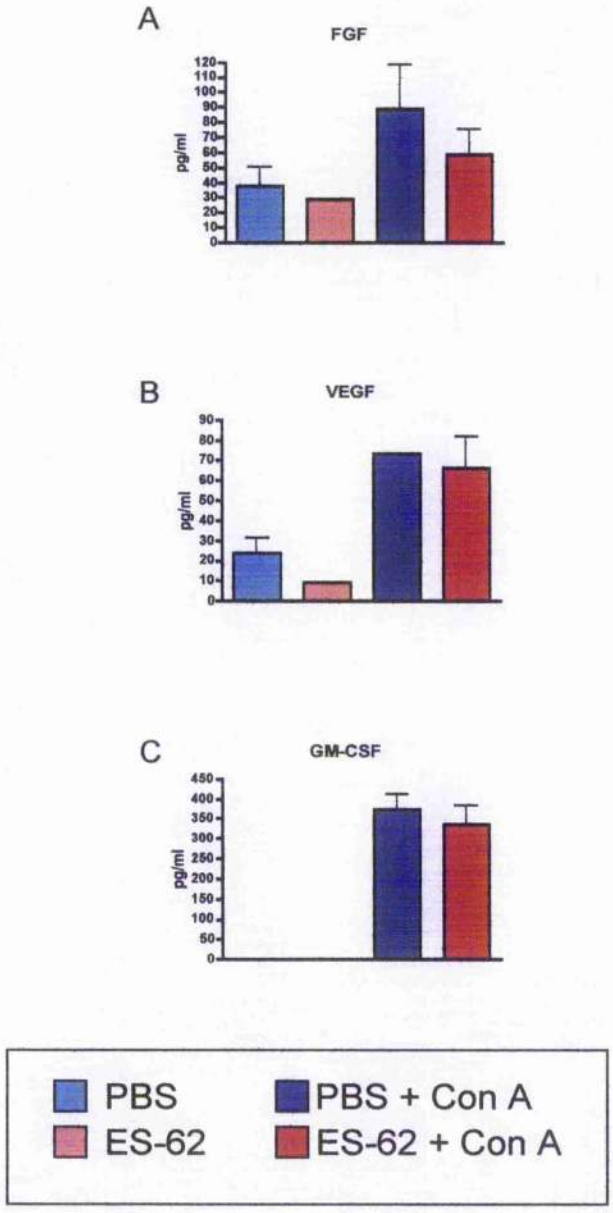

A
FGF
pg/ml
120
110
100
90
80
70
60
50
40
30
20
10
0
B
VEGF
pg/ml
90
80
70
60
50
40
30
20
10
0
C
GM-CSF
pg/ml
450
400
350
300
250
200
150
100
50
0
PBS
ES-62
PBS + Con A
ES-62 + Con A

Figure 5.11 Surface expression profile of bone marrow-derived DC from SLE model mice

SLE model mice were treated as described in the legend for figure 5.1. Femoral bone marrow was obtained from each mouse and pooled by treatment group. Bone marrow-derived DC were cultured *in vitro* for 7 days and then stimulated with media (panel A) or LPS (1μg/ml; panel B) for 24h. Expression levels of MHCII, CD40, CD54, CD80 and CD86 on CD11c+ DC from PBS (blue line) and ES-62 (pink line) treated mice were analysed by flow cytometry. This analysis was conducted using bone marrow derived DC from one SLE model experiment.

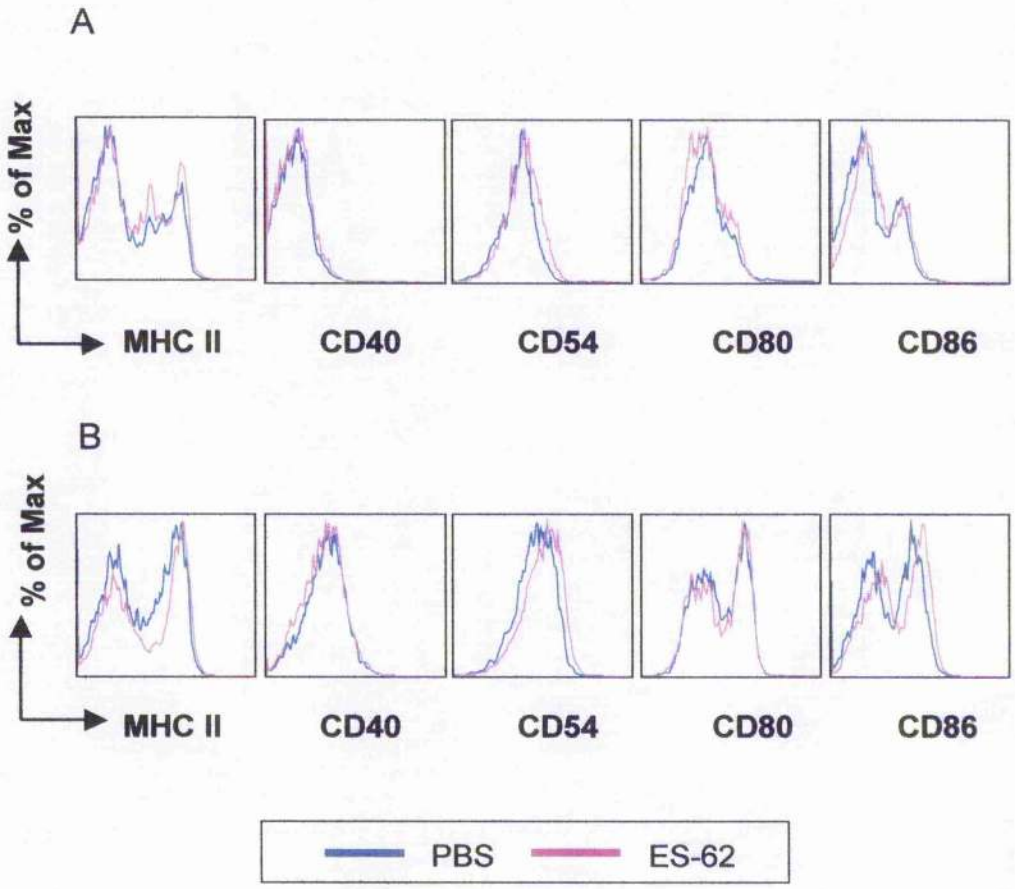

A
% of Max
MHC II
CD40
CD54
CD80
CD86
B
% of Max
MHC II
CD40
CD54
CD80
CD86
PBS
ES-62

Figure 5.12 ES-62 Inhibits LPS-induced cytokine production by bone marrow-derived DC from SLE model mice

SLE model mice were treated as described in the legend for figure 5.1. Femoral bone marrow was obtained from each mouse and pooled by treatment group. Bone marrow-derived DC were cultured *in vitro* for 7 days and then stimulated with media (Control) or LPS (1μg/ml) for 24h. Culture supernatants were analysed for IL-12 (A), TNFα (B), IL-6 (C), IL-1 (D) and IL-10 (E) by Luminex. Cytokine data are expressed as the mean of duplicate samples from a single culture well and are representative of one experiment.

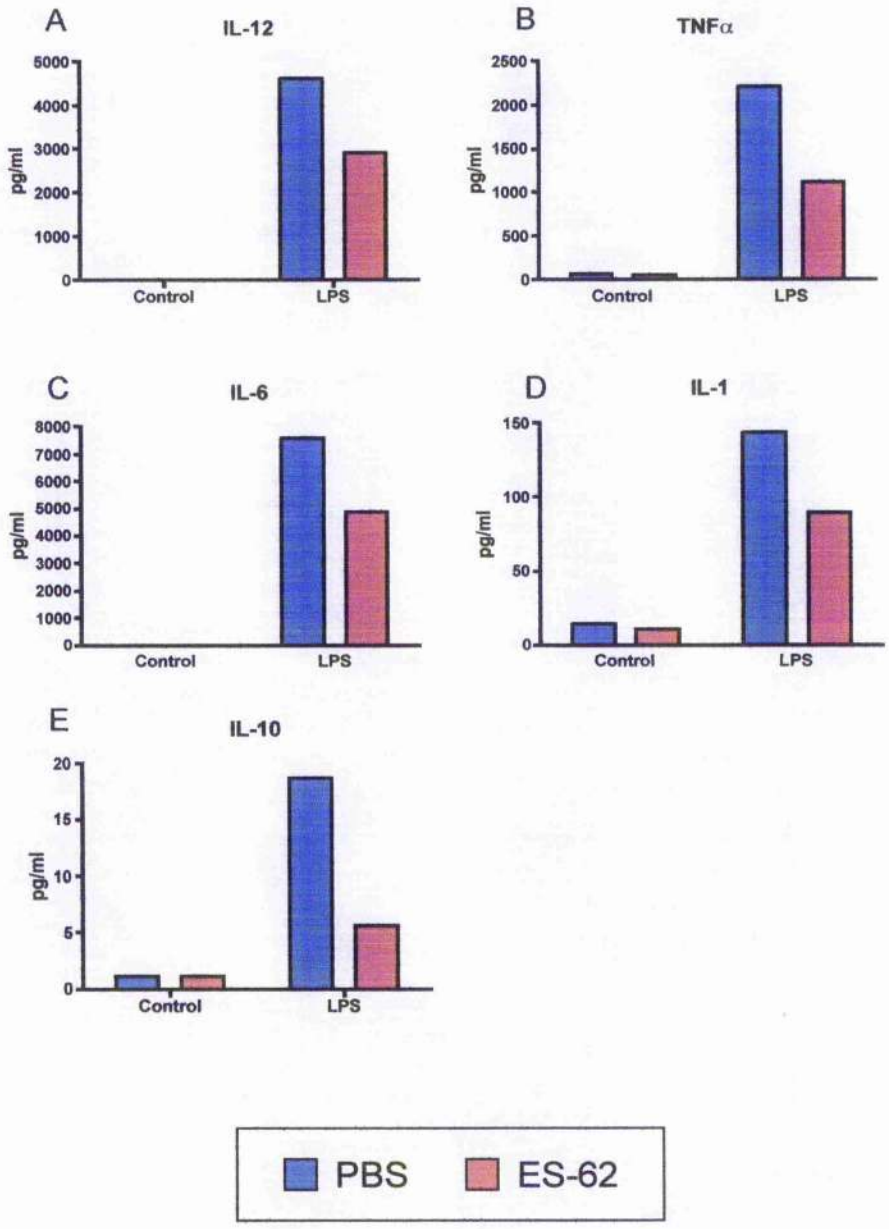

A
IL-12
pg/ml
5000
4000
3000
2000
1000
0
Control
LPS
B
TNFα
pg/ml
2500
2000
1500
1000
500
0
Control
LPS
C
IL-6
pg/ml
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
LPS
D
IL-1
pg/ml
150
100
50
0
Control
LPS
E
IL-10
pg/ml
20
15
10
5
0
Control
LPS
PBS
ES-62

Figure 5.13 The IL-17 effector pathway

IL-17 secreted by activated T cells induces production of pro-inflammatory cytokine TNFα. In turn, TNFα induces production of VEGF, an angiogenic growth factor and KC, an inflammatory chemokine which potently recruits neutrophils. Nevertheless, IL-17 can induce production of VEGF and KC directly (in the absence of TNFα) however, when IL-17 co-operates with TNFα to induce secretion of e.g. VEGF, a synergistic effect results. Thus, IL-17 is an angiogenic and neutrophilic cytokine and therefore, has an important role in pathogenesis of SLE. It has been suggested that IL-23 induces IL-17. ES-62 treatment of SLE inhibited T cell production of IL-17, TNFα, KC and VEGF, suggesting that ES-62 might be targeting a common link, which might be IL-23.

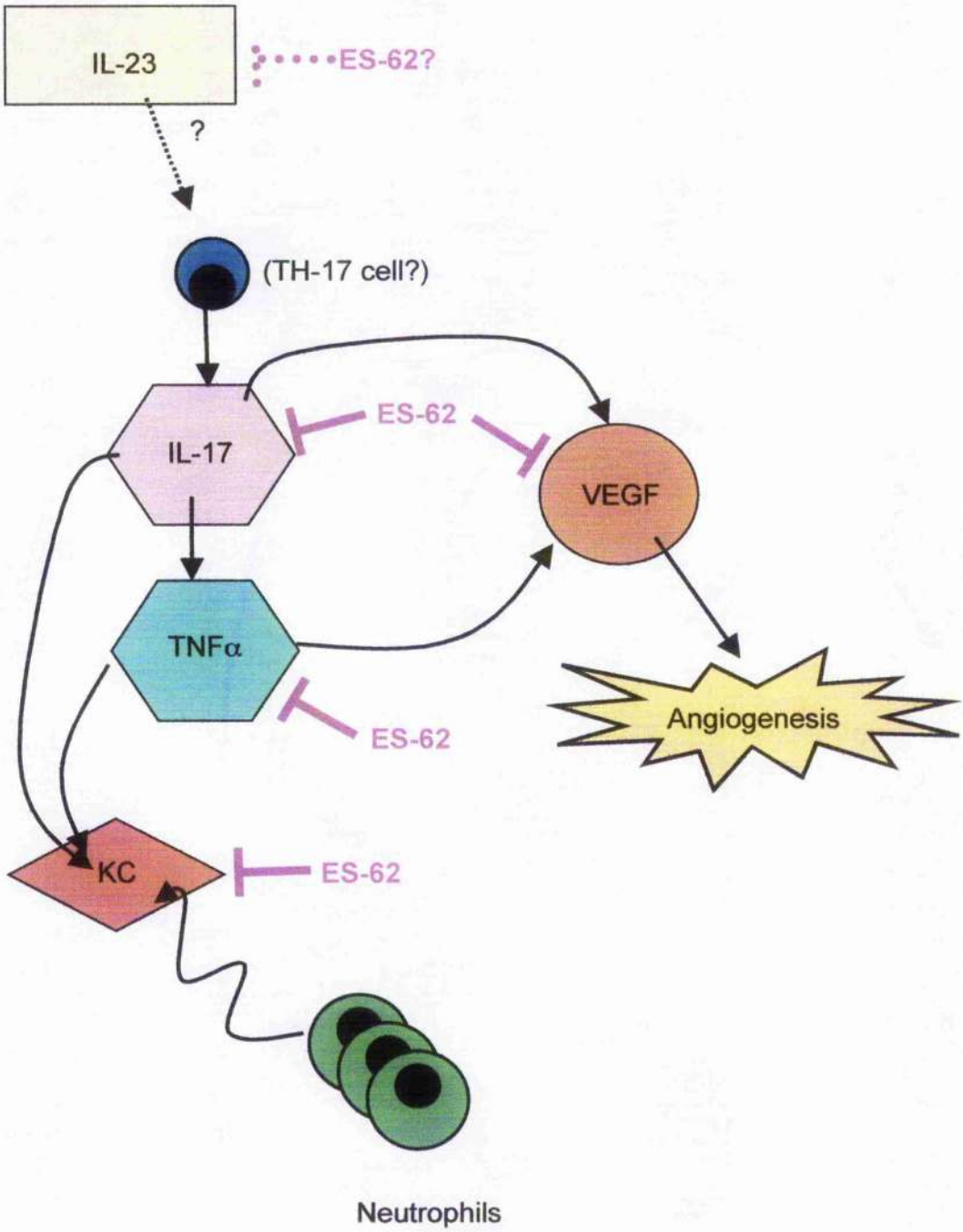
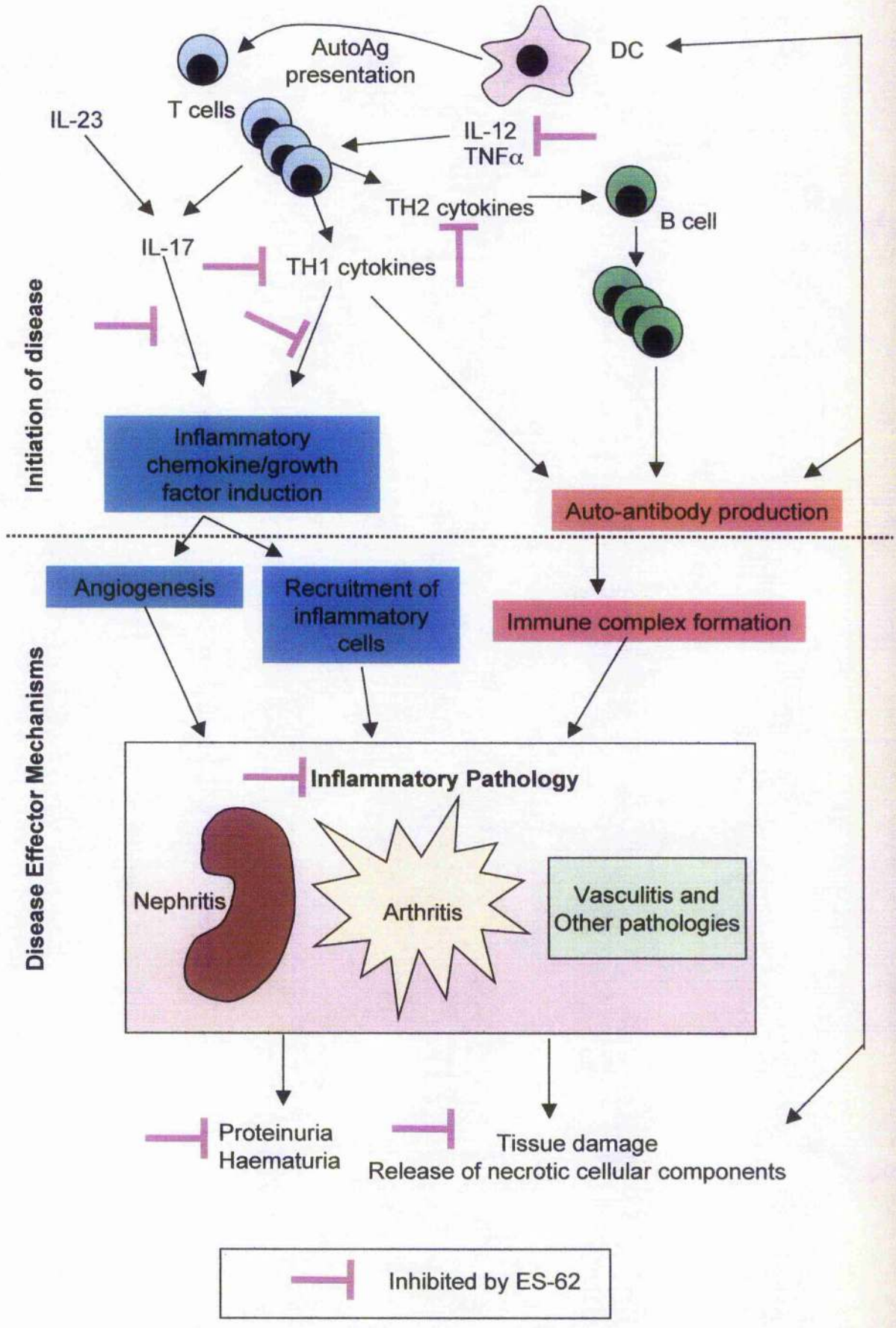

IL-23
ES-62?
?
(TH-17 cell?)
IL-17
ES-62
VEGF
TNFα
Angiogenesis
ES-62
KC
ES-62
Neutrophils

Figure 5.14 Development of pathology in MRL/lpr mice

In the MRL/lpr mouse pathology appears to be brought about by activation of multiple inflammatory pathways running in series and in parallel. Activated auto-reactive B cells undergo hyperplasia and initiate hypersecretion of Autoantibodies, which leads to development of gross immune complexes and ultimately, organ damage, such as nephritis. In addition, secretion of inflammatory TH1 and TH2 cytokines, chemoattractant chemokines and angiogenic factors help to promote the developing inflammatory pathology. Furthermore, positive feedback is set up as target organs become inflamed and necrotic tissue is released, leading to generation of further auto-antibody development.

ES-62 treatment of MRL/lpr mice does not target T or B cell hyperplasia or production of autoantibodies. However, ES-62 does inhibit production of inflammatory chemokines and angiogenic growth factors required to help develop the pathology initiated by the effects of autoantibody production and immune complex formation. Thus, ES-62 inhibits development of inflammatory autoimmune pathology, such as nephritis and arthritis, exhibited in this model, which is demonstrated by reduction of proteinuria and arthritic score


AutoAg
presentation
DC
T cells
IL-23
IL-12
TNFα
TH2 cytokines
B cell
IL-17
TH1 cytokines
Initiation of disease
Inflammatory
chemokine/growth
factor induction
Auto-antibody production
Angiogenesis
Recruitment of
inflammatory
cells
Immune complex formation
Disease Effector Mechanisms
Inflammatory Pathology
Nephritis
Arthritis
Vasculitis and
Other pathologies
Proteinuria
Haematuria
Tissue damage
Release of necrotic cellular components
Inhibited by ES-62

6 Dissecting the immunomoduiatory components of parasite derived substances

6.1 Introduction

6.1.1 Phosphorylcholine, an immunomodulatory component of ES-62

As described previously (Chapter 3), phosphorylcholine (PC) is a common component of many pathogens in which it exhibits dual immunostimulatory and immunomodulatory functions [177]. Published investigations from this laboratory, which indicate that PC (alone, or conjugated to albumin) mimics multiple immunomodulatory actions of ES-62 [167, 183, 191], suggest that PC may be an active component of ES-62. Furthermore, the data presented in Chapter 3 demonstrated that the anti-inflammatory action of ES-62 *in vivo* could also be largely mimicked by OVA-PC. Nevertheless, PC-independent immunomodulatory actions of ES-62 were also uncovered, indicating that PC is not the sole immunomodulatory feature of ES-62. It was therefore prudent to examine, in more detail, the immunomodulatory properties of ES-62 substructures. Indeed, PC attached to a carbohydrate backbone, as exhibited within the structure of ES-62, is a structural feature conserved amongst molecules derived from filarial nematodes [179].

6.1.2 A glycosphingolipid component of nematode, *Ascaris suum*, with immunomodulatory properties

A PC-containing glycosphingolipid (GSL) component of the porcine parasite, *Ascaris suum* [468], has previously been investigated by this laboratory and found to exhibit several immunomodulatory properties similar to ES-62 (reviewed in [281]). To review, this GSL induced human peripheral blood mononuclear cells to produce low-levels of TH1 and pro-inflammatory cytokines [195] and inhibited murine B cell proliferation, resulting from ligation of the antigen receptor [468]. *Ascaris* GSL also inhibited the normal response to the potent inflammatory agent, LPS in both murine B cells and macrophages [468]. *A. suum* GSL comprises carbohydrate and ceramide moieties in addition to phosphorylcholine (PC) (which is in phosphodiester linkage with N-acetylglucosamine) [195]. Thus, with similar actions and analogous structural components to the PC-glycoprotein, ES-62, it was assumed that the PC component of GSL was responsible for its immunomodulatory properties.

6.1.3 *Ascaris suum* glycosphingolipid exhibits PC-dependent immunomodulatory action

To test whether PC was indeed the immunomodulatory moiety, the immunomodulatory properties of intact *Ascaris* glycosphingolipid were compared with glycosphingolipid from which PC was removed by treatment with hydrofluoric acid. As predicted, PC was found to be essential for production of cytokines by human peripheral blood mononuclear cells

[195] and inhibition of both LPS-induced murine B cell proliferation and macrophage Th1 cytokine production [468]. However unexpectedly, glycosphingolipid-induced inhibition of antigen receptor dependent B cell proliferation was found not to be dependent on PC [468]. This latter finding suggested that PC is not the only immunomodulatory substructure present in *A. suum* glycosphingolipid.

6.1.4 Aims

In Chapters 3, 4 and 5, it was demonstrated that ES-62 treatment *in vivo* consistently reduced disease-associated inflammation, at least in part via the induction of immunomodulatory effects on bone marrow-derived cells of the innate immune system (e.g. dendritic cells and macrophages). Furthermore, previous investigations conducted in this laboratory have demonstrated that ES-62 targets these cell types primarily, for direct or indirect modulation of the immune response [189-191]. As mentioned previously, the nematode product, GSL contains similar PC-glycan structure to those of ES-62 and displays analogous immunomodulatory effects on macrophages. Therefore, the core aim of this investigation was to determine the actions of parasite-derived PC-containing substructures on dendritic cells and macrophages. It was intended that this would aid dissection of the mechanism of action of ES-62 in models of inflammatory disease.

With the aid of collaborators (R Geyer et al, Giessen, Germany), the role of PC, glycans or their combination in the immunomodulatory actions of ES-62 and other PC-containing parasite products were dissected. In particular, three synthetic mimetics of *A. suum* GSL with specific components of the native structure removed or replaced (as detailed in Figure 6.1) were synthesised. In addition, complete and PC-free versions of native GSL were prepared. The specific aims of this investigation were as follows:

1. To establish the, as yet undetermined, immunomodulatory effect of native *Ascaris* GSL on DC phenotype and cytokine production.
2. To extend the previous investigations conducted with *Ascaris* GSL by determining the respective actions of the ceramide, carbohydrate and PC components of the GSL structure on macrophage function.
3. To determine the respective actions of ceramide, carbohydrate and PC components of the GSL structure on normal DC phenotype and function.

6.2 Results

6.2.1 Native *A. suum* glycosphingolipid does not modulate DC cytokine production or cell surface expression

As mentioned in Section 6.1, the effects of native *A. suum* GSL treatment on macrophages, but not DC, have previously been determined [468]. Thus, DC were cultured with native *Ascaris* glycosphingolipid and their phenotype determined in terms of cytokines secreted and cell surface molecule expression. However, treatment of DC with native glycosphingolipid alone did not significantly modulate DC IL-12 or TNFα production or surface molecule expression (Figure 6.2). The observed lack of immunostimulatory action of native *Ascaris* glycosphingolipid on DC was therefore similar to the results of this analysis in macrophages.

6.2.2 Native A. suum glycosphingolipid inhibits LPS-induced cytokine production and up-regulation of cell surface expression by DCs

Stimulation with LPS induced marked maturation of DC. This was demonstrated by elevated secretion of IL-12 and TNFα (Figure 6.3b, c) and up-regulation of cell surface markers: MHCII, CD40, CD86, CD80 and CD54 (Figure 6.3a). Pre-treatment of DC with *Ascaris* glycosphingolipid significantly inhibited LPS-induced IL-12, but not TNFα, production. This effect was found to be PC-independent as demonstrated by maintenance of the inhibitory effect when DC were pre-treated with PC-free glycosphingolipid. Interestingly, this inhibitory action had been demonstrated to be PC-dependent in macrophages [468], indicating that the action of *Ascaris* glycosphingolipid was not identical in DC and macrophages. Consistent with the PC-independent inhibitory effect on cytokine production, native and PC-free glycosphingolipid also slightly inhibited LPS-induced up-regulation of CD86 (Figure 6.3), but had negligible effects on MHC II, CD40, CD80 and CD54.

6.2.3 Effect of synthetic glycosphingolipids on cytokine production in macrophages

To extend the analysis of glycosphingolipid action on macrophages and DC and determine more comprehensively the structural components necessary for the PC-dependent and independent immunomodulatory action of *Ascaris* glycosphingolipid, the immunomodulatory potential of three synthetic glycosphingolipids (Figure 6.1) was explored.

Previously, it was determined that native *Ascaris* glycosphingolipid did not have any cytokine-inducing action on bone marrow-derived macrophages [468]. To determine whether the same was true of the synthetic glycosphingolipids, bone marrow-derived

macrophages were cultured with them and the resulting supernatants analysed for cytokines. Treatment with Compound 1 or Compound 2 did not induce production of IL-12p40 or TNFα (Figure 6.4). Interestingly however, Compound 3 markedly induced production of IL-12p40 and TNFα by macrophages (Figure 6.4), indicating that it possessed modest immunostimulatory properties, similar to ES-62 in this cell type [190].

6.2.4 Effect of synthetic glycosphingolipids on LPS/IFNγ-induced IL-12 production in macrophages

The immunomodulatory potential of the synthetic glycosphingolipids on the macrophage response to potent inflammatory stimuli was also determined. Thus, macrophages were pre-treated with synthetic glycosphingolipid, before stimulation with LPS plus IFNγ and culture supernatants were analysed for cytokines. As expected, treatment of macrophages with LPS/IFNγ alone induced markedly elevated production of the TH1-promoting cytokine, IL-12 and the pro-inflammatory cytokine TNFα (Figure 6.4). Pre-treatment with Compound 1 or Compound 2 did not significantly modulate LPS/IFNγ-induced Th1 cytokine production. These findings were somewhat unexpected, as the structure of PC-containing Compound 1 exhibited the greatest similarity of the three synthetic compounds to native *Ascaris* GSL (Figure 6.1), which had previously been demonstrated to inhibit the response to LPS in this cell type. However, pre-treatment with Compound 3, significantly reduced LPS/IFNγ-induced macrophage IL-12p40 production (Figure 6.4). Macrophage TNFα production induced by LPS/IFNγ was also routinely found to be slightly reduced by prior treatment with Compound 3, but this was not found to be statistically significant. These results indicated that exposure to Compound 3, but not Compounds 1 or 2, inhibited the subsequent pro-inflammatory cytokine response of macrophages to LPS. Again, this was similar to the action of ES-62 on this cell type.

6.2.5 Synthetic glycosphingolipids induce TH1-promoting cytokine production by, and maturation of, dendritic cells

Following observation of the somewhat unexpected effects of the three synthetic mimetics of *Ascaris* GSL on macrophage cytokine production reponses, it was of particular interest to also determine the action of these three compounds on DC phenotype and function. Thus, DC were cultured with synthetic glycosphingolipid and their phenotype assessed as described above. Treatment with Compound 1 or 2 did not significantly modulate DC TH1-promoting cytokine production (Figure 6.6). However, similar to the results for synthetic glycosphingolipid treatment of macrophages, Compound 3 stimulated DC to produce elevated IL-12 and to a lesser extent TNFα. This maturation effect of Compound 3 was reflected by up-regulation of MHC II and CD54 expression (Figure 6.5) although, interestingly, Compound 1 also showed a little activity in terms of up-regulation of MHCII and CD54 expression on these $CD11c^+$ DC.

6.2.6 Synthetic glycosphingolipids inhibit LPS-induced IL-12 production in dendritic cells

To determine the action of various structural components of glycosphingolipid on the dendritic cell response to LPS, DC were treated with synthetic glycosphingolipid before stimulation with LPS and the cytokines produced were analysed. As demonstrated previously, LPS induced markedly increased IL-12 cytokine production by DC (Figure 6.6). In accordance with their effects on cell surface expression, LPS-induced IL-12 production was not significantly modulated by pre-treatment with Compounds 1 or 2. Confirming that although Compounds 1 and 2 exhibited the greatest similarity to intact and PC-free native Ascaris glycosphingolipid respectively, their actions did not reflect the immunomodulatory effects of their native counterparts on DC phenotype and function (Section 6.2.2). However, inhibition of LPS-induced DC IL-12 production was observed when DC were pre-treated with Compound 3. In combination with the results presented in section 6.2.5, this observation confirmed that PC-containing Compound 3 exhibited similar inhibitory actions to those of native *Ascaris* GSL and furthermore, similar immunomodulatory (stimulatory and inhibitory) effects to those of PC-containing glycoprotein, ES-62 [190], on DC.

6.3 Discussion

In summary, it has been demonstrated that synthesis of viable small molecule derivatives of parasite products (with immunomodulatory capacity) is possible in the laboratory. The results described in this chapter demonstrate how, by utilising an *in vitro* system, it has also been made possible to determine the immunomodulatory potential of distinct structural components common to many PC-containing parasite products. Furthermore, new information about the action of nematode-derived GSL on DC has been uncovered.

6.3.1 PC is not sufficient for immunomodulatory action of parasite-derived substances

It has previously been shown that native *Ascaris* glycosphingolipid has similar properties to ES-62, such as inhibition of macrophage responses to LPS and IFNγ, in particular, IL-12 production [468]. Furthermore this effect appeared to be dependent on the PC moiety of the glycosphingolipids. It is perhaps not surprising therefore that PC-free Compound 2 (Figure 6.1), was unable to inhibit the LPS/IFNγ-induced IL-12 production (Figure 6.6). Curiously however, of the two PC-containing compounds (Compound 1 and Compound 3), only compound 3 was able to inhibit IL-12 production. This is perhaps all the more surprising as Compound 1 might be considered to resemble the native material more than Compound 3. Perhaps the structure of Compound 1 may have unforeseen effects on the availability of the PC group for interaction with macrophages. However it should be noted that it has not formally been demonstrated that PC is the inhibitory component of Compound 3. Whatever the case it can be concluded that simply containing PC is insufficient for parasite-derived substances to modulate cytokine responses.

6.3.2 Compound 3: a PC-containing synthetic glycolipid that exhibits immunostimulatory actions similar to ES-62.

Compound 3 demonstrated a property that was distinct from the native glycosphingolipids in that it was able to induce the secretion of TNFα and IL-12 by murine macrophages and DC *in vitro*. This result was, however, reminiscent of the effect of the native material on human peripheral blood mononuclear cells [195]. However, the dual stimulatory and inhibitory actions of Compound 3 appear to most closely resemble the actions of PC-containing glycoprotein, ES-62 [190, 191]. As mentioned previously, the effects of ES-62 are largely PC-dependent suggesting that PC may indeed be the important moiety with respect to Compound 3. Curiously however, PC is attached to ES-62 via an N-type glycan and more specifically, as with Compound 1, to an *N*-acetylglucosamine residue (reviewed in [469]). Thus once again the lack of any immunomodulatory activity in Compound 1 can perhaps be considered surprising.

6.3.3 *Ascaris suum* GSL exhibits inhibitory action with distinct PC-dependencies in DC and macrophages.

The results of the current study indicate that intact native glycosphingolipid also significantly inhibits the DC response to LPS, demonstrated by reduced cytokine production and suppression of LPS-mediated up-regulation of DC surface marker expression. This result suggested that glycosphingolipid of *A. suum* has similar immunomodulatory action on both, macrophages and DC. ES-62 generally also has similar immunomodulatory action on macrophages and DC with again PC playing a prominent role [193]. It is surprising therefore that the inhibitory effect of *Ascaris* glycosphingolipid on LPS-induced DC IL-12 production, is apparently PC-independent. This suggests that the PC-free portion of the *Ascaris* molecule contains the immunomodulatory entity responsible for inhibition of LPS-induced cytokine production by DC, but not by macrophages. Previously, it has been shown that this portion of the glycosphingolipid has immunomodulatory capacity in B cells [468]. More specifically, it was responsible for inhibition of surface antigen receptor-induced B cell proliferation.

6.3.4 A non-PC immunomodulatory component of glycosphingolipid

The discovery of more than one immunomodulatory moiety within the glycosphingolipid structure, triggered speculation as to what the undefined, PC-independent immunomodulatory substructure might be. Ceramide, present in Compound 2 and native *Ascaris* GSL, has previously been identified as an immunomodulator [470] and has been shown to be apoptotic in many cell types [471, 472]. Furthermore, it was postulated that this apoptotic action of ceramide was responsible for GSL-mediated PC-independent inhibition of B cell proliferation [468]. However as Compound 2 has been shown to have no effect on DC responses clearly the ceramide moiety (consisting of C18-sphingosine and an amide-linked palmitic acid) is unable or insufficient to promote inhibition of cytokine production. The question remains open as to whether native ceramide moieties of Ascaris glycosphingolipids consisting of a long-chained 2-hydroxy fatty acid and iso-branched C17-sphingosine or C17-sphinganine derivatives may promote inhibition of cytokine production.

Carbohydrate, the remaining PC-independent component of glycosphingolipid has long been known to possess immunosuppressive activity [473, 474]. If it is, as seems likely, responsible for the PC-independent inhibitory effect observed then results obtained with synthetic Compounds 1 and 2 dictate that the intact carbohydrate chain and/or one or both of the two terminal sugars (galactose and N-acetyl galactoamine; Figure 2.1) are necessary for activity.

6.3.5 A receptor for the carbohydrate moiety of glycosphingolipid?

If a carbohydrate component is responsible for the immunosuppressive action of the glycosphingolipids, consideration of the mechanism of action at the surface of DC and macrophages is permitted. CD83 is an Ig-like lectin adhesion receptor that appears to be present on DC, but not on macrophages and hence, has an expression distribution consistent with the differential effects of PC-free Ascaris glycosphingolipid. However, CD83 appears to bind sialic acid [475], which is absent from the glycosphingolipids. The data obtained with the three synthetic glycosphingolipids might argue a case for N-acetylgalactosamine and/or galactose being important in recognition, as virtually no activity was observed in their absence (Compound 1). Furthermore, the only carbohydrate component of Compound 3 is galactose, although, as mentioned earlier, the PC dependence of this glycolipid is unknown. With respect to N-acetylgalactosamine/galactose binding, a C-type lectin (macrophage lectin specific for galactose/N-acetylgalactosamine; MGL) has been described with specificity for these two sugars. Studies on cells derived from humans indicated that MGL was expressed on immature DC but not mature DC, monocytes or macrophages [476]. Nevertheless, recently published studies contradict these findings by indicating that MGL is expressed on macrophages in mice and, in addition suggesting that it may not recognise galactose, but only N-acetylgalactosamine [477]. Thus, clearly at this stage further information is required before speculation can be made as to the receptor for the carbohydrate component of Compound 3.

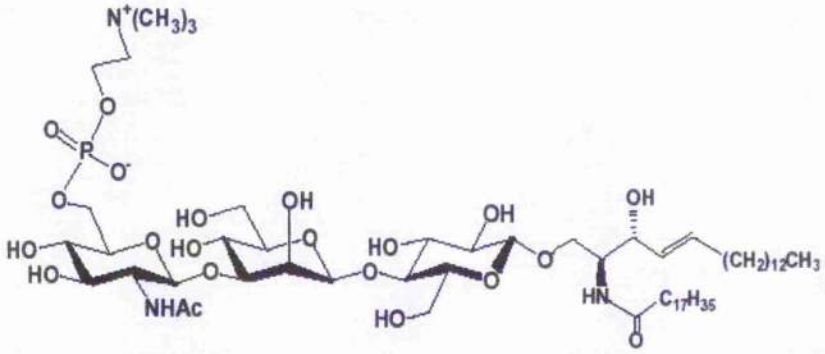
6.3.6 What do these findings mean in terms of ES-62 action *in vivo*?

In summary, both carbohydrate and PC components of the Ascaris glycosphingolipid structure exhibit immunomodulatory action. In Chapter 3 it became clear that non-PC components of ES-62 also exhibited immunological action because PC-deficient rES-62 did not lack all of the immunomodulatory actions of its parasite-derived intact homologue. Similarly, PC conjugated to OVA did not mimic the actions of ES-62 entirely. Thus, it was concluded that non-PC (i.e. glycan) components of ES-62 were also important for the anti-inflammatory and immunomodulatory actions of ES-62 in CIA. Given the results presented in this chapter it is clear that the carbohydrate component of ES-62 represents a viable candidate for mediation of PC-independent ES-62 action in CIA.

Figure 6.1 Structures of parasite-derived and synthetic mimetics of A. suum glycosphingolipid

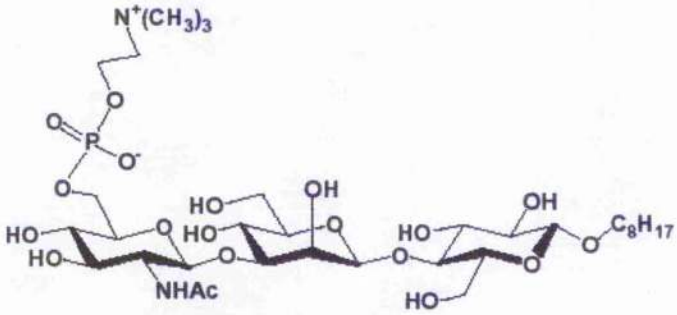
Compound 1 (B) is resembles native GSL (A) without the ceramide (Cer) moiety (which has been replaced with an octyl group (Oct)) and the two N-terminal sugars (Galactose and N-acetylgalactosamine). Compound 2 (C) contains ceramide, but lacks PC and the two N-terminal sugars. Compound 3 (D) resembles a related PC-glycolipid and consists of PC and two galactose sugars, with PC attached to one and an octyl group to the other.

A



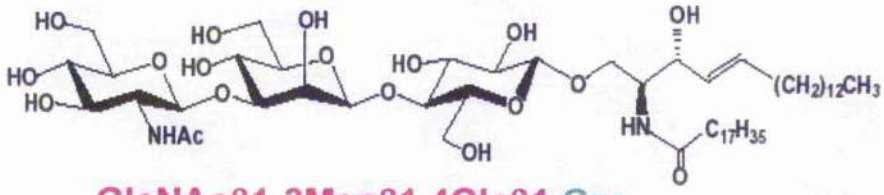
PC -6Galα1-3GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1-1-Cer

B



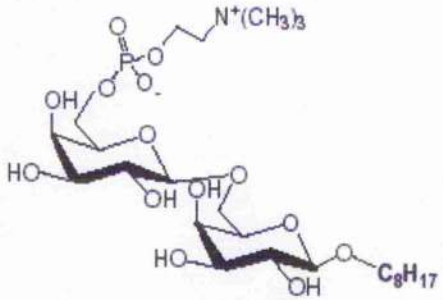
PC-6GlcNAcβ1-3Manβ1-4Glcβ1-Oct

C



GlcNAcβ1-3Manβ1-4Glcβ1-Cer

D



PC-6Galβ1-6Galβ-1-Oct

Figure 6.2 Native Ascaris glycosphingolipid does not modulate DC surface expression profile or stimulate DC cytokine production.

Bone marrow-derived dendritic cells were treated with media (Control), native *Ascaris* glycosphingolipid (GSL, 5 μg/ml) or PC-free *Ascaris* glycosphingolipid (GSL-PC, 5 μg/ml) and cultured for 24h. Control cells (Control) received media instead of glycosphingolipid treatment.

Panel A Cells were stained for flow cytometry and analysed as described in Chapter 2. Data from only $CD11c^+$ cell populations are expressed and are representative of 4 independent experiments. Cell surface expression levels are depicted as % of maximum comparing control (black line) vs intact glycosphingolipid (blue line) or PC-free glycosphingolipid (green line) for the cell surface markers MHC class II, CD40, CD54, CD80 and CD86 and were gated positive related to the relevant isotype control.

B, C Culture supernatants were harvested and analysed for IL-12p40 (B) and TNFα (C) cytokine content by ELISA. Data are expressed as mean ± SD (n=3) and are representative of 3 independent experiments. No statistically significant differences were observed between cytokine production levels (student's T-test).

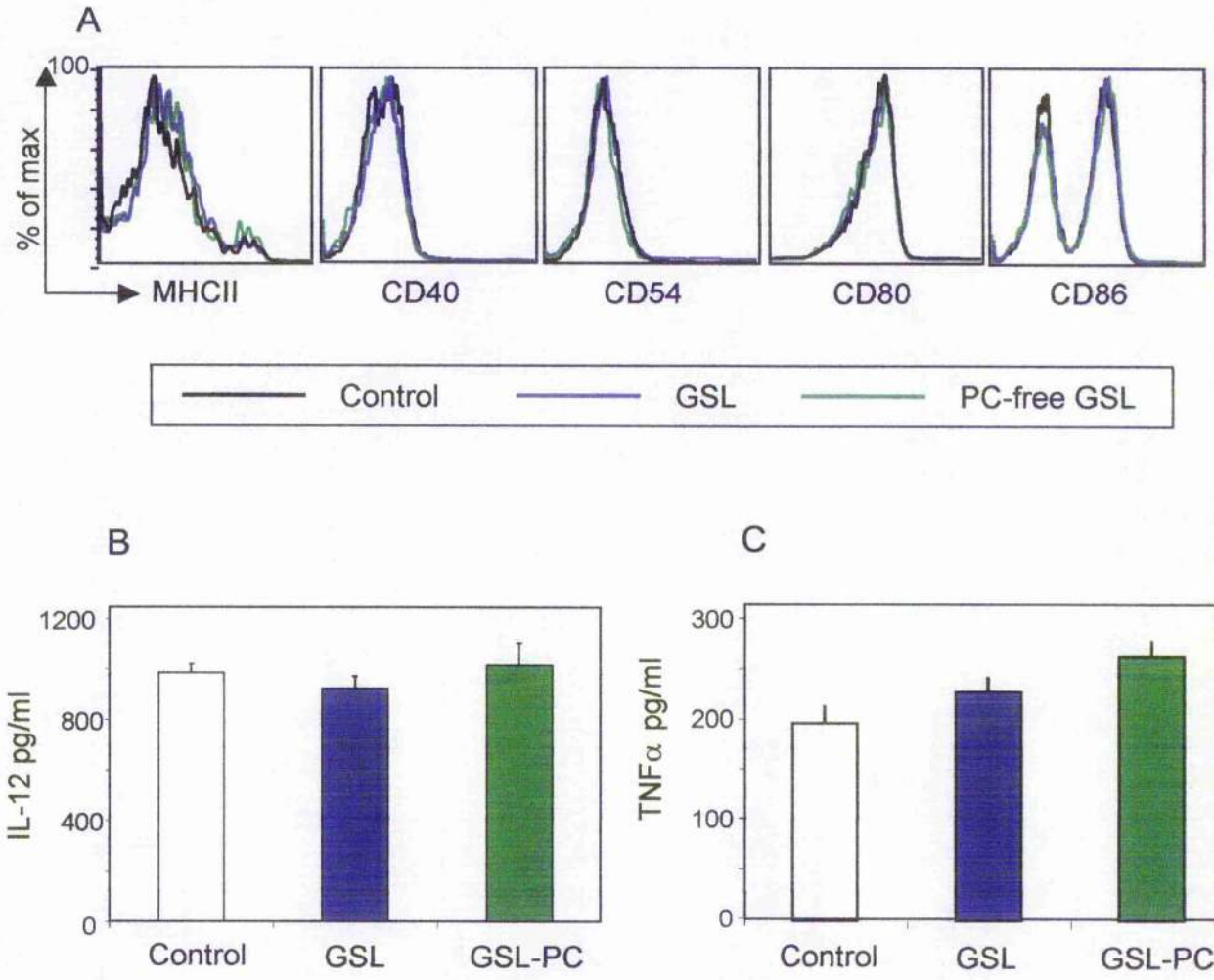

A
100
% of max
MHCII
CD40
CD54
CD80
CD86
Control
GSL
PC-free GSL
B
1200
800
400
0
IL-12 pg/ml
Control
GSL
GSL-PC
C
300
200
100
0
TNFα pg/ml
Control
GSL
GSL-PC

Figure 6.3 Native Ascaris glycosphingolipid inhibits dendritic cell responses to LPS in a PC-independent manner.

Bone marrow-derived dendritic cells were pre-treated with native *Ascaris* glycosphingolipid (5μg/ml) or PC-free *Ascaris* glycosphingolipid (panel B, 5 μg/ml) for 18h prior to treatment with LPS (1 μg/ml) for a further 24h. Control cells ((-)) received media instead of glycosphingolipid pre-treatment or LPS treatment.

Panel A Cells were stained for flow cytometry and analysed as described in Chapter 2. Data from only $CD11c^+$ cell populations are expressed and are representative of 4 independent experiments. Cell surface expression levels are depicted as % of maximum comparing control vs native glycosphingolipid or PC-free glycosphingolipid for the cell surface markers MHC class II, CD40, CD54, CD80 and CD86 and were gated positive related to the relevant isotype control.

B, C Culture supernatants were harvested and analysed for IL-12p40 (B) and TNFα (C) cytokine content. Data are expressed as mean ± SD (n=3) and are representative of 3 independent experiments. Significance is calculated by student's t-test and illustrated by *p<0.05, ***p<0.001 compared with LPS alone levels (unless otherwise indicated). +LPS indicates LPS treatment.

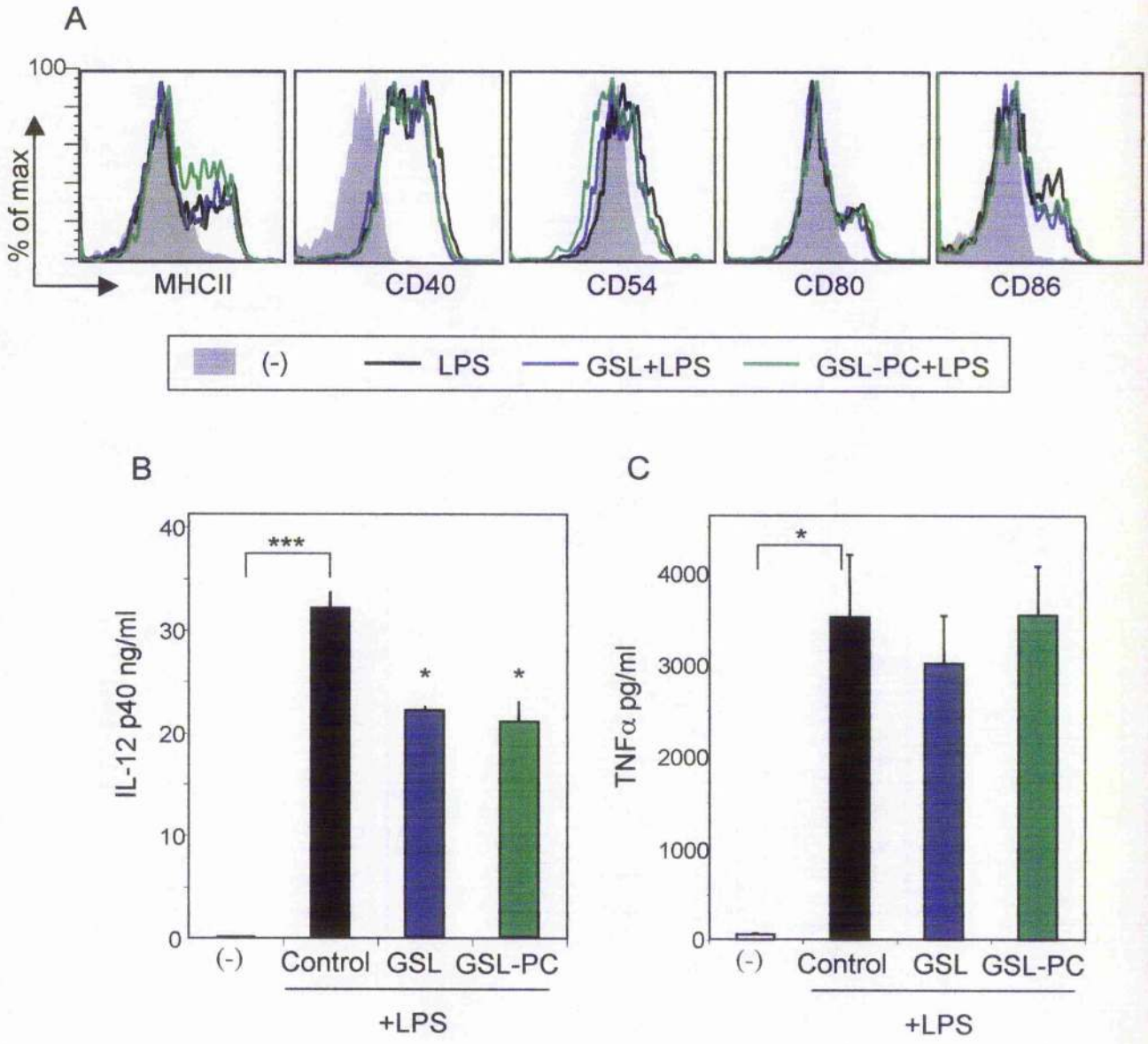

A
100
% of max
MHCII
CD40
CD54
CD80
CD86
(-)
LPS
GSL+LPS
GSL-PC+LPS
B
IL-12 p40 ng/ml
40
30
20
10
0
***
*
*
(-)
Control
GSL
GSL-PC
+LPS
C
TNFα pg/ml
4000
3000
2000
1000
0
*
(-)
Control
GSL
GSL-PC
+LPS

Figure 6.4 Compound 3 induces immunomodulatory action on bone marrow-derived macrophage cytokine production

A, B Bone marrow-derived macrophages were cultured for 42h in media (Control), Compound 1 (GSL1, 10 μg/ml), Compound 2 (GSL2, 10 μg/ml) or Compound 3 (GSL3, 10 μg/ml). Supernatants were harvested and analysed for IL-12p40 (A) and TNFα (B) cytokine content. Data are expressed as mean ± SD (*n*=3) and are representative of 3 independent experiments. Significance calculated by student's t-test and illustrated by **p<0.01, *p<0.05 compared with control levels.

C, D Bone marrow-derived macrophages were pre-treated with media (Control), Compound 1 (GSL1, 10 μg/ml), Compound 2 (GSL2, 10 μg/ml) or Compound 3 (GSL3, 10 μg/ml) for 18h prior to treatment with LPS (100 ng/ml) and IFNγ (100 U/ml) for a further 24h. Control cells were cultured in media only for the entire 42h culture period. Supernatants were harvested and analysed for IL-12p40 (C) and TNFα (D) cytokine content. Data are expressed as mean ± SD (*n*=3) and are representative of 3 independent experiments. Significance is calculated by student's t-test and illustrated by **p<0.01, *p<0.05 compared with LPS alone levels (unless otherwise indicated). +LPS/IFNγ indicates LPS and IFN-γ treatment.

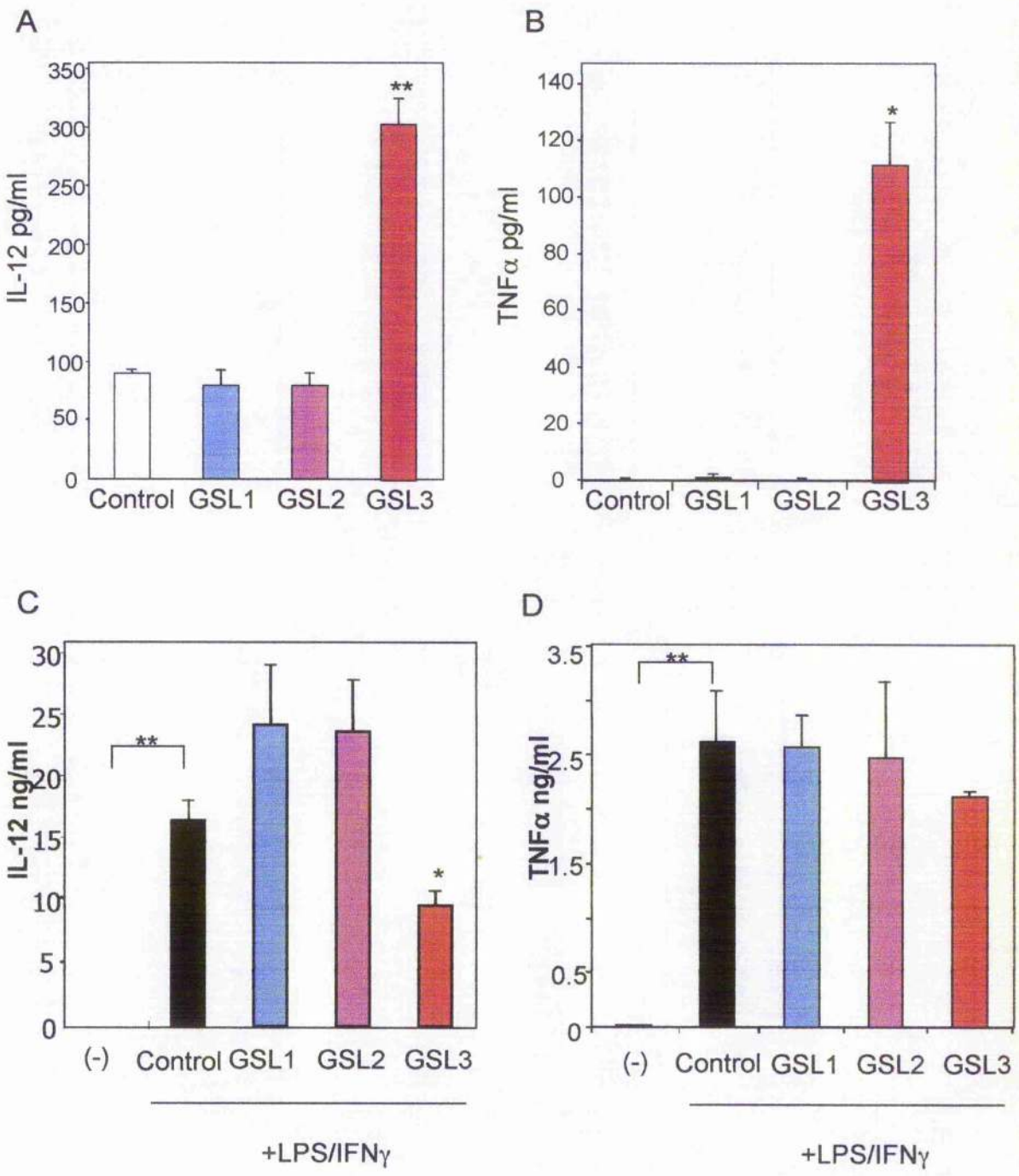

A
IL-12 pg/ml
350
300
250
200
150
100
50
0
**
Control
GSL1
GSL2
GSL3
B
TNFα pg/ml
140
120
100
80
60
40
20
0
*
Control
GSL1
GSL2
GSL3
C
IL-12 ng/ml
30
25
20
15
10
5
0
**
*
(-)
Control
GSL1
GSL2
GSL3
+LPS/IFNγ
D
TNFα ng/ml
3.5
2.5
1.5
0.5
0
**
(-)
Control
GSL1
GSL2
GSL3
+LPS/IFNγ

Figure 6.5 Compound 3 induces upregulation of dendritic cell surface expression.

Bone marrow-derived dendritic cells were cultured for 42h in media (Control), Compound 1 (panel A, 10 μg/ml), Compound 2 (panel B, 10 μg/ml) or Compound 3 (panel C, 10 μg/ml). Cells were stained for flow cytometry and analysed as described in Chapter 2. Data for the CD11c+ cell populations are expressed and cell surface expression levels are depicted as % of maximum comparing control (solid line) and glycosphingolipid-treated (dotted line) cells for the cell surface markers MHC class II, CD40, CD54 and CD86 and were gated positive related to the relevant isotype control.

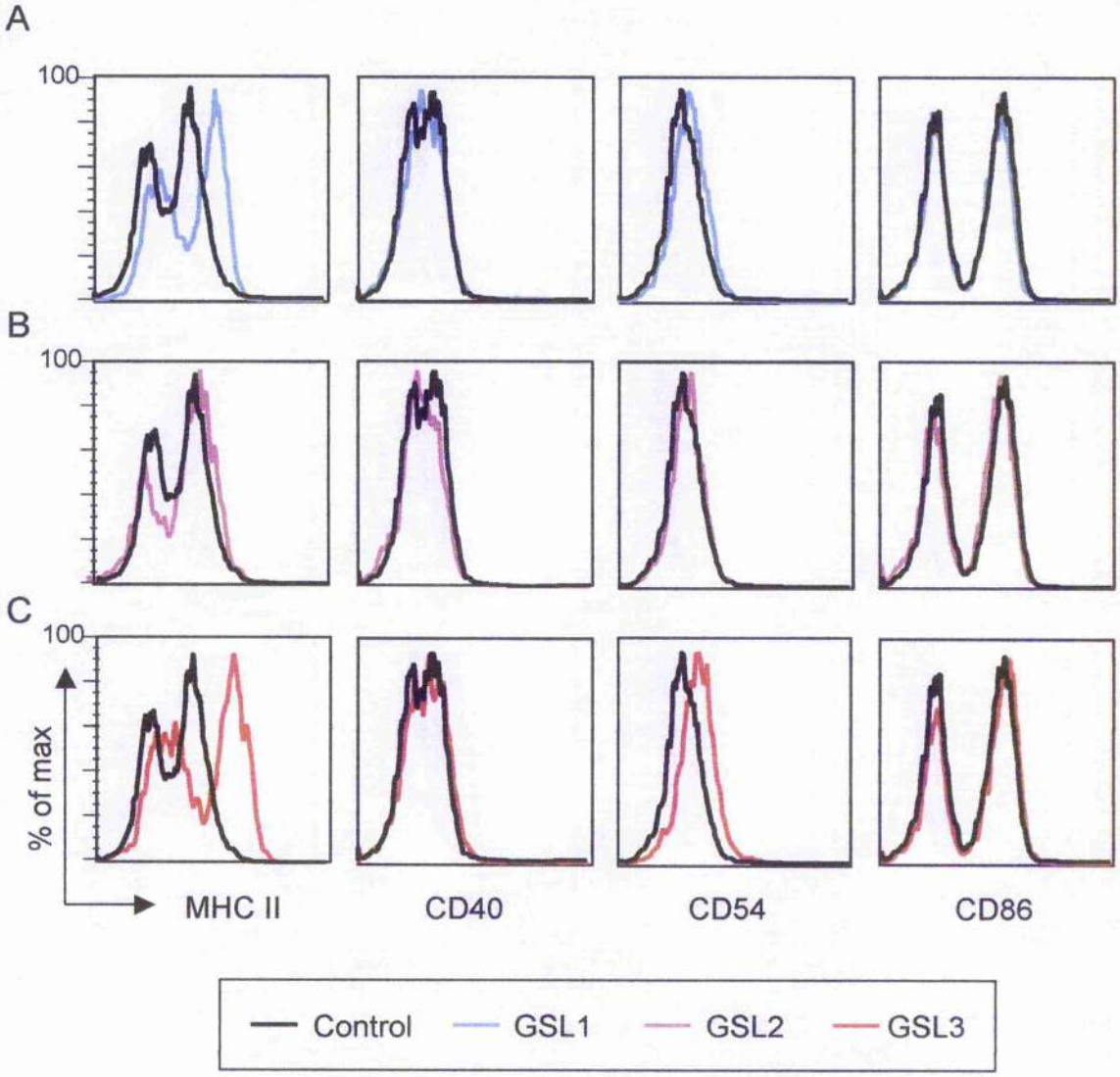
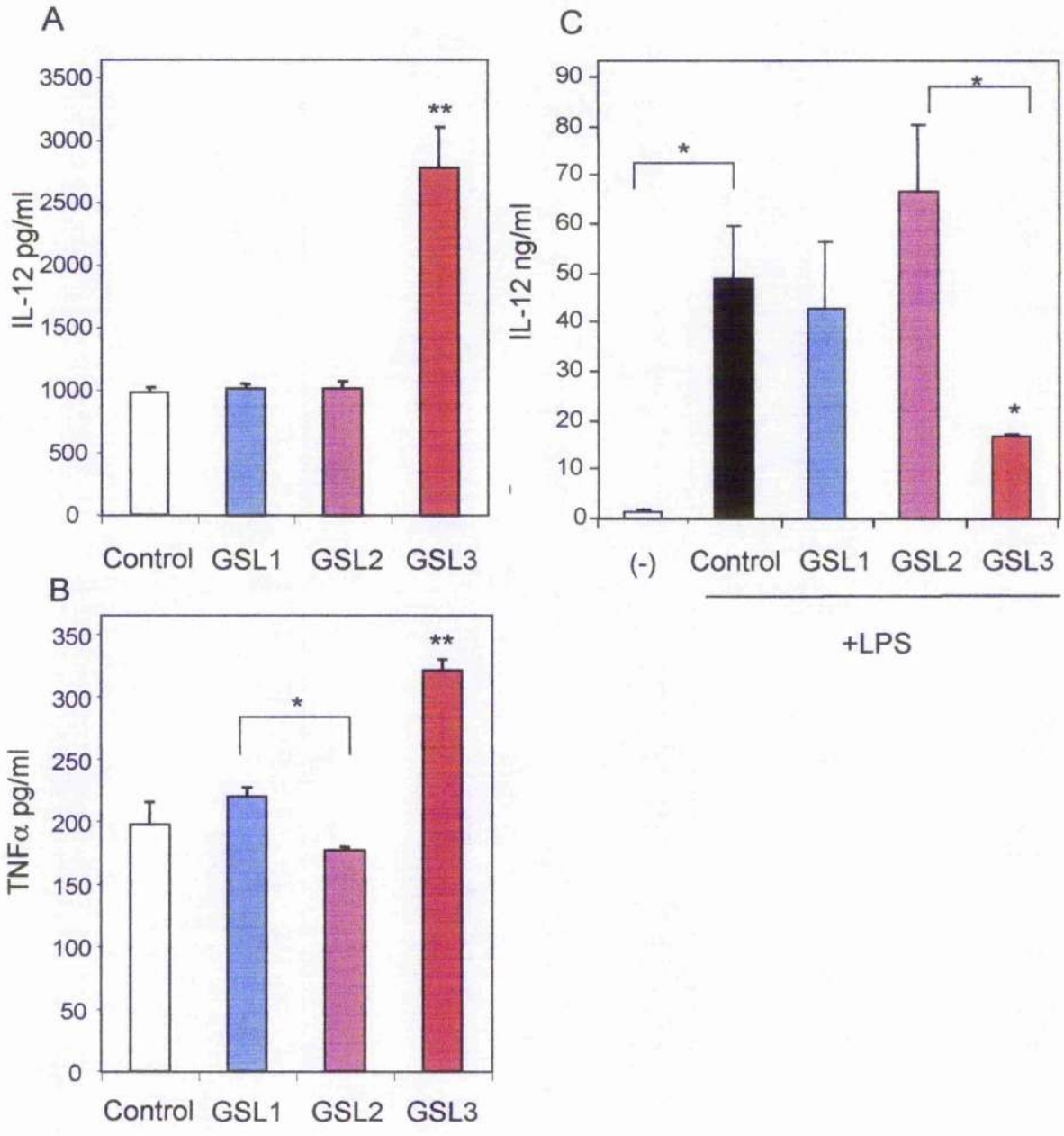

A
B
C
100
100
100
% of max
MHC II
CD40
CD54
CD86
Control
GSL1
GSL2
GSL3

Figure 6.6 Compound 3 exhibits immunomodulatory action on bone marrow-derived dendritic cell cytokine production.

A, B Bone marrow-derived dendritic cells were cultured for 42h in media (Control, open bars), Compound 1 (GSL1, 10 μg/ml), Compound 2 (GSL2, 10 μg/ml) or Compound 3 (GSL3, 10 μg/ml). Supernatants were harvested and analysed for IL-12p40 (A) and TNFα (B) cytokine content. Data are expressed as mean ± SD (*n*=3) and are representative of 3 independent experiments. Significance is calculated using student's t-test and illustrated by *p<0.05, **p<0.01 compared with control levels (unless otherwise indicated).

C Bone marrow-derived dendritic cells were pre-treated with media (Control), Compound 1 (GSL1, 10 μg/ml), Compound 2 (GSL2, 10 μg/ml) or Compound 3 (GSL3, 10 μg/ml) for 18h prior to treatment with LPS (1 μg/ml) for a further 24h. Negative control cells ((-)) were cultured in media only for the entire 42h culture period. Supernatants were harvested and analysed for IL-12p40 content. Data are expressed as mean ± SD (*n*=3) and are representative of 3 independent experiments. Significance is calculated using student's t-test and illustrated by *p<0.05 compared with LPS alone levels (unless otherwise indicated). +LPS indicates LPS treatment.


A
IL-12 pg/ml
3500
3000
2500
2000
1500
1000
500
0
**
Control
GSL1
GSL2
GSL3
B
TNFα pg/ml
350
300
250
200
150
100
50
0
*
**
Control
GSL1
GSL2
GSL3
C
IL-12 ng/ml
90
80
70
60
50
40
30
20
10
0
*
*
*
(-)
Control
GSL1
GSL2
GSL3
+LPS

7 Promotion of differential T cell-mediated immune responses by plant 2S seed albumins, via modulation of dendritic cell phenotype.

7.1 Introduction

Dendritic cells form an essential part of the immune system responsible for distinguishing pathogenic from non-pathogenic stimuli. Indeed, the previous chapters of this thesis have provided evidence to demonstrate modulatory effects of parasite-derived glycoprotein (Chapter 4) and glycolipids (Chapter 6) on DC phenotype. Thus, it has been suggested that via the modulatory actions of these parasite-derived substances on DC phenotype, inflammatory immune responses to parasite infections can be subverted. In allergic individuals, aberrant TH2-mediated inflammatory immune responses are normally triggered in response to recognition of non-self, non-pathogenic proteins. However it is not yet clear how an allergen is distinguished from a non-allergenic protein and furthermore, how a TH2-mediated immune response is initiated in response to allergen recognition by cells such as DC [478]. Therefore, it was of particular interest to determine whether allergenic proteins induce modulation of DC, hence distinguishing allergen from non-allergen at this stage in immune response initiation.

7.1.1 Allergy to consumed antigen

Many research studies have investigated the allergic response to otherwise harmless antigens that are inhaled, eaten or absorbed. In certain susceptible individuals there is a breakdown of tolerance to specific components (allergens) of food products. In allergic reaction to food proteins, TH2-type, IgE-mediated immune responses are most prevalent [479]. IgE antibodies specific for allergenic antigen are bound to the high-affinity FcεRI receptors on the surface of mast cells present in the mucosa of many body systems (for example: the oral, pulmonary or gut mucosae). When the bound IgE is ligated by its specific antigen (or allergen); mast cell degranulation is initiated and a cascade of inflammation (direct hypersensitivity reaction) ensues. This acute inflammatory immune response can range from mild and temporary to severe (or fatal) anaphylactic pathology. As mentioned in Chapter 1, allergy to foodstuffs, such as fish or nut proteins, manifest the most severe allergic responses, when compared with other inhaled or injested allergens [65]. In particular, several plant proteins (e.g. 2S seed albumins) present in nuts and seeds have been reported to trigger an allergic response.

7.1.2 2S seed albumins as allergenic proteins

Historically, 2S albumins from seeds have been associated with allergy in the oil milling (castor and rapeseed) and baking industries and several proteins from this group have been characterised as clinically important allergens [480-484]. As a class, the 2S

albumins are water-soluble proteins, products of multi-gene families and are present in seeds of a wide range of flowering plant species [485] with an as yet unknown specific biological function. Structurally the 2S albumins belong to the bifunctional inhibitor/lipid transfer protein/ storage 2S albumin or plant prolamin superfamily [486] of proteins, characterised by their conserved anti-parallel bundle of four α-helices held together by four disulphide bonds in a distinctive right-handed fold. A large number of known food allergens such as plant α-amylase/trypsin inhibitors, 2S storage proteins and lipid transfer proteins (LTPs) belong to the prolamin superfamily and seem to have evolved from the same ancient gene [486].

7.1.3 Comparison of two 2S seed albumins of differing allergenicity

To examine initiation of the direct hypersensitivity allergic response and the components of plant proteins involved, recombinant 2S seed albumins from Brazil nut seeds (Ber e 1) and sunflower seeds (SFA 8) have been compared. Ber e 1 has been identified as a major allergen [487], whilst the allergenicity of SFA 8 is yet to be conclusively established. The allergenicity of Ber e 1 was established when it was genetically engineered into soybean to improve its nutritional value and found to induce allergic reactivity when tested in allergic individuals [484]. By contrast, whilst there are reports of SFA8-binding IgE in the sera of some patients who are allergic to sunflower seeds [488-490], the overall incidence of sunflower seed allergy is very low, even in areas of high consumption such as Spain, Germany and Greece. The majority of cases of allergy to sunflower seed proteins are reported to be via inhalation instead of ingestion by, for example, caged bird owners [491, 492].

It has been suggested that stability in the gastrointestinal tract is a pre-requisite for allergenicity [479] and many allergens, especially those derived from plant foods, are consistently more resistant to degradation by pepsin than other proteins [493]. The native and the recombinant forms of both Ber e 1 and SFA8 have previously been shown to possess similar stability to pepsin-containing simulated gastric fluid (SGF) [200, 494] and also resistance to high temperatures, guanidinium chloride and acidic pH conditions [494, 495]. Moreover, SFA8 and Ber e 1 proteins also have similar structures. The tertiary structure of SFA8 in solution has recently been determined [496] and agrees with the tertiary structures of other 2S albumins and the model structure proposed for Ber e 1 [200]. This conservation of the 3D structure was exploited in the design of chimeric proteins for the mapping of the human structural IgE epitope of Ber e 1 whereby proteins containing exchanged domains were produced [497]. Therefore, the stability or structure of these proteins does not appear to account for their different allergenicities. In summary, the precise mechanisms underlying initiation of allergy to antigens such as seed albumins remains unclear.

As mentioned above, it is well known that DC can form the first point of contact for foreign antigens and play an essential and important role in initiation and direction of immune responses. It has previously been demonstrated that DC phenotype can direct immune response phenotype via interaction with naïve $CD4^+$ T cells [189]. Furthermore, it has been identified that substances present in the local environment at the time of DC activation can influence the phenotype of the subsequent immune response activated [189]. Using this system, the action of recombinant seed albumins Ber e 1 and SFA 8 on DC phenotype could be identified and hence, the subsequent immune phenotype these DC would confer on naïve $CD4^+$ T cells could potentially be deduced. Therefore, the core aim of this investigation was to determine whether the distinct allergenic properties of Ber e 1 and SFA8 were reflected in their effects, if any, on DC phenotype.

7.1.4 Aims

It was hypothesised that allergenic Ber e 1 would promote activation of DC that would drive a type II immune response; therefore the aims of this investigation were as follows:

1. To determine the effects of recombinant Ber e 1 and SFA 8 on the activation of bone marrow derived dendritic cells (DC)
2. To determine whether the TH-phenotype of the mouse strain from which the DC were derived influenced the effects of Ber e 1 and SFA 8 on bone marrow derived DC
3. To determine whether, via differential modulation of DC phenotype, Ber e1 and SFA 8 would promote development of T cell-mediated immune responses with distinct phenotypes, in terms of cytokine production and transcription factor expression.

7.2 Results

7.2.1 The effects of 2S seed albumins on cytokine production by BMDC from C57 BL/6 mice.

It has been well established that the immunological nature of different substances present at the time and place of DC activation can influence the phenotype of the resultant immune response, by modulation of DC phenotype. To determine the action of recombinant seed albumins on dendritic cell (DC) phenotype and function, bone marrow-derived DC were cultured with media containing increasing concentrations of SFA 8 or Ber e 1 for 24h. DC cultured from bone marrow of C57 BL/6 mice secreted the TH1-promoting cytokine IL-12 and the pro-inflammatory cytokine TNFα in response to treatment with SFA 8, but not Ber e 1 recombinant seed albumins (Figure 7.1a, b). Moreover, this response to SFA 8 treatment was dose-dependent, indicating that whilst non-allergenic SFA 8 potently induced secretion of pro-inflammatory cytokines by DC (that was proportional to the concentration of protein), allergenic Ber e 1 did not. Based on the results of this assay, a concentration of 50 μg/ml 2S seed albumin was considered optimum for further *in vitro* experiments.

LPS is a well-known inducer of TH1-promoting cytokine production by DC. Pre-exposure with seed albumins (SFA 8 or Ber e 1) did not significantly modulate the IL-12 or TNFα response to LPS treatment in these bone marrow-derived DC (Figure 7.1c, d). Therefore, although SFA 8 and Ber e 1 treatment alone stimulated different DC cytokine production profiles they were not immune response - dampening substances (such as ES-62), in terms of the normal cytokine response to LPS. The LPS cytokine response is initiated by ligation of pattern-recognition Toll-like receptor 4 (TLR4) on the surface of DC and facilitated by down-stream adapter molecule MyD88 [498]. To determine whether SFA 8-induced DC production of IL-12 and TNFα employed this mechanism, DC were cultured from MyD88-deficient mice, treated with SFA 8 and cytokine production was analysed. It was found that the observed cytokine response to SFA 8 was not mediated via activation of this signalling pathway, because SFA 8-induced IL-12 and TNFα production was maintained in DC from MyD88-deficient mice (Figure 7.1e, f).

7.2.2 SFA 8 seed albumin action is maintained in BMDC from BALB/c mice.

It has been well documented that C57 BL/6 mice are more likely to develop TH1-mediated immune responses. Thus, to determine any influence of strain TH-bias, DC were cultured from bone marrow of BALB/c mice, which are pre-disposed to generate TH2-polarised immune responses. Similarly to C57 BL/6 DC, SFA 8, but not Ber e 1, induced IL-12 and TNFα secretion by BALB/c DC (Figure 7.1g, h). This result indicated that the cytokine response to the 2S seed albumins was not dependent on the TH-bias of the mouse strain.

In addition to secretion of TH1-promoting cytokines, DC can also produce the TH2 / anti-inflammatory cytokine, IL-10. Thus, IL-10 is an important mediator of anti-inflammatory signals and TH2-mediated immunity; both of which can down-regulate TH1-mediated immune responses. Given that Ber e 1 did not induce TH1-promoting pro-inflammatory cytokine production by DC, it was therefore postulated that Ber e 1 might induce IL-10. Furthermore, as an anti-inflammatory cytokine, IL-10 can act to induce suppression of TH2-mediated immune responses also. Therefore, as SFA 8 had been demonstrated to induce production of TH1-promoting cytokines and hence, promotion of TH1-mediated immune responses, it was additionally proposed that SFA 8 might also induce IL-10 to reduce TH2-mediated immunity and further polarise the immune response towards a TH1-phenotype. However, following analysis of DC culture supernatants, it was concluded that SFA 8 or Ber e 1 did not induce IL-10 production by BMDC from either mouse strain (results not shown).

7.2.3 SFA 8 induces moderate upregulation of cell surface molecules on DC

Following recognition of antigen and activation, DC upregulate cell surface expression of co-stimulatory, adhesion and antigen-presentation molecules necessary for priming naïve T cells and initiation of an antigen-specific immune response [3]. Thus, using flow cytometry, cell surface expression on DC cultured from bone marrow of C57 BL/6 mice was analysed. SFA 8 treatment induced a small upregulation of MHC II, CD40, CD80 and CD86 expression on $CD11c^+$ DC (Figure 7.2). The expression of adhesion molecule, CD54, was also measured, but this was not modulated by SFA 8 treatment. The upregulation of surface expression induced by SFA 8 was much more subtle than that induced by the potent inflammatory agent, LPS. Consistent with the cytokine production analysis, Ber e 1 treatment of DC did not modulate expression levels of the five cell surface molecules examined.

These studies were repeated using DC cultured from bone marrow of BALB/c mice. Surprisingly, in BALB/c DC, SFA 8 induced greater upregulation of MHC II, CD40, CD80 and CD86 (Figure 7.3a), compared to its action on DC from C57 BL/6 mice. Furthermore, unlike the effect of SFA 8 on C57 BL/6 DC, CD54 expression was upregulated by SFA 8 in BALB/c DC. The reasons for the difference in SFA 8-induced upregulation of cell surface molecule expression on BALB/c and C57 BL/6 DC were not clear. However, because DC from both mouse strains were cultured and treated in an identical manner, it was postulated that these differences might be due to the distinct TH-bias of BALB/c and C57 BL/6 mice. Pretreatment with Ber e 1 or SFA8 did not alter LPS-induced BALB/c DC maturation (Figure 7.3b).

The results of cell surface expression analysis in both DC types indicated that DC treated with SFA 8 exhibited a more mature phenotype than those treated with Ber e 1 and these phenotypes were consistent with the cytokine production patterns discussed above.

7.2.4 The effect of 2S seed albumins on priming and polarisation of naïve T cell responses.

As mentioned previously, DC activation precedes priming of $CD4^+$ T cells, to enable initiation of an adaptive antigen-specific immune response. In vivo, activated DC, presenting portions of antigen at the cell surface (in complex with MHCII) activate naïve T cells specific for the antigen presented. Depending on the signals received from the antigen-presenting DC, at the time of priming, a TH1 or TH2-mediated immune response results. To determine, *in vitro*, the phenotype of the adaptive immune response that would be mounted, following T cell priming with seed albumin-treated DC, the DO.11.10 transgenic OVA-specific TCR system was employed. BMDC derived from BALB/c bone marrow were pre-treated with 2S seed albumin or LPS (as a positive control fro driving TH1-mediated immune responses) before pulsing with OVA and then co-culturing with naïve DO.11.10 $CD4^+$ T cells (specific for OVA) in fresh media. DC treatment with SFA 8, Ber e 1 or LPS did not significantly modulate T cell proliferation in response to OVA presentation, however, LPS-stimulated DC induced slightly elevated proliferation of T cells at reduced OVA concentrations (Figure 7.4a). Interestingly, all co-cultures produced significant levels of IFNγ. Surprisingly, given their apparent immature phenotype, DC pre-exposed to Ber e1 induced significantly more IFNγ than those matured with LPS (Figure 7.4b). DC treated with SFA8 also appeared to induce higher levels of IFNγ than those exposed to LPS but this effect was not statistically significant. Pre-treatment of DC with Ber e 1 induced markedly elevated IL-5 and IL-4 production by co-cultured T cells compared to those co-cultured with LPS- or SFA 8-treated DC (Figure 7.4c and d). IL-10 production was also significantly higher in T cells co-cultured with Ber e 1-treated DC compared to cultures containing LPS or SFA 8 treated DC (Figure 7.4e).

7.2.5 The profile of T cell transcription factors induced by 2S seed albumins

Initiation, development and maintenance of antigen-specific immune responses by T cells is under the direction of key T cell transcription factors. Development of TH1 and TH2-mediated immune responses is induced by expression of T-bet and GATA-3, respectively [499]. Anti-inflammatory antigen-specific T cell-driven immune responses can also be generated and function to regulate the action of TH1 or TH2-type immune responses. These *regulatory* T cells can be distinguished by their expression of transcription factor, Foxp3 [500]. To determine the phenotype of T cell present in the seed albumin-treated

DC-T cell co-cultures, T cell transcription factor profiles were analysed by RT-PCR. Cultures were analysed for TH1 (T-bet), TH2 (GATA-3) and T regulatory (Foxp3) transcription factors. Consistent with the IFNγ production by all cultures, T cells from cultures containing Ber e 1-, LPS- or SFA 8-treated DC expressed elevated levels of T-bet mRNA, with Ber e 1-treated cultures showing the highest levels (Figure 7.4f). Similarly, and consistent with the elevated IL-4 and IL-5 observed, GATA-3 levels were highest in T cells cultured with Ber e1-treated DCs (Figure 7.4g). By contrast, there was no apparent induction of T-regulatory transcription factor, Foxp3 in any of the cultures (Figure 7.4h), indicating that Ber e 1-induced IL-10 detected in these co-cultures was not produced by anti-inflammatory T-regulatory cells.

7.3 Discussion

It has previously been determined that recombinant seed albumins, Ber e 1 and SFA 8 show similar degradation resistance properties to their native counterparts [501]; an important characteristic of albumins. Based on the results presented above, it was possible to extend this analysis of recombinant seed allergens to include proposals for initiation of the allergic response, involving direct action of seed albumins on dendritic cells and subsequent direction of an adaptive immune response.

SFA 8, a sunflower seed component of, as yet, undefined allergenicity induced maturation and TH1-promoting cytokine production by BMDC derived from two mouse strains of different Th-bias. Conversely, Ber e 1, a Brazil nut albumin renowned for its allergenic properties, did not appear to activate DC in this way. Further, Ber e 1-treated DC induced priming and activation of naïve T cells into TH2-type T helper (Th) cells. These Th cells were defined as such because they produced elevated levels of IL-4, IL-5 and IL-10 and expressed increased levels of GATA-3 (a TH2-type transcription factor), compared to T cells primed by SFA 8- or LPS-treated DC.

7.3.1 Recombinant Brazil nut allergen fails to induce a TH1-mediated immune response in DC

These results provide novel information concerning how these two albumins of potentially different allergenicity, have corresponding different actions on cells of the innate and (indirectly) adaptive immune responses. As mentioned in Chapter 1, when B cells receive help from TH2 cells, secretion of IgE is induced. IgE is a key immune mediator in allergy, therefore, allergic immune responses are facilitated by the TH2 arm of the immune system. It is clear from the results of this study that Ber e 1 is capable of inducing a TH2-mediated immune response. Conversely, SFA 8 appears to promote a TH1-mediated immune response. Given that Ber e 1 is certainly more allergenic than SFA 8, it would appear that allergy to Brazil nut may be the result of an inability to induce a TH1 immune response. DC action is key in the development and direction of an adaptive immune response. In this study it appears that DC are induced to produce IL-12 and TNFα by stimulation with SFA 8 which (combined with changes in cell surface expression), leads to a TH1-type adaptive immune response. Conversely, Ber e 1 does not induce DC IL-12 or TNFα production or cell surface expression changes and these DC promote induction of a TH2 adaptive immune response. Consistent with this theory, Reider and colleagues demonstrated that DC isolated from atopic patients exhibited a reduced IL-12 response to CD40 ligation (simulating T cell activation) when compared to DC from non-atopic donors. [502]. Overall, this would suggest that induction of allergy (in terms of DC response) is the consequence of not inducing a TH1-promoting response in DC. The reverse scenario would therefore be that induction of a TH1-promoting response in DC ensures failure to

promote an allergic response. In other words, SFA 8, a protein that does not appear to induce allergy, induces a TH1-promoting immune response in DC. Consistent with this proposal, a study by Smart and colleagues revealed that oral consumption of sunflower seed albumin (after prior sensitisation) promoted induction of an IgG2a (TH1) antibody response in a model of experimental asthma. It also attenuated the induction of a delayed-type hypersensitivity response in this model [503].

7.3.2 Brazil nut allergen-treated DC induce TH2 cell differentiation.

The results of the DC-T cell co-culture revealed that not only does Ber e 1 fail to induce a TH1 immune response in DC, it also promotes development of a TH2-type immune response. This was demonstrated by secretion of TH2 cytokines and expression of GATA-3 (a TH2 transcription factor). Consistent with the opposing action of SFA 8 to Ber e 1 in DC, SFA 8 maintains its TH1-promoting action in this co-culture system. It has previously been demonstrated that different DC phenotypes (induced by different *in vitro* treatments) can direct T cell phenotype in the ensuing adaptive immune response [189]. LPS-treated DC induced differentiation of TH1-type T cells producing high levels of IFNγ and low levels of IL-4. Contrastingly, ES-62-treated DC induced differentiation of T cells of a different phenotype, producing high levels of IL-4 and low levels of IFNγ. The data presented here demonstrate that seed albumins can also induce distinct DC phenotypes that direct resulting T cell phenotypes. It is noteworthy that all groups of T cells in this study produced high levels of IFNγ and expressed T-bet, suggesting IFNγ or T-bet do not absolutely determine T cell phenotype in this system. It may be production of TH2 cytokine, e.g. IL-4 or IL-5 or more likely, the ratio of IL-4 to IFNγ production that dictates the observed immune response phenotype. Further investigation is required to dissect this theory. However, support for these findings was provided by a recent study that demonstrated differences between allergen-treated DC from atopic and non-atopic donors and their effect on autologous naïve and memory T cells. Here, T cells from atopic donors produced increased TH2 cytokines (IL-4, IL-5 and IL-10) in response to co-culture with allergen-pulsed DC than T cells from non-atopic donors. In accordance with the findings presented in this chapter, T cell IFNγ production and proliferation rates were not significantly different between groups, however it was suggested that differences were not due to DC IL-12 production [504].

7.3.3 Strain-specific differences in DC surface expression response

SFA 8 induced upregulation of cell surface co-stimulatory molecule expression on DC from BALB/c mice more than on DC from C57BL/6 mice. SFA 8 also conferred a TH1-promoting phenotype on such DC, demonstrated by TH1-promoting cytokine production. As this TH1-inducing action of SFA 8 was observed to a lesser degree in TH-1 prone C57 BL/6 DC, this may indicate that less action was required by SFA 8 to obtain a TH1

outcome in this strain. Using this postulation, the reverse is true for BALB/c DC. BALB/c mice are TH2-prone, therefore SFA 8 'needed' to induce a more marked response in DC from BALB/c mice to ensure a TH1 phenotype. Consistent with this proposal, in a recent study, using a murine model of TH2-mediated allergic airway inflammation, BALB/c mice demonstrated significantly more perivascular pulmonary inflammation than C57 BL/6 mice, indicating that the former were more prone to development of TH2 mediated inflammation than the latter [505].

7.3.4 Activation of DC TH1-promoting cytokine production by SFA 8 was independent of MyD88

DC production of IL-12 and TNFα in response to SFA 8 occurred independently of the important Toll signalling adapter protein, MyD88. The majority, but not all, Toll-like receptors (TLRs) signal via MyD88 [498]. Therefore, this result suggests SFA 8 was either recognised by a TLR that does not use MyD88 for downstream signalling or it was detected by an entirely different pattern-recognition signalling system within the DC.

It is well understood that, *in vivo*, allergens are recognised by their specific IgE antibody, bound to FcεRI on the surface of mast cells in allergic individuals. The results of this study provide interesting data on the interaction between allergens and DC, another cell type of the 'first line of defence' that would be encountered by allergen upon entering the body. It has been recently demonstrated that DC from the peripheral blood of atopic patients express FcεRI on their surface [506]. This observation is consistent with a role for DC in mediation of the allergic immune response, following allergic sensitisation. Therefore the interaction between allergens and DC demonstrated in this study may be a viable allergy-initiating process in susceptible individuals *in vivo*.

7.3.5 Production of IFNγ and expression of T-bet does not necessarily determine TH cell phenotype.

As described above, pre-treatment of DC with SFA 8, LPS or Ber e 1 resulted in similar production of IFNγ and expression of T-bet by DC-primed T cells. Therefore, induction of distinct immune response phenotypes by SFA 8 and Ber e 1 appears independent of IFNγ and T-bet. However, in addition to IFNγ production, Ber e 1 treatment of DC induced production of IL-4 and IL-5 and expression of GATA-3 in cognate T cells. This was not observed in T cells primed by SFA 8- or LPS-treated DC and it therefore appears that in this system a TH1-type immune response is the default and TH2 signals need to be induced additionally, to promote a TH2 immune response. Nevertheless, instead of an 'either/or' TH-phenotype, there appears to be a sliding scale of TH1 to TH2-like responses depending on conditions. Thus, the ratio of TH2:TH1 signals, such as transcription factor expression and cytokine secretion etc, (instead of absolute production) may underlie the

nature of the allergic immune response in this system. Consistent with this, in a recent study, atopic patients diagnosed with allergic rhinitis were treated with a TH1-promoting anti-tumour agent. This treatment reduced nasal inflammation by reducing allergy-promoting TH2 cytokines, IL-4 and IL-5, and by increasing production of IL-12, but not IFNγ[507]. This study therefore provides support for development of a TH2-mediated immune response regardless of IFNγ levels. Furthermore, it has been demonstrated that amelioration of TH2-mediated allergic airway inflammation can be induced by treatment with bacterial lipopeptide, due to promotion of a TH1-mediated immune response that was dependent upon IL-12 production [508]. Therefore, it is likely that IL-12 secreted by DC in response to culture with SFA 8 (and more potently, in response to LPS) promoted development of the T cell-mediated immune response that was biased toward a TH1 phenotype. On the other hand, Ber e 1 did not induce IL-12 and, therefore, the result was development of a more TH2-like immune response.

In this investigation, development of TH1 cells, secreting IFNγ and expressing T-bet, appeared to be default and independent of the nature of the DC-stimulating factor. The action of Ber e1 on DC was unusual because it additionally induced development of TH2 cells, secreting IL-4 and IL-5 and expressing GATA-3. Based on the findings of this investigation it might be postulated that, due to its structural homology to SFA 8, rather than passively *failing* to induce DC cytokine production and upregulation of cell surface expression, Ber e 1 may have actively *prevented* these events that occur as part of the default maturation process of TH1-promoting DC. This alternate action of Ber e 1 resulted in development of TH2-promoting DC that would induce development of a TH2-mediated immune response *in vivo*.

7.3.6 Lack of allergenic action of SFA 8 contradicts reported incidence of sunflower seed allergy

Based on the data presented in this investigation, recombinant SFA 8 does not appear to induce allergy-promoting TH2-mediated immune responses. However, as mentioned previously, sunflower seed albumins have been demonstrated as IgE binding proteins in a small set of susceptible individuals, which would support an allergenic role for this seed protein. Thorough explanation of the difference between these apparently contradictory findings is not possible based on the results of this study. Epidemiologically, reported cases of sunflower seed allergy are relatively rare compared with e.g. peanut allergy cases, suggesting that these individuals exhibit an atypical response to sunflower seed albumin. Furthermore, the majority of cases of allergy to sunflower seed proteins are reported to be via inhalation instead of ingestion by, for example, caged bird owners, who experience abnormally high exposure to this protein [491, 492]. It is possible, however, that this allergy may be the result of a inappropriate immune response to sunflower seed

in patients with a dysfunctional TH1 immune system. However, this hypothesis cannot be tested based on the results of this small study. In addition, the protein investigated here may be different to the proteins investigated in previous studies inferring allergenic properties of sunflower seed albumins. Further study is required to explain the apparent discrepancy.

7.3.7 Conclusion

As mentioned above, the findings presented here represent the overall results of a relatively small study. Due to time restraints this study could not be continued further. However, if made possible, future studies should concentrate on investigation of the molecular mechanisms of DC interaction with SFA 8 or Ber e 1, including the mechanism(s) by which DC recognise these proteins; and identification of T cell subsets (by analysis of T cell surface expression) induced by allergen-treated DC.

The actions of these recombinant albumins, observed in this study, provide interesting theories about how the native seed albumins could interact with the immune system to initiate (or not) allergy (illustrated in Figure 7.5). Further investigation of their properties and actions will help to reveal an understanding of the processes leading to allergic sensitisation and hopefully, methods by which it can be prevented.

Figure 7.1 Recombinant sunflower seed 2S albumin, SFA 8, induces production of TH1 cytokines by bone marrow-derived dendritic cells

Dendritic cells cultured from bone marrow of C57BL/6 (a-d), MyD88 knock-out (e, f), BALB/c (g, h) mice were treated with recombinant Ber e 1 or SFA 8 for 24h. SFA 8 and Ber e 1 were added to culture wells at 50 μg/ml unless otherwise stated. In c and d, cells were further stimulated for 24h with 1μg/ml LPS (*E. coli*) after seed albumin treatment. Supernatants were harvested and cytokines measured by ELISA. Results are expressed as mean±SD n=3 and are representative of at least 4 independent experiments. **p<0.01, ***p<0.001 vs untreated DC.

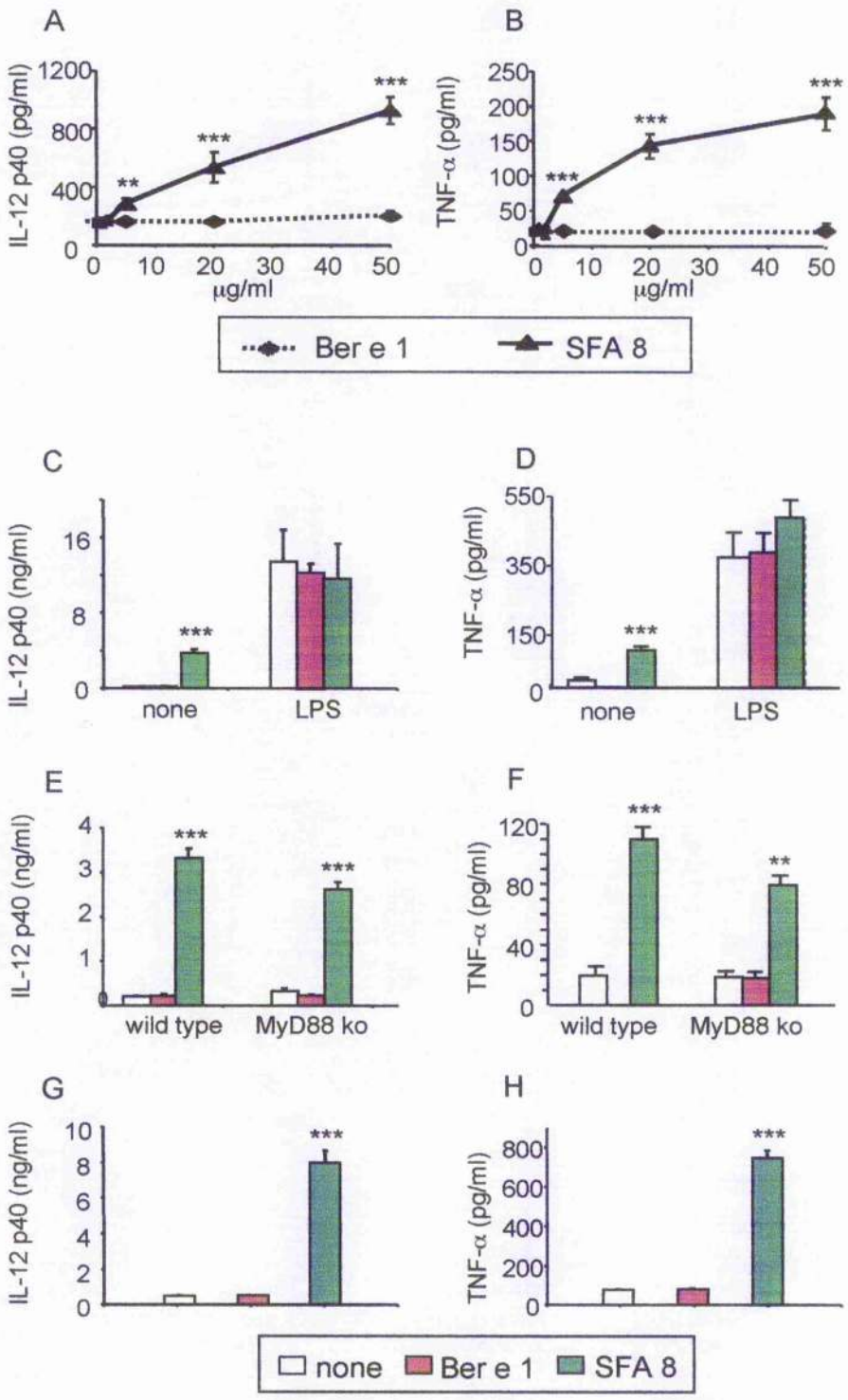

A
B
IL-12 p40 (pg/ml)
TNF-α (pg/ml)
μg/ml
Ber e 1
SFA 8
C
D
IL-12 p40 (ng/ml)
TNF-α (pg/ml)
none
LPS
E
F
wild type
MyD88 ko
G
H
none
Ber e 1
SFA 8

Figure 7.2 Recombinant sunflower seed 2S albumin, SFA 8, induces moderate upregulation of cell surface expression on C57BL/6 bone marrow-derived dendritic cells

Dendritic cells cultured from bone marrow of C57BL/6 mice were treated with Ber e 1(50 μg/ml), SFA 8 (50 μg/ml) or LPS (1 μg/ml; *E. coli*) for 24h. After the culture period, DC were stained for CD11c and MHC class II, CD40, CD54, CD80 or CD86 and analysed by flow cytometry. Histograms represent expression levels of $CD11c^+$ cells. Results are representative of at least 2 independent experiments.

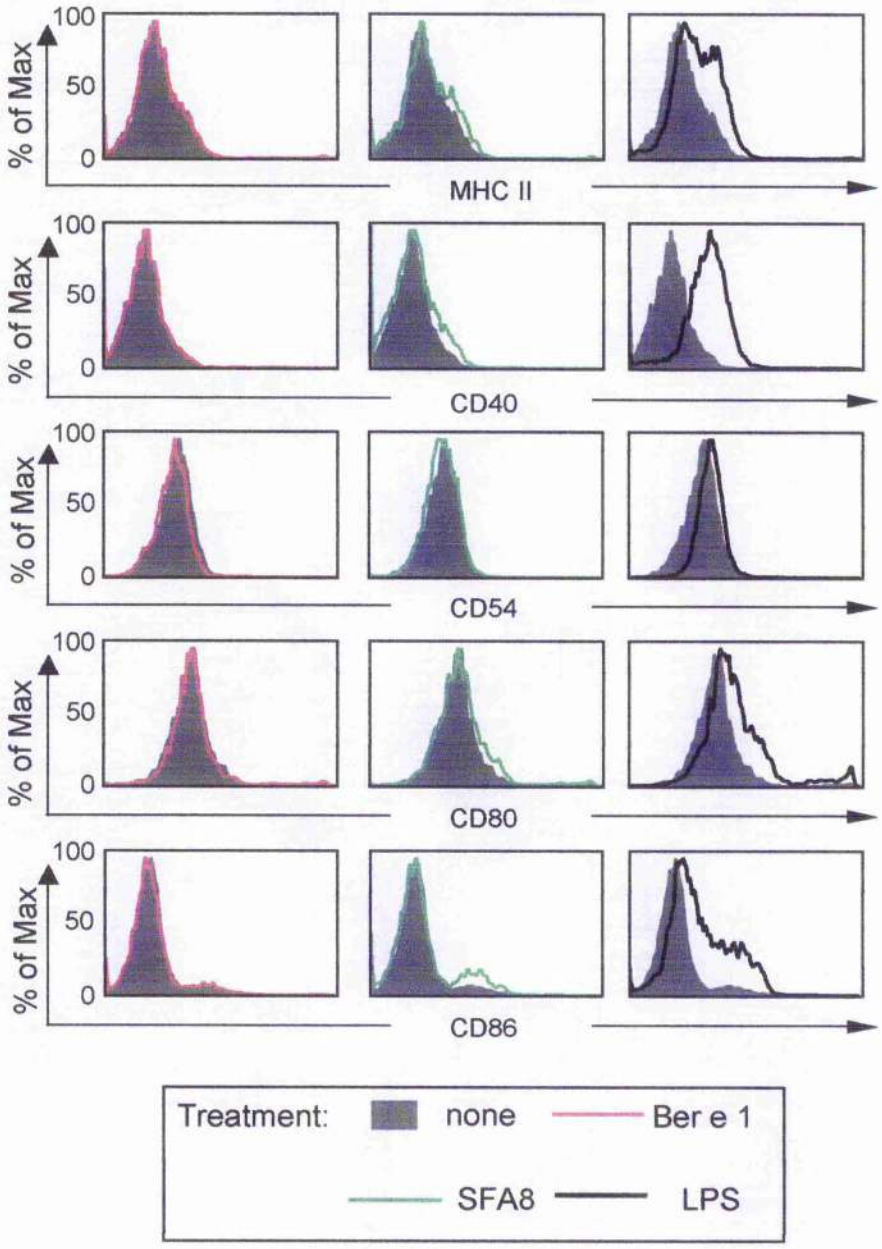

100
50
0
% of Max
MHC II
CD40
CD54
CD80
CD86
Treatment:
none
Ber e 1
SFA8
LPS

Figure 7.3 Recombinant sunflower seed 2S albumin, SFA 8, induces upregulation of cell surface expression on BALB/c bone marrow-derived dendritic cells

Dendritic cells cultured from bone marrow of BALB/c mice were pre-treated with Ber e 1(50 μg/ml) or SFA 8 (50 μg/ml) for 24h, followed by stimulation with media (A) or LPS (1 μg/ml; *E. coli*) (B) for 24h. After the culture period, DC were stained for CD11c and MHC class II, CD40, CD54, CD80 or CD86 and analysed by flow cytometry. Histograms represent expression levels of $CD11c^+$ cells. Results are representative of at least 2 independent experiments.

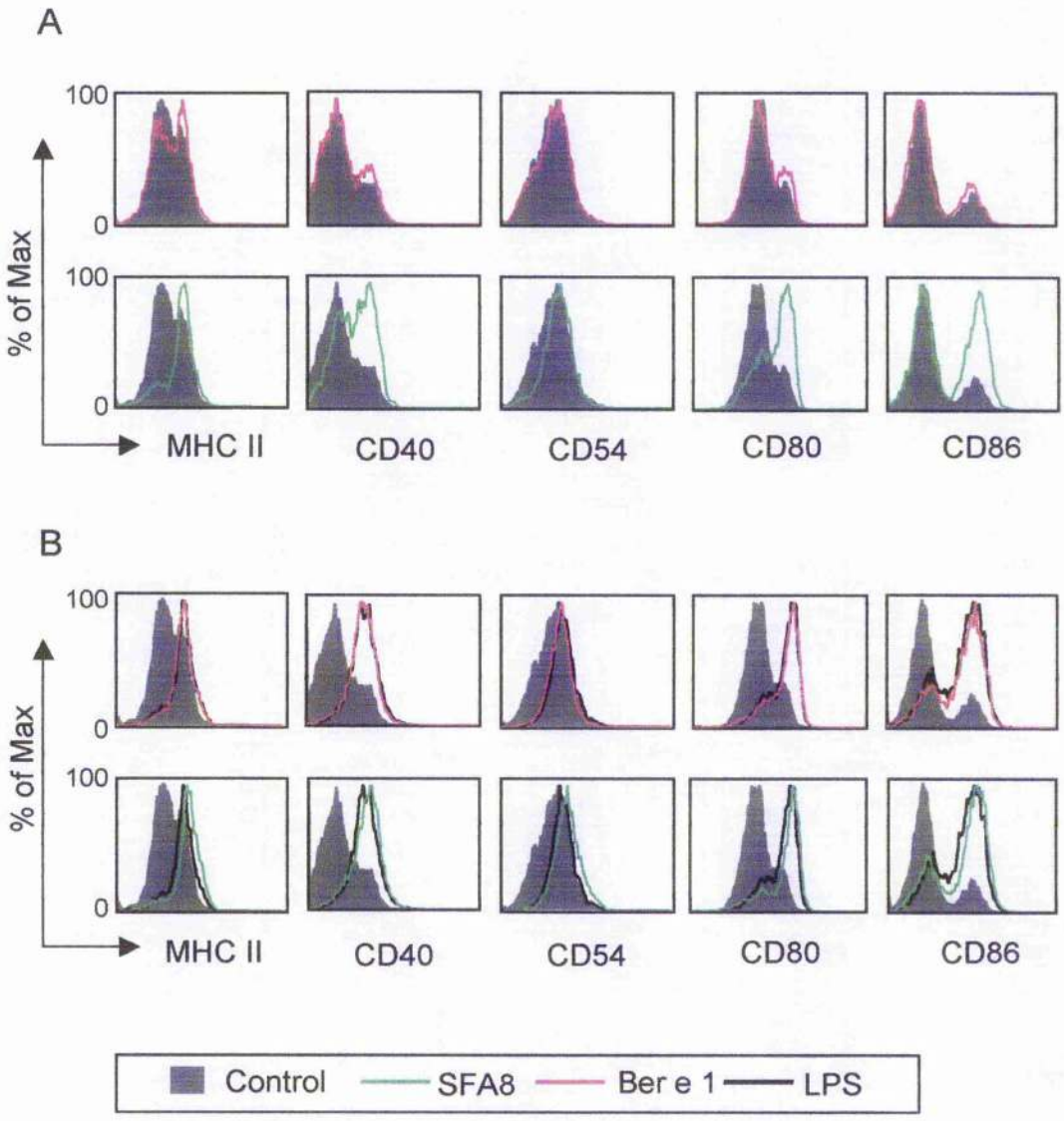
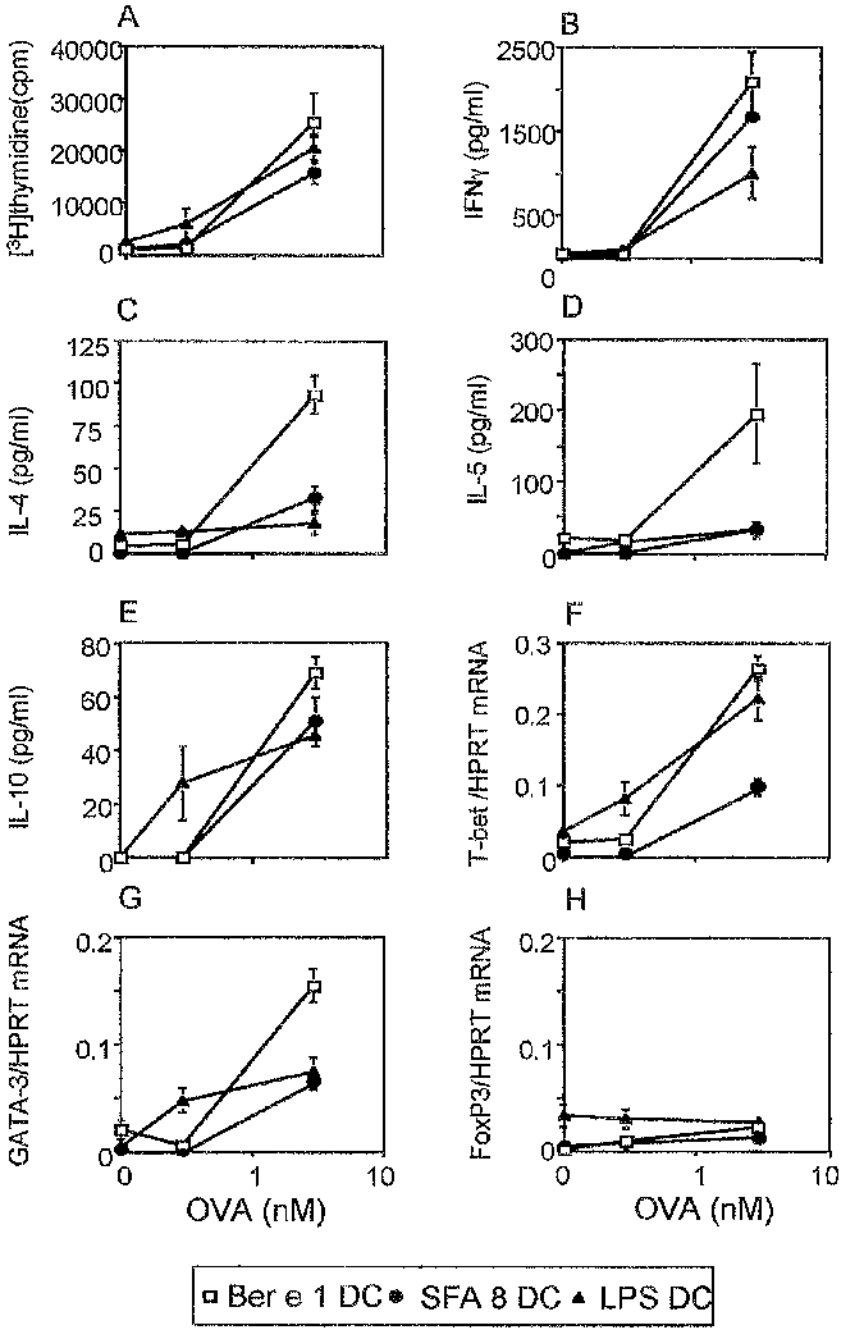

A
100
0
100
0
% of Max
MHC II
CD40
CD54
CD80
CD86
B
100
0
100
0
% of Max
MHC II
CD40
CD54
CD80
CD86
Control
SFA8
Ber e 1
LPS

Figure 7.4 Co-culture of recombinant 2S seed albumin-treated bone marrow-derived dendritic cells and naïve OVA-specific $CD4^+$ T cells

Dendritic cells cultured from bone marrow of BALB/c mice were pre-treated with Ber e 1(50 μg/ml), SFA 8 (50 μg/ml) or LPS (1 μg/ml; *E. coli*) for 24h before co-culturing with naïve DO.11.10 $CD4^+$ T cells. (1 DC:10 T cells) and OVA (0.3 or 3 nM) for 72h. Proliferation (a) was determined by measuring [^{3}H]-thymidine incorporation during the last 8 hours of culture. Culture supernatants were analysed for IFNγ (b), IL-4 (c), IL-5 (d) and IL-10 (e) by ELISA. Cells were analysed for T-bet (f), GATA-3 (g) and Foxp3 (h) transcription factor mRNA levels by RT-PCR (levels expressed relative to HPRT levels). In b, LPS $p<0.02$ vs Ber e 1. In c, LPS $p<0.001$ vs Ber e 1, SFA 8 $p<0.002$ vs Ber e 1. In d, Ber e 1 $p<0.02$ vs SFA 8 and LPS. In e, LPS $p<0.01$ vs Ber e 1. Data are representative of 3 independent experiments and expressed as mean ± SD n=3.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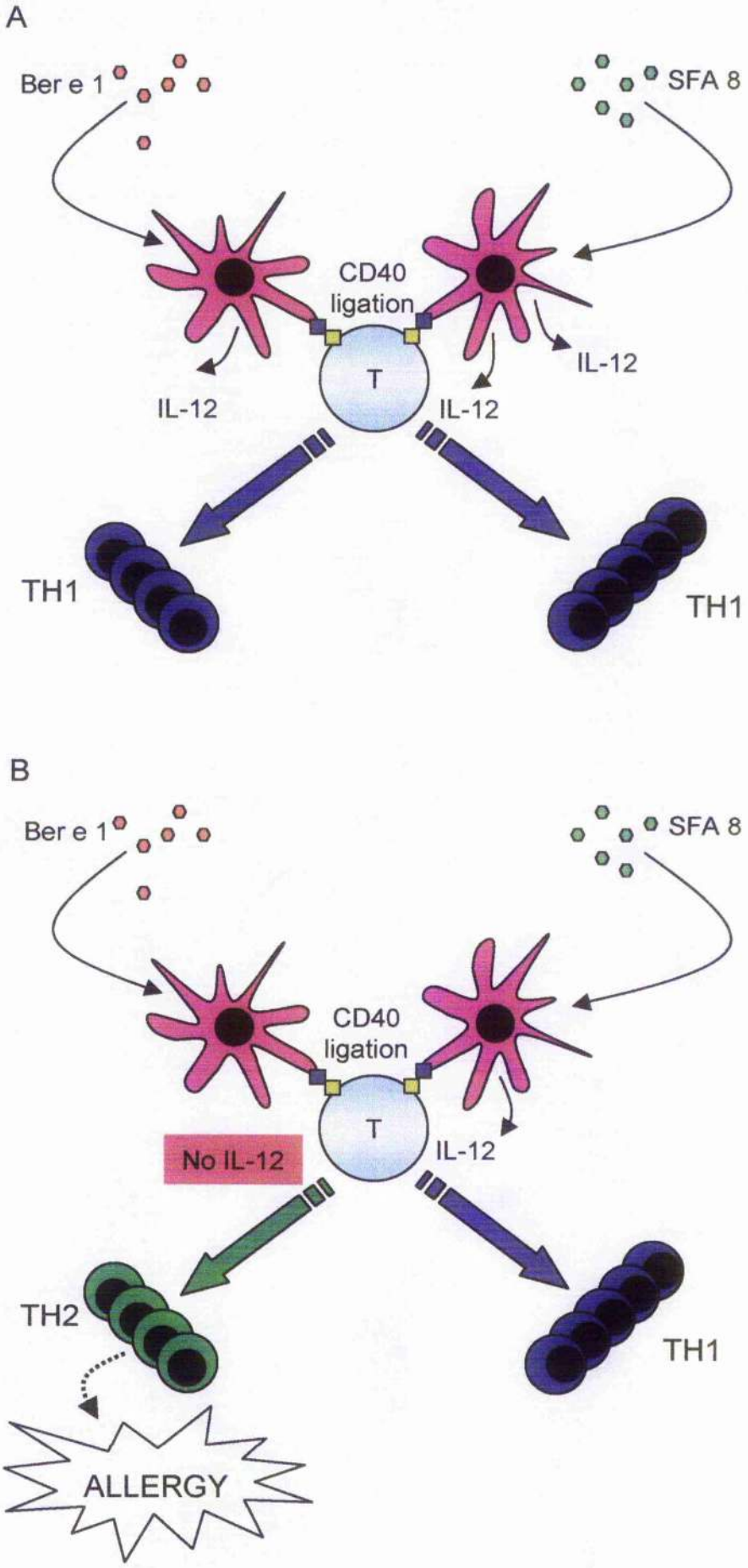
[3H]thymidine(cpm)
40000
30000
20000
10000
0
B
IFNγ (pg/ml)
2500
1500
500
0
C
IL-4 (pg/ml)
125
100
75
50
25
0
D
IL-5 (pg/ml)
300
200
100
0
E
IL-10 (pg/ml)
80
60
40
20
0
F
T-bet /HPRT mRNA
0.3
0.2
0.1
0
G
GATA-3/HPRT mRNA
0.2
0.1
0
H
FoxP3/HPRT mRNA
0.2
0.1
0
0
1
10
OVA (nM)
0
1
10
OVA (nM)
Ber e 1 DC
SFA 8 DC
LPS DC

Figure 7.5 Model of response of atopic and non-atopic individuals to 2S seed albumin exposure

Data presented in this chapter has demonstrated that SFA8, but not Ber e 1, induces IL-12 production from bone marrow-derived DC. Moreover, a recent report demonstrates that PBMC-derived DC from atopic patients produce less IL-12 in response to CD40 ligation than those from non-atopic donors [502]. This recent finding is therefore likely to further impact on the potential functional outcome of T cells primed with DC exposed to *either* SFA8 *or* Ber e 1. Thus, the IL-12 shown to be associated with Ber e1 in the non-atopic panel (A) refers to that potentially produced by Ber e1 in conjunction with T cell-mediated CD40-signalling in non-atopic patients. Such IL-12 production is not likely to be observed in atopic patients (B), a prediction consistent with the data presented here, showing that Ber e 1 does not upregulate CD40 on DC. By contrast, SFA8, which has been shown to be capable of inducing IL-12, in the absence of CD40 signalling, could therefore induce IL-12 even in atopic patients whilst in non-atopic patients, could induce enhanced levels of IL-12 in conjunction with CD40 signalling. Thus, in conclusion, as SFA8 can induce IL-12 by DC, it biases the immune response of both non-atopic and atopic individuals away from a TH2 allergic phenotype. By contrast, as atopic individuals produce little CD40-induced IL-12 relative to that generated by non-atopics, they would be rendered more likely to develop a TH2-biased allergic response to Ber e 1.


A
Ber e 1
SFA 8
CD40
ligation
T
IL-12
IL-12
IL-12
TH1
TH1
B
Ber e 1
SFA 8
CD40
ligation
T
No IL-12
IL-12
TH2
TH1
ALLERGY

8 General Discussion

It has been observed that the prevalence of asthma, allergic diseases and autoimmunity is reduced in parasite-infected individuals [110, 509]. Indeed, parasite infection has been proposed as protective against development of allergy and autoimmunity [118, 140]. It is clear that parasites have evolved to mount self-protective mechanisms to ensure longevity of infection in the host, which can involve secretion of immunomodulatory substances. This action, whilst beneficial to the parasite, can also benefit the host by protecting them not only from development of pathology, but also from development of allergy or autoimmune disease

ES-62 is an ES product generated by rodent filarial nematode, *A. viteae,* and has previously been demonstrated to exhibit immunomodulatory properties when examined *in vivo* and *in vitro* on specific cells of the immune system [190, 191, 193, 281]. The results described in this thesis have extended the current understanding of ES-62 action to include anti-inflammatory actions in models of inflammatory disease. Moreover, evidence has been provided to establish that ES-62 treatment of two distinct models of autoimmunity and a model of allergic airway inflammation induces amelioration of inflammatory symptoms. In more detail, ES-62 inhibited TH1-mediated inflammation in collagen-induced arthritis, TH2-mediated inflammation in ovalbumin-induced asthma and development of heterogeneous lupus-like inflammation in MRL/lpr mice. These combined findings highlight novel anti-inflammatory properties of ES-62 that are mediated unreservedly, independent of inflammation source, phenotype or manifestation.

8.1 How does ES-62 manipulate the immune system in inflammatory disease?

Clearly, ES-62 modulates the normal action of several different mechanisms necessary for mediation of the inflammatory immune response in the three models described above. For example, an interesting pattern was observed in ES-62-mediated modulation of serum antigen-specific antibody responses during analysis of the CIA and asthma models. Moreover, it appeared that ES-62 mediated modulation of the serum antibody response to antigen sensitisation in both models was most likely not necessarily directly related to ES-62-mediated reduction of inflammation. This was evidenced by inhibition of antigen-specific IgG2a in the CIA model and antigen-specific IgE in the asthma model, induced when ES-62 was administered prophylactically, but not when it was administered therapeutically, despite maintenance of the anti-inflammatory effect of ES-62 in both treatment protocols. Furthermore, ES-62 mediated inhibition of collagen-specific IgG2a in the prophylactic treatment CIA model appeared to be PC-independent and therefore, did not correlate with the anti-inflammatory effect of ES-62 in this model. This was confirmed by maintenance of the inhibitory effect on collagen-specific IgG2a when mice were treated

with PC-deficient rES-62, despite loss of anti-inflammatory action, leading to the suggestion that ES-62-mediated inhibition of collagen-specific IgG2a was mediated by a non-PC component of ES-62. Therefore, considering that modulation of the serum antibody response does not appear to correlate directly with modulation of inflammation, it might be predicted that if an antigen-specific antibody response could be measured in the MRL/lpr SLE model, it would not be directly related to the anti-inflammatory effects of ES-62 in this model either.

Nevertheless the difference in the effects of prophylactic and therapeutic ES-62 treatment on the serum antibody responses in the CIA and asthma models did reveal differences in the action of ES-62 in each treatment protocol. For example, when administered prophylactically, ES-62 most likely inhibits immune response initiation mechanisms (reflected by antibody production) and when administered therapeutically, ES-62 disrupts inflammatory effector mechanisms (which do not influence the antibody response). Therefore, the serum antibody profile reflects the nature of the immune response generated but not necessarily the inflammatory status of the model. In summary, it appears that ES-62 has probably two major modes of action on the models of inflammation investigated in this thesis, namely modulation of immune response initiation mechanisms and inhibition of inflammation effector mechanisms

8.1.1 ES-62 mediated disruption of immune response initiation

It has been clearly established that DC are integral in development of an antigen-specific immune response. As it is currently understood, initiation of an immune response normally involves recognition of antigen (in the presence of a 'danger signal') to induce DC activation, migration of activated antigen-presenting DC to the secondary lymphoid organs and priming of naïve T cells to initiate an antigen-specific adaptive immune response [3]. It has previously been suggested that DC comprise a primary target for ES-62-mediated modulation of the immune response [467] and as demonstrated by this laboratory, ES-62 can modulate DC phenotype and function at several stages of this immune response initiation process. Indeed, ES-62 treatment *in vivo* (delivered via implanted osmotic pumps) modulates the phenotype and function of DC that are subsequently differentiated from bone marrow progenitor cells [191]. Furthermore, ES-62 treatment of pre-differentiated bone marrow-derived DC *in vitro* results in modulation of the normal cytokine production and cell surface expression response of these cells to stimulation with 'danger signals' such as bacterial LPS [191], indicating that ES-62 treatment of immature DC in peripheral tissues *in vivo* would disrupt their activation and hence, their ability to activate naïve T cells.

Therefore, the results presented in this thesis have consolidated these previous findings by providing evidence to suggest that such modulatory effects of ES-62 on immune response initiation mechanisms are also mediated in models of inflammatory disease. For example, in the CIA model (Chapter 3), the action of ES-62 on bone marrow-derived DC phenotype, whilst modest, was apparent, the effects being enhanced by increasing the number of doses of ES-62 *in vivo* and reducing the time between ES-62 application and the beginning of bone marrow cell culture *ex vivo*. This was exemplified by the more potent effects being observed on DC derived from the therapeutic relative to the prophylactic model. Therefore it appears that the modulatory effect of ES-62 is positively associated with the quantity of treatment and negatively associated with the time period between the application of ES-62 *in vivo* and the beginning of DC culture period. Interestingly, DC have been implicated in treatment of autoimmunity, therefore modulation of DC phenotype or function represents an effective mechanism for disruption of immune response initiation.

Nevertheless, DC activation is only the first stage of antigen-specific immune response development. Following activation, DC migrate to lymph nodes in order to trigger activation of antigen-specific T cells, which normally leads to clonal expansion of antigen-specific T cells, heralding the beginning of the adaptive immune response. As a result of communication between activated antigen-specific T cells and B cells in the lymph node follicles, B cell antigen-specific antibody production is switched to an appropriate isotype and initiation of the adaptive immune response is complete. Indeed, as mentioned above, prophylactic ES-62 treatment of the CIA and asthma models inhibited development of the appropriate antigen-specific antibody response, indicating that ES-62 action must disrupt the mechanisms resulting in antigen-specific antibody production by B cells. Indeed, it has recently been identified (using an adoptive transfer system) that T cells from ES-62-treated mice exhibit reduced clonal expansion and antigen-specific pro-inflammatory cytokine production [467]. Furthermore, it has been demonstrated that such T cells exhibit reduced migration into B cell follicles, a process necessary for induction of the antigen-specific antibody response. Consistent with this, antigen-specific IgG2a responses were inhibited in this TH1-biased model, therefore, it might be postulated that this mechanism is employed by ES-62 in the CIA and asthma models for inhibiting production of antigen-specific IgG2a and IgE respectively. In summary, by combining the published results from this laboratory and the findings presented in this thesis it has been demonstrated that the action of ES-62 influences the function of different cell types, at several stages during initiation of an immune response, which contributes to development of an inhibited inflammatory immune response in models of inflammatory disease (Figure 8.1).

8.1.2 ES-62 mediated disruption of immune response effector mechanisms

At the inflammatory site, a number of different cell types work in unison, secreting inflammatory factors (cytokines and chemokines) to recruit and stimulate survival of other cells and enhance or amplify the inflammatory environment. Usually the cellular infiltrate is representative of the phenotype of the inflammatory response. Moreover, TH1-type responses are largely mediated by phagocytes (macrophages and neutrophils) and cytotoxic T cells, whereas mast cells and eosinophils are most commonly associated with TH2-type/allergic immune responses.

Cytokine production by antigen-specific T cells in response to antigen challenge can be considered an primary step in the effector stage of an immune response. In response to antigen-specific TH1 or TH2 cytokine production by activated TH cells, inflammation is triggered in TH1- and TH2-mediated immune responses respectively. Thus, ES-62 mediated inhibition of antigen-specific cytokine production by TH cells from the CIA and asthma models can be considered contributory to its anti-inflammatory action in both these models. However, it is important to note that this is unlikely to be a direct effect of ES-62 treatment on T cells, as the direct immunomodulatory effects of ES-62 on T cells are minimal [167]. It is more likely that ES-62 modulates DC phenotype, which becomes translated, *in vivo*, into augmented lymphocyte and effector cell responses. This DC-facilitated anti-inflammatory action has previously been identified in a model of arthritis, where IL-10-treated DC administered peri-articularly ameliorated inflammation [510].

In both, TH1-mediated RA and TH2-mediated asthma, macrophages located in the inflammatory site act to maintain and promote inflammation. In particular, it has been proposed that contact-dependent communication between activated T cells and adjacent macrophages in the joint is necessary for progression of synovial inflammation in RA [245]. Indeed, by employing an *in vitro* system it was demonstrated that ES-62 treatment *in vitro* or *in vivo* can disrupt contact-dependent communication between T cells and macrophages, thus it was proposed that this might be an important method employed by ES-62 for inhibition of the effector mechanisms of inflammation in the CIA model. Similarly, macrophages are the major cell type present in the inflammatory infiltrate of the asthmatic lungs [363] and hence, represent an important effector cell for mediation of TH2-type inflammation in asthma. ES-62 induced differentiation of macrophages from asthma model mice with reduced activation, evidenced by reduced overall cytokine production rates. Therefore, in TH1-mediated and TH2-mediated inflammation, it is likely that macrophages are targeted by ES-62 for inhibition of existing inflammation.

Associated with TH2-mediated inflammatory immune responses, eosinophils are a major component of the inflammatory cell infiltrate and therapeutic ES-62 treatment was

demonstrated to inhibit recruitment of this cell type in the asthma model. As recruitment of eosinophils can be induced by production of TH2-type cytokine, IL-5, it is likely that ES-62-mediated inhibition of antigen-specific TH2 cell cytokine production may have been responsible for this effect on the pulmonary cellular infiltrate. In addition, another important effector cell of the inflammatory response in TH2-mediated immunity is the mast cell. Recently it has been demonstrated *in vitro* that mast cell degranulation in response to cross-linking of FcεRI, an important effector step in mediation of the inflammatory response in TH2-mediated inflammation, was inhibited by direct ES-62 treatment (A Melendez, M Harnett and W Harnett, unpublished observations). Thus, similar to its action in TH1-mediated inflammation, ES-62 appears to target distinct, appropriate cell types for mediation of anti-inflammatory action in the effector stage of TH2-mediated inflammation. Figure 8.2 depicts the proposed pattern of ES-62 mediated action in effector mechanisms of inflammation.

In summary, it appears that ES-62 acts to inhibit development or progression of inflammation, independent of source, cell type or phenotype. In particular it is interesting that the modulatory effect of ES-62 on DC appears to be dependent on the extent of application as observed in the CIA model. Furthermore, the anti-inflammatory action of therapeutic ES-62 treatment on asthma model mice was dose-dependent. These findings suggest that infection with *A. viteae* (resulting in consistent exposure to ES-62) would mediate more potent effects on the cells responsible for mediation of the inflammatory immune response. Indeed, there are several homologues of ES-62 secreted by human filarial nematodes [161], thus it might be suggested that infection with filarial nematodes may protect these infected individuals from development of aberrant inflammatory immune responses (i.e. autoimmunity or allergy), which might be viewed as a beneficial side-effect of nematode infection, certainly by sufferers of autoimmunity or allergic asthma in the Western world.

8.2 How are the immunomodulatory effects of ES-62 mediated?

The molecular mechanism(s) of the interaction between immune cells and ES-62 (resulting in modulation of their normal cellular responses) is currently under investigation in this laboratory. As part of their innate immune functions, DC and macrophages express Toll-like receptors (TLRs) on their extracellular surface. As mentioned previously, TLRs are a family of pattern recognition receptors that recognise specific pathogen-associated molecular patterns (PAMPs) and ligation of TLRs results in activation of innate immune cells [511]. For example, TLR2 and TLR4 recognise Gram-positive and Gram-negative bacteria, respectively [511]. In response to ligation of TLRs, downstream intracellular signalling pathways are activated which induce secretion of pro-inflammatory cytokines, to recruit other innate immune cells and upregulation of cell surface molecules, required by

DC for activation of T cells. Recently, it has been identified that ES-62 requires TLR4 and a downstream adaptor protein/signal transducer of several TLR signalling cascades, MyD88, for mediation of its immunomodulatory effects on DC and macrophage cytokine production [374]. However interestingly, ES-62 appears to interact with TLR4 on DC and macrophages utilising a novel mechanism, evidenced by maintenance of its immunomodulatory action in C3H/HeJ mice, which do not express a functional TLR4. It has been suggested that ES-62 preferentially targets cells of the innate immune response for mediation of its anti-inflammatory action and, indeed, this suggestion has not been disputed by the results presented in this thesis. Therefore, via TLR4 (and MyD88), ES-62 may modulate the function of innate immune cells thereby indirectly augmenting the ensuing adaptive immune response in inflammatory diseases. For example, the anti-inflammatory effects of ES-62 observed in Chapter 3 may be initiated by interaction of ES-62 and TLR4 expressed by macrophages and DC, a theory that remains to be investigated.

TLR4 is also expressed on innate immune cells associated with TH2-type inflammation, such as mast cells [512]. As mentioned above, it has been demonstrated that ES-62 treatment of mast cells inhibits their de-granulation in response to cross-linking of the IgE receptor (FcεRI). Thus, it might be proposed that TLR4 also acts as a receptor for ES-62-mediated anti-inflammatory action in this cell type. In particular, the anti-eosinophilic action of ES-62 in the OVA-induced airway inflammation model, described in Chapter 4, was the predominant anti-inflammatory action of ES-62 in this model. However, the expression of TLR4 on eosinophils is currently a topic of debate [513-515]. Nevertheless, it has recently been identified that LPS-induced enhancement of eosinophilia in the OVA-induced airway inflammation model is dependent on TLR4 expression by mast cells [516]. Thus in response to ligation of TLR4 (by LPS), mast cells release TH2-promoting soluble mediators, including TH2 cytokines and eotaxin 2, which act to recruit eosinophils. Therefore, it could be hypothesised that, via TLR4 expressed on mast cells, ES-62 acts (in an alternative method to LPS) to stimulate negative signalling, resulting in reduced mast cell de-granulation (in response to cross-linking of FcεRI) and hence, inhibited release of eosinophil-recruiting factors, ultimately reducing eosinophilia in this model. This hypothesis represents an entirely new investigation that could be conducted as part of the future directions of this project.

8.3 Future Work

The therapeutic actions of ES-62 (after the onset of established inflammation) in the TH1-mediated CIA model and TH2-mediated asthma model have been demonstrated in Chapters 3 and 4. Thus, by direct action of ES-62 on inflammatory effector mechanisms, inhibition of established inflammation could be enabled. Thus, it would be interesting to

examine the effect of ES-62 treatment of MRL/lpr mice after establishment of lupus-like immunopathology. It might be proposed that T and B cell responses, triggered during initiation of the inflammatory immune response in MRL/lpr mice, would be minimally modulated by therapeutic ES-62 treatment. Moreover, ES-62 cannot 'reverse' established immune pathways when administered after the onset of inflammation, but prevents amplification of inflammatory signals and maintenance of inflammation by direct action on effector cells.

Ultimately, based on the results presented in this thesis, it may be prudent to suggest that a therapy for inflammatory conditions might be designed, using ES products, such as ES-62, as a template. Indeed, the PC component of ES-62 has been demonstrated to mimic several of the anti-inflammatory effects of ES-62, in the CIA model, therefore an interesting first step in this process would be to additionally determine the PC-dependency of the anti-inflammatory effects of ES-62 in the asthma and SLE models. As discussed in Chapter 6, PC was deemed responsible for immunomodulatory action, similar to ES-62, induced by mimetics of another helminth product, GSL. However, designing a anti-inflammatory drug based on the structure of PC alone would be ill-advised as PC has also been shown to have immunogenic properties [177], depending on how it is presented to cells of the immune system. Furthermore, immunomodulatory effects of native ES-62 and GSL that were PC-independent were uncovered, indicating that a distinct combination/structure of carbohydrate, protein and PC are required to mimic the anti-inflammatory and non-immunogenic effects of native parasite products. As yet, it has not been possible to precisely mimic the biosynthesis of parasite-derived products synthetically [199], however small molecule derivatives of ES products, such as ES-62 and GSL, are currently under investigation in this laboratory with a view to designing anti-inflammatory therapy for inflammatory disease.

8.4 Conclusion

ES-62-mediated anti-inflammatory action, in the OVA-induced airway inflammation model (Chapter 4), has been mirrored by treating a model of allergic airway inflammation with extracts of filarial nematode parasite, *Ascaris suum* [375], or by infection of such model mice with gastrointestinal nematode, *Heligmosomoides polygyrus* [139]. In addition, it has previously been demonstrated that infection with malarial parasite, *Plasmodium chabaudi*, ameliorates inflammation in the NZB/W murine model of SLE [517], an effect induced by prophylactic treatment with ES-62 (Chapter 5). Similar to the action of ES-62 observed in the murine CIA model (Chapter 3), infection with protozoan parasite, *Trypanosoma brucei brucei*, inhibits development of inflammation in a rat model of CIA [282]. Therefore, parasite-mediated inhibition of inflammation in the host is not a novel theory. However, ES-62 represents the first example of a defined secreted parasite product that exhibits

anti-inflammatory action across a panel of inflammatory diseases of different immunological phenotype. Indeed, as mentioned previously, ES-62 has several homologues secreted by filarial nematodes that infect humans [161, 518]. Thus, the combined findings presented in this thesis may be extrapolated to provide an explanation for the observed immunosuppression and reduced prevalence of inflammatory disease in countries endemic for filarial nematode infection. The evidence put forward here does not directly support the hygiene hypothesis as it was originally stated (lack of exposure to TH1-inducing bacterial infection predisposes TH2-skewed atopy; [102]). However, in terms of infection modifying disposition to inflammatory disease (and taking into consideration the role of genetic and environmental factors) an alternative hypothesis can be supported. That is, the reduced prevalence of inflammatory autoimmune and allergic disease in many developing countries is a direct result of anti-inflammatory action mediated by parasite-derived immunomodulatory products.

Figure 8.1 ES-62 disrupts inflammatory immune response initiation mechanisms

Following differentiation of immature (dendritic cells) DC from bone marrow progenitor cells they are released into the peripheral tissues, where they perform a surveillance function as part of the innate immune system. In this state, immature DC continually take up and process antigen until they are activated by antigen in the presence of a danger signal (e.g. an adjuvant). In response to activation, DC antigen uptake ceases and antigen-presentation becomes a major function. Furthermore, the activated DC are deemed 'mature' in phenotype, evidenced by a morphological change (which increases their surface area), enhanced secretion of cytokines and upregulation of cell surface co-stimulatory, antigen-presentation and adhesion molecules necessary for activation of T cells. Following migration to a secondary lymphoid organ the activated DC prime antigen-specific T cells. Such T cell activation heralds the start of an antigen-specific adaptive immune response and begins with clonal expansion of antigen-specific T cells. ES-62 treatment *in vivo* and *in vitro* has been demonstrated to disrupt this immune response initiation pathway at multiple stages. For example, it has been demonstrated that exposure of bone marrow progenitors to ES-62 *in vivo* results in differentiation of DC that preferentially stimulate production of a less inflammatory immune response [191]. Furthermore, pre-treatment of differentiated bone marrow-derived 'immature' DC with ES-62 inhibits their activation, and disrupts subsequent DC-mediated T cell activation [189, 191]. Thus, *in vivo*, in a model of inflammatory disease, exposure to ES-62 acts to inhibits the initiation mechanisms leading to development of antigen-specific inflammation.

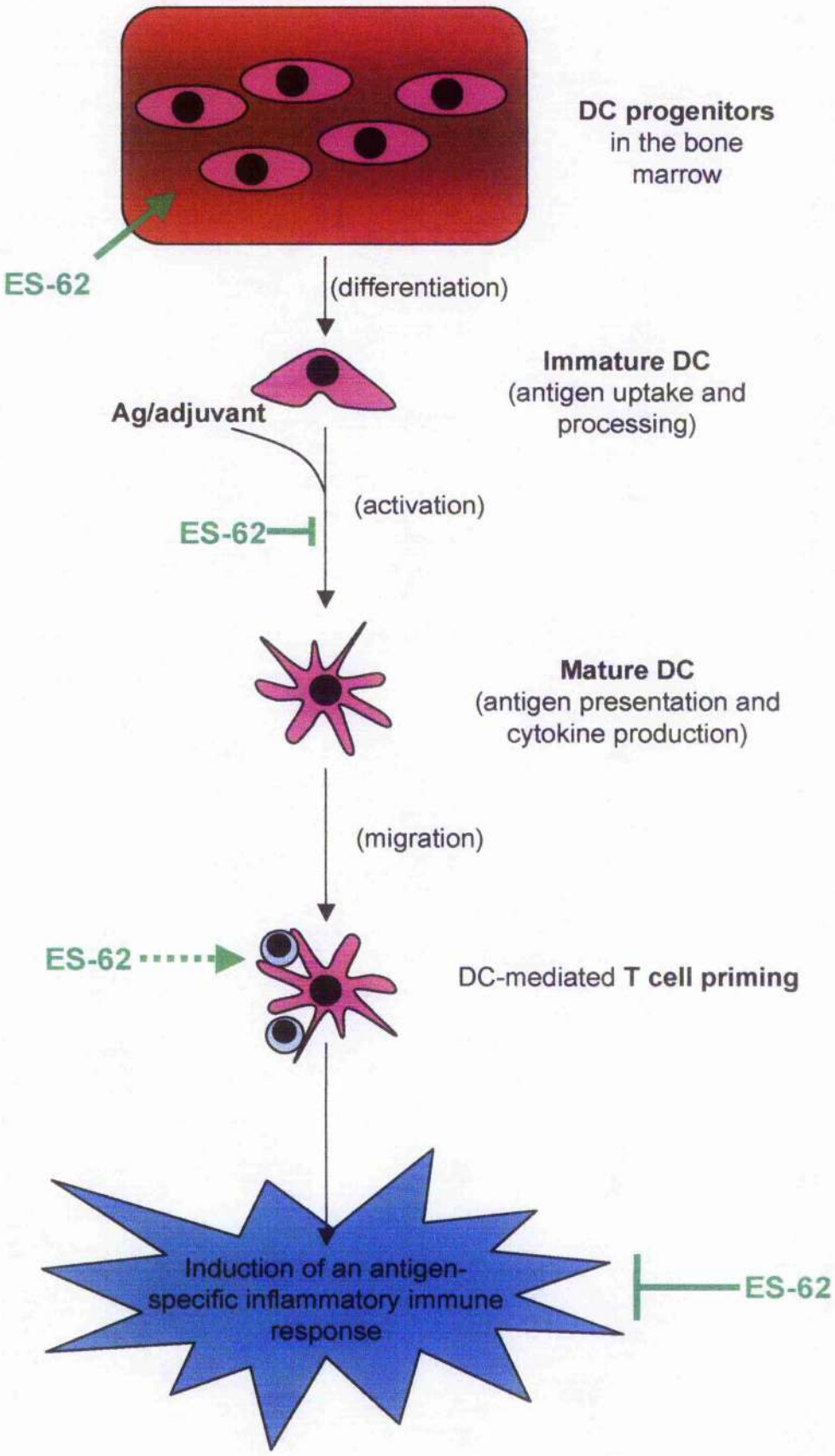
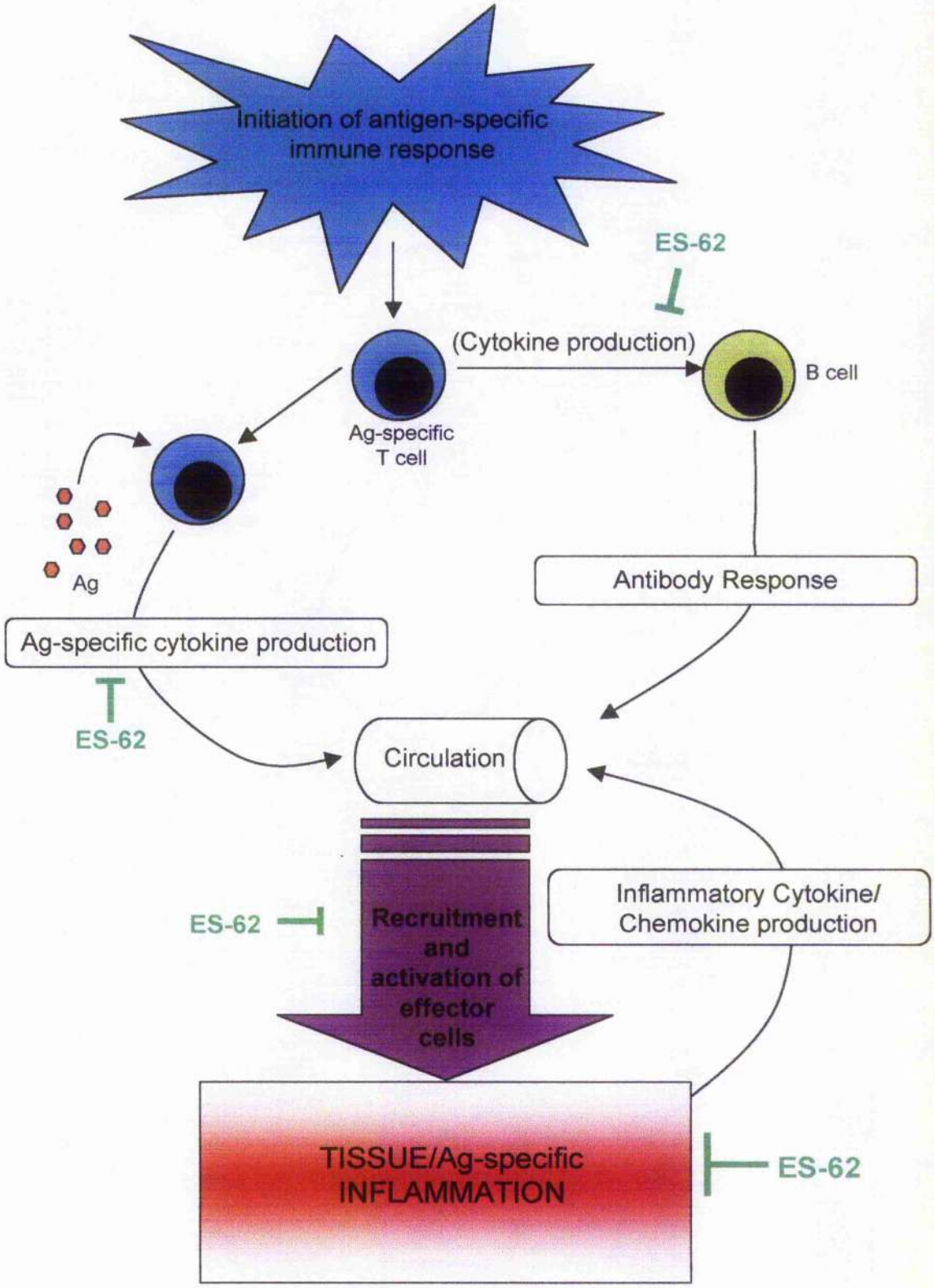

DC progenitors
in the bone
marrow
ES-62
(differentiation)
Immature DC
(antigen uptake and
processing)
Ag/adjuvant
(activation)
ES-62
Mature DC
(antigen presentation and
cytokine production)
(migration)
ES-62
DC-mediated T cell priming
Induction of an antigen-
specific inflammatory immune
response
ES-62

Figure 8.2 ES-62 treatment inhibits effector mechanisms of inflammatory disease

In general terms, following initiation of an inflammatory immune response (Figure 8.1), antigen-specific T helper (TH) cells secrete cytokines specific for the phenotype of the immune response, which help to induce switching of B cell antibody production to a corresponding phenotype. In response to antigen stimulation, antigen-specific T cells produce further TH-phenotype specific cytokines, which help to recruit and activate appropriate effector cells at the inflammatory site. In response to activation, such effector cells secrete inflammation-promoting chemokines and cytokines, which circulate in the bloodstream and help to recruit further inflammatory cells, inducing an positive feedback cycle of inflammation and potential tissue destruction. ES-62 treatment of established inflammation in models of autoimmune or allergic disease appears to induce inhibition of antigen-specific cytokine production by activated T cells and, via inhibitory action on effector cells in the inflammatory site, reduces recruitment of cells and ultimately inhibits inflammation and tissue destruction.


Initiation of antigen-specific immune response
ES-62
(Cytokine production)
B cell
Ag-specific T cell
Ag
Antibody Response
Ag-specific cytokine production
ES-62
Circulation
ES-62
Recruitment and activation of effector cells
Inflammatory Cytokine/ Chemokine production
TISSUE/Ag-specific INFLAMMATION
ES-62

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Publications

D. E. Kean, I. Ohtsuka, K. Sato, N. Hada, T. Takeda, G. Lochnit, R. Geyer, M. M. Harnett & W. Harnett. Dissecting Ascaris glycosphingolipids for immunomodulatory moieties – the use of synthetic structural glycosphingolipid analogues. Parasite Immunology 2006; 28: 69 – 76