

# **The Effects of ES-62 on DC Maturation and Effector Function**

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**Declaration**

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

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## **Publications**

Harnett MM, Kean DE, Boitelle A, McGuiness S, Thalhamer T, Steiger CN, Egan C, Al-Riyami L, Alcocer MJ, Houston KM, Gracie JA, McInnes IB, Harnett W.; The phosphorycholine moiety of the filarial nematode immunomodulator ES-62 is responsible for its anti-inflammatory action in arthritis. *Ann Rheum Dis.* 2007 Aug 17; [Epub ahead of print]

## Abbreviations

<b>Ab,</b>	antibody
<b>Ag,</b>	antigen
<b>APC,</b>	antigen presenting cell
<b>BAL,</b>	broncho-alveolar lavage
<b>BCR,</b>	B cell receptor
<b>bm,</b>	bone marrow
<b>BSA,</b>	bovine serum albumin
<b>CCR,</b>	chemokine receptor
<b>CD,</b>	cluster of differentiation
<b>cDC,</b>	conventional DC
<b>CIA,</b>	collagen induced arthritis
<b>CLR,</b>	C-type lectin receptors
<b>CRP,</b>	C-reactive protein
<b>cSMAC,</b>	core supramolecular activation cluster
<b>CTL,</b>	cytotoxic T cells
<b>CTLA-4</b>	cytotoxic T lymphocyte Ag
<b>DC,</b>	dendritic cell
<b>DN,</b>	double negative
<b>DP,</b>	double positive
<b>EAE,</b>	experimental autoimmune encephalomyelitis
<b>EBI,</b>	Epstein-Barr virus-induced gene
<b>ELISA,</b>	enzyme-linked immunosorbance assay
<b>ES,</b>	excretory-secretory
<b>FACS,</b>	fluorescence-activated immunosorbent assay
<b>FAM,</b>	6-carboxy-fluorescein
<b>FCS,</b>	foetal calf serum
<b>FITC,</b>	fluorescein isothiocyanate
<b>FSC,</b>	forward scatter
<b>GM-CSF,</b>	granulocyte-macrophage colony-stimulating factor
<b>HIGM,</b>	hyper IgM immune syndrome
<b>HPRT,</b>	hypoxanthine-guanine phosphoribosyltransferase
<b><i>i.n.</i>,</b>	intra-nasal

<b><i>i.p.</i>,</b>	intra-peritoneal
<b><i>i.v.</i>,</b>	intravenous
<b>ICAM,</b>	intracellular adhesion molecule
<b>iDC,</b>	immature DC
<b>IFN,</b>	interferon
<b>Ig,</b>	immunoglobulin
<b>IL,</b>	interleukin
<b>IRAK,</b>	IL-1R associated kinase
<b>IRAK,</b>	IL-1R-associated kinase
<b>IRF,</b>	IFN response factor
<b>JAK,</b>	Janus kinase
<b>JNK,</b>	c-Jun N-terminal kinase
<b>kD,</b>	kilo Daltons
<b>LC,</b>	Langerhans cell
<b>LCMV,</b>	lymphocytic choriomeningitis virus
<b>LPS,</b>	lipopolysaccharide
<b>LT,</b>	lymphotoxin
<b>mAb,</b>	monoclonal antibody
<b>MAP,</b>	mitogen activated protein
<b>MF,</b>	microfilariae
<b>MHC,</b>	major histocompatibility complex
<b>MIP,</b>	macrophage inhibitory protein
<b>MLN,</b>	mesenteric lymph node
<b>MR,</b>	mannose receptor
<b>mRNA,</b>	messenger RNA
<b>MyD88,</b>	myeloid differentiation primary-response protein 88
<b>NF-<math>\kappa</math>B,</b>	nuclear factor- $\kappa$ B
<b>NFAT,</b>	nuclear factor of activated T cells
<b>NK,</b>	natural killer
<b>OD,</b>	optical density
<b>OVA,</b>	Ovalbumin
<b>PAF,</b>	platelet activating factor
<b>PAMP,</b>	pathogen-associated molecular pattern

<b>PBMC,</b>	peripheral blood mononuclear cells
<b>PBS,</b>	phosphate buffered saline
<b>PC,</b>	phosphorylcholine
<b>PCR,</b>	polymerase chain reaction
<b>pDC,</b>	plasmacytoid DC
<b>PE,</b>	phycoerythrin
<b>PI,</b>	propidium iodide
<b>PI-3K,</b>	phosphoinositide-3 kinase
<b>PKC,</b>	protein kinase C
<b>PLC,</b>	phospholipase C
<b>PRR,</b>	pattern recognition receptor
<b>pSMAC,</b>	peripheral supramolecular activation cluster
<b>RNA,</b>	ribonucleic acid
<b>RT-PCR,</b>	reverse transcriptase polymerase chain reaction
<b>s.c.,</b>	subcutaneous
<b>SA,</b>	streptavidin
<b>sp,</b>	spleen
<b>SSC,</b>	side scatter
<b>STAT,</b>	signal transducer and activator of transcription
<b>TAB,</b>	TAK-binding protein
<b>TAK,</b>	transforming-growth-factor- $\beta$ -activated-kinase
<b>TAMRA,</b>	6-carboxy-tetramethyl-rhodamine
<b>TBS,</b>	Tris buffered solution
<b>TCR,</b>	T cell receptor
<b>TEC,</b>	thymic epithelial cell
<b>TGF,</b>	transforming growth factor
<b>Th,</b>	T helper cell
<b>TLR,</b>	Toll-like receptor
<b>TNF,</b>	tumour necrosis factor
<b>TRAF,</b>	TNF-receptor-associated factor
<b>TRAF-6,</b>	TNF-R-associated factor 6
<b>Treg,</b>	regulatory T cell
<b>UBC,</b>	ubiquitin-conjugating enzyme

## Summary

Parasitic filarial nematodes are often tolerated in their human hosts for decades, with little evidence of pathology, due to parasite-induced immune modulation. The consensus of opinion, to date, is that such parasites achieve prolonged survival as a result of their active modulation of the host's immune system by secreted immunomodulatory molecules. Consistent with this, the excretory-secretory protein of *Acanthocheilonema vitae*, ES-62, has been shown to exhibit immunomodulatory activities that are broadly Th2/anti-inflammatory in nature.

Dendritic cells are specialised antigen (Ag)-presenting cells critical for initiating and regulating immune responses. They stimulate T cells and produce cytokines that have important immunoregulatory functions. To date, more than 6 different DC subtypes have been identified that arise from at least two different cell lineages. These subtypes are not only phenotypically distinct but they are also thought to perform different functions and be restricted to certain lymphoid organs. Thus, it was proposed that ES-62 might differentially affect individual DC subtypes and thereby polarise the immune response towards a Th2/anti-inflammatory phenotype.

Consistent with this hypothesis, when splenic cDCs were exposed to ES-62, slightly different cytokine profiles were produced by the individual DC subtypes. However, when such cDC subtypes were used to prime Ag-specific T cells, although minor differences could be detected, the overall T cell response displayed a Th2/Treg phenotype. The ability of prior exposure to ES-62 to prevent LPS-matured DC subtypes from inducing Th1 polarisation was also investigated. Although such DCs showed reduced development of a Th1/pro-inflammatory phenotype, only weak Th2 effector responses could be seen suggesting that whilst ES-62 induced a Th2/anti-inflammatory-promoting phenotype with all cDC subsets tested, this maturation could be at least partially reversed/overcome by a subsequent strong pro-inflammatory signal.

In addition, the effect of ES-62 on maturation of the pDC phenotype, and consequent ability to induce Th2/anti-inflammatory responses, was investigated. Similarly, the effects of CpG, which is known to activate pDCs, and LPS, a TLR4 ligand which is reported to have no effect on such pDCs, and the ability of such

differentially activated pDC phenotypes to prime Ag-specific T cells were studied. Whilst only low levels of cytokines were produced by pDC upon stimulation with any of these molecules, ES-62-exposed pDCs were able to induce Th2-like responses, whereas both CpG- and LPS-matured pDCs induced pro-inflammatory phenotypes. Therefore to investigate the ability of ES-62 to inhibit such responses, pDCs were pre-exposed to ES-62 and subsequently matured with either CpG or LPS. Interestingly, while ES-62+LPS-matured pDCs could promote a Th2 phenotype, pre-exposure to ES-62 could only partially inhibit the pro-inflammatory phenotype induced by GpG.

DCs are the main Ag-presenting cells in the body. They express a variety of receptors, including pattern recognition receptors, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and also receptors belonging to the Mannose Receptor group. Furthermore, DCs also express the platelet activating factor receptor (PAF receptor). ES-62 has previously been shown to depend on the presence of TLR4 for exhibiting its effects on bmDC and macrophages, although it has been determined that the receptor does not need to be functional. Thus, it was believed that ES-62 might signal through a different co-receptor or might even be able to utilize a different receptor in cells, such as pDCs, which naturally lack expression of TLR4. Thus, in addition to further investigating the role of TLR4-signalling in the transduction of ES-62-mediated immunomodulation, signalling through two other receptors, namely the mannose receptor (MR) and the PAF receptor, were also investigated. In these studies, it was shown that, in cells expressing TLR4, ES-62 depends on the presence of this receptor, and its downstream adaptor molecule MyD88, to activate DCs and to subsequently induce T cell priming. Whilst no loss of function, in terms of cytokine secretion, could be detected in DCs deficient for the MR or the PAF receptor, ES-62-matured DCs from PAF receptor KO mice appeared to prime slightly stronger Th2/anti-inflammatory responses. Furthermore, whilst LPS treatment of such PAF receptor KO-DCs induced cytokine production similar to that seen with WT DCs, they were unable to prime T cells. Thus, these results indicated that LPS-driven Th1-polarisation is at least partially dependent on signalling through the PAF receptor

and that this receptor might be a possible target for ES-62 mediated inhibition of pro-inflammatory signalling.

Finally the potential therapeutic role of ES-62 in two murine models of disease, namely collagen-induced arthritis (CIA) and asthma was investigated. In the CIA model, both prophylactic and therapeutic application of the parasite product *in vivo* has been shown to be beneficial, resulting in delayed onset and reduced severity of disease or a reduction in the severity of existing inflammation, respectively. Thus, it was decided to further investigate the mechanisms underlying such immunomodulation by studying the effects of ES-62 on individual DC subtypes from mice in this disease model. One of the most interesting findings during these experiments was the apparent lack of double negative (DN) DCs recovered from spleens from CIA model mice. However, investigations of the effects of ES-62 on the maturation of the individual subtypes did not reveal any significant differences between individual DC subtypes. Furthermore, when these cells were cultured with T cells from CIA model mice, no significant Th1 or Th2 cytokine production could be found, and collectively the cytokine profile looked very similar to that of disease-associated DCs cultured on their own.

By contrast, investigation of the effect of ES-62-treatment in asthma model mice showed that ES-62 was able to drive two different responses. In the lung, draining lymph nodes and the spleen, ES-62 inhibited inflammation and Th2 development, by reducing Th2 cytokines, whereas it promoted Th2 development in peripheral lymph nodes. Interestingly although clinical improvement has been associated with an increase in lung levels of IL10, a Treg-associated cytokine, and increased levels were found in spleen cell cultures following ES-62 treatment of “asthma” model mice, no increase in natural Tregs could be found. Thus to further dissect the mechanisms by which ES-62 mediates its anti-inflammatory action in this disease model, bone-marrow derived DCs from “asthma” model mice treated with ES-62 or PBS *in vivo* were analysed for cytokine production and for their ability to prime Th cells, either in an immature or LPS-matured state. The results of this study showed that whilst “asthma” group bmDCs did not induce production of pro-inflammatory cytokines, they were able to prime T cells towards a Th2 phenotype. Interestingly,



this Th2-like response was reduced if bmDCs had been treated with ES-62 *in vivo*. By contrast LPS maturation of such “asthma” group bmDCs, and, to a much lesser extent, ES-62 treated “asthma” bmDCs, resulted in increased production of pro-inflammatory cytokines. Furthermore while these “asthma” group DCs induced a slightly reduced Th2 response, the reverse was true for bmDCs from “asthma” mice exposed to ES-62 *in vivo*. Collectively, the data obtained from the asthma model showed that ES-62 is capable of driving differential immune responses at distinct sites within an animal.

In summary, the results presented in this thesis provide novel information about the mechanisms underlying the modulation of T cell responses by ES-62-exposed DC subtypes, and may have identified a putative pathway by which ES-62 might inhibit pro-inflammatory signalling. Furthermore, these studies provide a better understanding of the therapeutic potential of this parasite molecule in inflammatory diseases such as CIA and asthma and collectively, they have demonstrated the potent immunomodulatory effects of ES-62.

# **Chapter 1**

## **General Introduction**

## 1.1 The Immune System

### 1.1.1 The Innate Immune System

The innate immune system is phylogenetically conserved in almost all multicellular organisms and is the body's first line defence against pathogens. It is a fast acting system utilising diverse mechanisms to protect against infection and disease. Among these mechanisms are anatomical barriers, secretory molecules and cellular components. The most obvious of these are the physico-chemical barrier mechanisms such as the desquamation of the skin, peristalsis, low pH and mucus generated in the intestinal tract as well as the oscillation of the broncho-pulmonary cilia and the lysozyme in oro-naso-pharyngeal and lachrymal secretions which collectively serve as shield from contaminated inhaled or ingested particles. However the innate immune system also comprises soluble mediators that act to protect against infection. The most important of these include the *interferons* which inhibit viral replication and activate cells which kill pathogens, *tumor necrosis factor alpha* (*TNF- $\alpha$* ) which suppresses viral replication and activates phagocytes, *acute phase proteins* such as C-reactive protein (CRP) which interact with the complement system and finally inflammatory mediators such as *leukotrienes*, *prostaglandins*, *histamine* and *plasmin* that contribute to inflammation. Generation of inflammation is one of the most important functions of the innate immune system in that it helps to recruit and activate different cells of the innate immune system such as dendritic cells (DC), mast cells, macrophages, monocytes, eosinophils and neutrophils.

Until recently, the innate immune system was considered to be relatively non-specific, with its main role being to destroy pathogens and to present (antigens) Ags to cells of the adaptive immune system. Although reliable recognition of microbial pathogens is a difficult task, because of their molecular heterogeneity and their rapid evolution, recent research has shown that the innate immune system has developed a pattern recognition strategy for detecting such microorganisms. In this strategy, pattern recognition receptors (PRRs) which are expressed on cells of the immune system recognise pathogen-associated

molecular patterns (PAMPs) that are unique to the microbial world and invariant among entire classes of pathogens [1, 2]. Once PAMPs are detected by PRRs, intracellular signalling pathways are switched on which lead to the production of inflammatory cytokines, chemokines, type I interferons, and result in the maturation of DCs and consequently the activation of the adaptive immune system.

#### **1.1.1.1 Toll like receptors**

The best characterised class of PRRs in mammalian species is the Toll-like receptor (TLR) family. Its discovery began with the identification of Toll, a transmembrane receptor expressed in drosophila flies. There it was found to be essential in the establishment of dorsoventral polarity during embryogenesis [3]. Subsequent studies in Toll mutant flies revealed that the receptor has an essential role in the insect's innate immune response against fungal infection [4-6]. Database searches revealed homologues in other species which were referred to as TLRs and to date, 11 members of the TLR family have been found in human and mouse [7] (Table 1.1). All TLRs possess leucine-rich repeats at their amino-termini which they use for recognising PAMPs [8-11], whilst intracellular signalling is conducted via the Toll-interleukin-1 (IL1) receptor (TIR) domain at the carboxy-terminal end [8, 12] (Figure 1.1). The first TLR characterised was TLR4 which detects bacterial lipopolysaccharide (LPS; endotoxin) [8]. Similarly, TLR2 has been shown to be the receptor for bacterial lipoprotein and peptidoglycan and appears to collaborate with TLR1 and TLR6, as well as with non-TLR receptors to diversify its recognition potential [8, 13, 14]. TLR5 binds flagellin, a protein monomer of bacterial flagella [15], whilst TLR9 recognises unmethylated CpG DNA of bacteria and viruses as well as hemozoin from *Plasmodium falciparum* [16-18]. TLR3 detects double stranded RNA and it has been shown that TLR3-deficient mice are more susceptible to certain viruses [19, 20]. Rather surprisingly therefore, it has also been shown that inflammatory responses to West Nile virus infection are suppressed in a TLR3-mediated manner [21]. TLR7 and TLR8 (human only) detect single stranded viral RNA [22]. Studies in TLR7 deficient mice determined that the receptor is necessary for inducing inflammatory cytokines, type I interferons and DC maturation in response to single stranded RNA or synthetic ligands [2, 22, 23].

TLRs- 1, 2, 4, 5 and 6 are expressed at the cell surface and act to recognise bacterial products that are unique to the bacteria and cannot be made by the host. By contrast, TLRs- 3, 7 and 9 are not expressed at the cell surface but rather in endosomal compartments of the cell [24-28] to allow them to recognise nucleic acids derived from internalised and degraded bacteria and viruses. Since the host's nucleic acids are not normally accessible in these compartments, these TLRs do not distinguish effectively between self and foreign. Thus, it has been shown that when host nucleic acids become available to TLRs following tissue damage, they can trigger inflammatory responses and contribute to the pathogenesis of autoimmune disease [29-32].

#### **1.1.1.2 Lectin receptors**

Another group of PRRs are C-type lectin receptors (CLR) [33]. So far more than 15 CLRs have been detected on DCs and macrophages [34] among them Dectin-1, the mannose receptor and DC-SIGN (Figure 1.2). In contrast to TLRs, CLRs have been shown to only recognize carbohydrate structures on Ags and to internalise these Ags without causing DC maturation [35, 36]. In order to differentially recognize not only monosaccharides but also more complex sugar moieties, CLRs have evolved different numbers of carbohydrate recognition domains [37]. Various studies have revealed that some pathogens use CLRs to modulate the immune response either by inhibiting Ag presentation or by modification of T cell responses [34, 38]. For example DC-SIGN has been shown to be targeted by various viruses such as HIV, Dengue, HCMV, HCV, Ebola and SARS [39-43] and also by *Mycobacterium tuberculosis* [44, 45], *Helicobacter pylori* [46], *Schistosoma mansoni* [47] and Leishmania [48]. Interestingly, recent research has demonstrated crosstalk between CLRs and TLRs [38]. Thus, one study revealed that Dectin-1 and TLR2 synergise to induce TNF $\alpha$  and IL12 production [14], whilst another showed that targeting the mannose receptor inhibited production of inflammatory cytokines by the TLR-4 ligand LPS [49]. Even though many functions of CLRs are as yet undiscovered or unexplained it is clear from the above mentioned data, and also from various other studies, that this receptor group plays an important part in regulating the immune system.

### 1.1.2 The adaptive immune system

The adaptive immune system is a highly sophisticated system which can only be found in vertebrates. The principal cells in this system are B and T lymphocytes. T cells can be divided according to their function as CD8<sup>+</sup> cytotoxic T cells (CTL) and CD4<sup>+</sup> helper T cells. CD4<sup>+</sup> T cells can further be divided into a number of different subsets including Th1, Th2, Th17 and Treg cells that can be distinguished by the array of cytokine genes they produce [50-54]. For example, the hallmark cytokines of Th1 cells include IFN $\gamma$  and lymphotoxin (LT) which can activate microbial activity, induce cytokine production in macrophages and are often accompanied by the activation of CTLs and natural killer cells (NK). Hence a Th1 response is important for the eradication of intracellular parasites, bacteria, yeasts and viruses [50-54]. By contrast, Th2 cells mainly produce IL4, IL5, and IL13 and are able to activate mast cells and eosinophils which are important in the elimination of extracellular parasites such as helminths [50, 53]. Functioning properly, Th1 and Th2 cells effectively protect the body against pathogens. However an uncontrolled Th1 response can lead to autoimmune disease [55, 56] and Th2 cells can promote allergic and atopic manifestations [53, 57, 58].

B and T cells collectively have the ability to recognize a vast array of Ags using Ag receptors on their surface. Due to somatic gene rearrangement and somatic mutation in the genes for Ag receptors, the lymphocyte population is able to produce over 10<sup>13</sup> different species of Ag receptors. On B cells these Ag-recognition receptors are called membrane immunoglobulins (MLG or BCR) and on T cells, the T-cell receptor (TCR). The TCR resembles the B-cell receptor (BCR) in general structure as they are both encoded by similarly organised genes. Thus, the variable domains of both BCRs and TCRs consist of a random combination of different variable (V), diversity (D; heavy and  $\beta$  chains) and joining (J) gene segments and hence an individual lymphocyte expresses receptors of a unique Ag-binding specificity. Since these receptors are randomly generated some progenitor cells of the adaptive immune system express receptors with specificity for self-Ag. Therefore B and T cells have to undergo rigorous selection processes in the bone marrow and the thymus, respectively, to generate mature cells which are tolerant

to self-Ags before they are released into the periphery. The importance of this selection process becomes very clear with regard to autoimmune disease where failures in such mechanisms direct an immune response against autologous components of the body.

## **1.2 Dendritic cells**

### **1.2.1 Development of Dendritic cells, phenotypes and characterization**

Dendritic cells (DC) are a rare and heterogeneous population of Ag-presenting cells (APC). During the last few decades several types of DCs with different biological features have been identified in different tissues, among them Langerhans cells (LC), interstitial DCs, thymic DCs and DC populations in other lymphoid organs. DCs derive from haematopoietic progenitor cells from at least two different lineages, namely the myeloid-related and the lymphoid-related lineages [59-61]. Lymphoid-restricted precursor populations can be found in the murine thymus as well as in the murine bone marrow [62, 63]. Myeloid progenitor cells however have only been found in the murine bone marrow [64].

All mature murine DCs express CD11c, the co-stimulatory molecules CD80, CD86 and CD40 and moderate to high levels of MHC II. Furthermore, some murine DCs have been shown to express the T cell markers CD4 and CD8, the latter in the form of a CD8 $\alpha\alpha$  homodimer [65, 66] rather than the CD8 $\alpha\beta$  heterodimer which is typically found on T cells. Other markers found on some DC populations are CD11b and CD205. Currently five different subtypes of conventional DCs (cDC) [66-68] and one plasmacytoid cell (pDC) type [69-71] that can function as DC precursors have been defined in the murine system, using the above mentioned surface markers (Table 1.2). Thus, cDCs can be subdivided into CD11c<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup>CD205<sup>+</sup>CD11b<sup>-</sup>, CD11c<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>CD205<sup>-</sup>CD11b<sup>+</sup>, CD11c<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD205<sup>-</sup>CD11b<sup>+</sup>, CD11c<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD205<sup>+</sup>CD11b<sup>+</sup> and CD11c<sup>+</sup>CD4<sup>-</sup>CD8<sup>lo</sup>CD205<sup>hi</sup>CD11b<sup>+</sup> subsets. By contrast, the surface phenotype of the plasmacytoid dendritic cell precursor is CD11c<sup>int</sup>CD45RA<sup>hi</sup>MHCII<sup>lo</sup> and it can additionally express CD4 and/or CD8 (not regarded as different subtypes). This precursor cell can be found in all

lymphoid organs including the thymus and it has been shown in culture that upon activation the pDC develops typical dendritic morphology [69, 72].

The spleen predominately contains three of the cDC subtypes, the  $CD4^-CD8^+CD205^+CD11b^-$ ,  $CD4^+CD8^-CD205^-CD11b^+$  and  $CD4^-CD8^-CD205^-CD11b^+$  DCs [66]. The  $CD8^+$  DCs are mainly found in the T cell areas, whereas the  $CD8^-$  subsets are concentrated in the marginal zones and only migrate to the T cell area upon stimulation with microbial products [66, 73, 74]. It has been shown that these spleen DCs derive from different developmental pathways and that the lifespan of cDCs in the spleen is relatively short with a half life of only 1.5 days [66, 69, 75, 76]. Spleen pDCs however have a lifespan of over 14 days [69].

In the LNs, five cDC subtypes can be found, although the  $CD4^+$  DC are of low incidence [67]. Of these five cDC subtypes, two have been identified as skin-derived subtypes and can be divided into dermal- and epidermal-derived populations [67]. Both of these DC subtypes have a mature phenotype and are thought to have arrived in the LN via the lymphatic system [77]. The dermal-derived population, which is  $CD4^-CD8^-CD205^+CD11b^+$ , has been found in cutaneous-draining LNs (CLN) and in mesenteric LNs (MLN), whereas the epidermal population which expresses  $CD4^-CD8^{lo}CD205^{hi}CD11b^+$  is restricted to CLNs.  $CD4^-CD8^{lo}CD205^{hi}CD11b^+$  cDCs also express very high levels of langerin and are believed to be the mature form of the Langerhans cells [67]. All skin-derived DCs appear to have a long lifespan, with epidermal DCs displaying a slower turnover rate than dermal DCs. However experiments conducted by Kamath have shown that the long lifespan of such skin-derived DCs reflects a long residence time in the skin, rather than in the LNs [78].

In the thymus three different DC subtypes, two cDCs and pDCs, have been found [79] with the majority of thymic DCs localised in the medulla or at the cortico-medullary junction [80-83]. Most of the cDCs are  $CD8^+CD4^-CD205^+CD11b^-$  but in contrast to the spleen and LN cDCs of this subtype, a large proportion of the  $CD8^+$  thymic cDCs also express BP-1 a cell surface glycoprotein mainly found on early B-lymphocytes [72, 84]. A minority of the thymic cDC population is  $CD8^-$  and these



express high levels of the signal regulatory protein- $\alpha$  (Sirp $\alpha$ ) [79]. Both of these thymic cDC populations have been shown to be able to acquire CD8 $\alpha\beta$  from thymocytes, an ability which initially made recognising and separating subtypes particularly difficult [66]. Thymic pDC closely resemble their counterparts in the periphery regarding their cell surface markers and their capacity to transform to dendritic morphology only upon stimulation [69, 70]. The lifespan of thymic DCs is relatively long as the turnover rate of the majority of thymic cDCs is approximately 10 days, which is roughly the lifespan of a thymic T-lineage cell [78]. However a minority of cDCs showed a spleen cDC-like turnover of about 3 days [78]. Similarly, pDCs in the thymus are fairly long lived with an average lifespan of around 14 days [69]. Thymic DCs have been proposed to be important in T cell development and the negative selection process and may also be involved in tolerogenic mechanisms such as the induction of regulatory T-cells. Hence their roles in the thymus will be discussed in context of T cell development.

Very recently a potential new DC subtype, termed interferon producing killer DC (IKDC), has been discovered (Table 1.2) but little is known about these cells. Interestingly these DCs show homology not only to pDCs with regard to their levels of CD11c and CD45R but also to NK cells, a property that has made classification of these cells very difficult. For example IKDCs express CD49b which corresponds to VLA-2 an integrin that is found on NK cells. Furthermore IKDCs express the NK-activating receptor NKG2D. Upon stimulation these DCs secrete IFN $\gamma$  in the presence of IL15 or IL2 and IL12. However, in contrast to classical NK cells but similarly to pDCs, IKDCs are sensitive to CpG-ODN a TLR9 ligand [85]. Thus, considering all the data it was proposed that IKDCs have a distinct function in immunity possibly linking the innate and the adaptive immune system. Although to date no specific role has been found for these cells, their involvement in tumour immunosurveillance has been suggested in a recent paper [86].

### **1.2.2 Toll like receptor expression on DC subsets**

Activation of TLRs provides critical signals for the maturation of DCs that is evidenced by cytokine production, up-regulation of co-stimulatory molecules and

the ability to activate naive T cells [11, 87, 88]. In both mouse and human, distinct DC subsets can be found in specific locations throughout the body. Studies of these DCs revealed distinct TLR expression patterns indicating potential differential functions for the subsets in pathogen recognition and in influencing adaptive immune responses.

For example, pDCs express a distinct set of TLRs namely TLR7 and TLR9 in mice and TLR7, TLR8 and TLR9 in humans [89-94] which enables them to recognise viral components as it has been shown that TLR9 recognises unmethylated CpG-rich DNA that is common in bacteria and in the genome of DNA viruses such as HSV-1 and 2 or MCMV [17, 95, 96]. Likewise, TLR7 mediates recognition of single stranded RNA viruses such as influenza and vesiculostomatitis virus (VSV) as well as synthetic single stranded RNA sequences and ribonucleotide homologues [2, 22-25, 97]. All three receptors require an acidic environment for activation and are confined to endosomal compartments. Signalling through TLR7, 8, and 9 is entirely MyD88 dependent and induces a strong IFN type I response which can even be seen upon infection with non-replicating and inactivated viruses [91]. Interestingly, for reasons yet to be defined, some viruses, among them lymphocytic choriomeningitis virus (LCMV), have found ways to evade detection by these TLRs and do not cause an IFN type I response [98].

In most cells, transcription of IFN type I genes is primarily controlled by the transcription factors, IFN regulatory factor 3 (IRF3) and IRF7 through an IRF7-dependent autocrine feedback loop and consistent with this, it has been shown that IRF7 deficient mice are almost incapable of producing these cytokines [99-102]. However in pDCs, IFN $\alpha$  secretion is not inhibited in the absence of such positive feedback signalling [103]. This appears to be due to constitutive expression of IRF7 in pDCs [104, 105] and the ability of MyD88 to recruit it through a molecular complex including TRAF6 and IRAK4 [106, 107]. Thus it seems that the activation of IRF7 and hence IFN $\alpha$  is independent of IRF3 activation in pDCs. cDCs can also express TLR7 and TLR9 but, in contrast to pDCs the activation of these receptors leads to the production and secretion of other pro-inflammatory cytokines and chemokines such as IL12, Mip-1 $\alpha$  and Rantes [108].

cDCs generally express a wider variety of TLRs than pDCs but the precise expression pattern appears to depend on the subgroup. Since very few reliable antibodies against TLRs are available, most studies concerning the TLR expression profile of cDCs have been conducted using RT-PCR analyses of cDC mRNA. Thus, for example, in these studies it was shown that freshly isolated CD4<sup>+</sup> cDCs expressed mRNA for all known TLRs with the exception of TLR3. Although CD8<sup>+</sup> cDCs exhibited a similar expression profile they lacked TLR7 and TLR5 but expressed TLR3 mRNA. Moreover CD4<sup>-</sup>CD8<sup>-</sup>DCs were found to express mRNA for all TLRs [109]. Interestingly, there seems to be a difference in the expression of TLRs and responsiveness to certain TLR-ligands between freshly isolated and *in vitro* derived cDCs. Thus, a study has shown that whilst freshly isolated splenic cDCs do not respond to LPS stimulation, even though they express low amounts of TLR4, bone marrow precursors matured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) express TLR4 at much higher levels and show a strong response to LPS [94].

### **1.2.3 The role of dendritic cells in the immune system**

Dendritic cells are professional Ag-presenting cells (APC) critical for bridging the innate and adaptive immune response. They are important both in the generation of effector cell types and in the maintenance of tolerance to self Ags. In the periphery, immature DCs (iDC) patrol the microenvironment for invading pathogens. They continuously sample their environment by endocytosis, macropinocytosis and phagocytosis and it has been suggested that these iDCs constantly process and present Ags *in vivo* [110, 111]. Furthermore, studies have shown that there is a constitutive traffic of iDCs through tissues and lymphoid organs and it has been proposed that these DCs are involved in the induction of peripheral tolerance [112]. However in response to a variety of stimuli, iDCs initiate a maturation programme during which they lose their capacity to take up Ag and transform into potent effector DCs (Figure 1.3). This process is linked with the up-regulation of MHC Class II molecules, co-stimulatory molecules such as CD80, CD86 and CD40 and adhesion molecules such as CD54 which are essential for activation of naïve T cells [113, 114]. Various signals have been described that

induce DC maturation, among them, pathogens such as viruses and bacteria or components of these microorganisms [115-117], heat shock proteins [118], necrotic cells [119, 120], components of the extracellular matrix [121], interaction with activated T cells [122-125] and mechanical manipulation [119]. During maturation DCs also secrete cytokines and chemokines which can attract additional innate cells, such as neutrophils and macrophages to the site of infection/inflammation. Furthermore maturing DCs undergo a change in their chemokine receptor expression profile enabling them to migrate to the T cell rich areas in the LNs.

Upon activation and maturation, DCs leave peripheral tissues via the efferent lymphatics and migrate to T cell areas of lymphoid organs to present Ag to cognate T cells. Such migration of DCs is coordinated by chemokines [126] and consistent with this, it has been shown that maturing DCs down-regulate expression of the chemokine receptors CCR5, CCR6 and CXCR1 which bind to the chemokines CCL3, CCL20 and CXCL8 that are normally secreted by cells in the peripheral tissues to recruit cells of the innate immune system to sites of infection and inflammation. By contrast, mature DCs express CCR7 which is thought to be the receptor for CCL19 and CCL21 [127-131]. Both of these chemokines [132-134] are constitutively expressed in the secondary lymphoid organs [135] and CCL21 is also secreted by endothelial cells in high endothelial venules and lymphatic vessels. CCL19 was found to be expressed on DCs in T cell areas of the LNs [136-140]. Thus it seems that these chemokines direct DCs to the paracortical regions of the secondary lymphoid organs where they can interact with naïve CD4<sup>+</sup>T cells which are targeted to these locations by the same mechanism [141-143].

#### **1.2.4 Dendritic cell-regulated immunoregulation**

DCs initiate immune responses by providing lymph node-based naïve T and B cells with pathogen-related information from the affected tissues and thus, they play a crucial role in linking the class of immune response to the invading pathogen. It has been determined that DCs use 3 different signals (referred to as signal 1, 2 and 3) to initiate a T cell response following the ligation of selected TLRs by pathogen

products (Figure 1.4). Signal 1 is provided by the expression of MHC II complexes presenting pathogen-derived peptides at the surface of the mature DC. These peptide-MHC complexes are recognised by T cell receptors (TCR) on cognate Ag-specific T cells and determine the specificity of the T cell response [144]. Furthermore DCs up-regulate co-stimulatory molecules such as CD80 and CD86 upon maturation informing T cells about the need to initiate specific immune responses (signal 2) [113]. Taken together, signal 1 and signal 2 initiate T cell priming. However recently it was realised that DCs also direct the type of immune response by expressing a selective set of T cell polarising molecules (signal 3). These molecules are either soluble or membrane bound and appear to determine the balance between Th1, Th2 and T regulatory cell development. The expression profile of these molecules depends on the DC subtype and its ability to recognise pathogens with its pattern recognition receptors such as the TLRs. However the initial assumption that specific DC subtypes are only able to induce either Th1 or Th2 or Treg cells appears to be wrong [59, 61]. For example, a study using human pDCs and cDCs, stimulated with the TLR7 ligands imiquod and R-848, has shown that although different cytokines were secreted by the distinct DC subsets, ultimately both promoted Th1 polarisation [145]. Furthermore, studies with murine cDCs have shown that stimulation with LPS or ES-62, an excretory-secretory nematode protein, can drive Th1 or Th2 immune responses, respectively [146]. Thus, although very little is currently understood regarding the role of DC subtypes in T cell polarisation, generally it can be said that DCs exposed to intracellular pathogens will promote a Th1 response whereas extracellular pathogens such as helminths drive the development of Th2 cells [147, 148]. However, other extracellular pathogens or their products such as bacterial LPS can drive a Th1 response. Interestingly recent studies have shown that Ag-specific regulatory cells are induced by semi-mature DCs [149, 150].

## **1.3 T-cells**

### **1.3.1 T-cell development**

T cell development is a complex stepwise process which mainly takes place in the thymus. The thymus is made up of lobes, each of which is organised into inner

medullary and outer cortical regions which in turn are generated by thymic epithelial cells (TEC), interdigitating DCs and macrophages. Lymphoid progenitor cells from the bone marrow which can give rise to B cells, T cells, NK cells and DCs start to migrate to the thymus as early as on embryonic day 11.5 (E11.5) where they accumulate in the cortical regions of the thymus before entering the thymus properly on E12.5 [151].

The first identifiable thymocytes are called double negative 1 (DN1) due to their lack of CD4 and CD8 expression and they are defined by their surface marker expression profile which is  $CD4^-CD8^-CD25^-CD44^+$  and can be found in the DN1 compartment of the thymus [152]. As these cells mature, they progress through three more stages. In the DN2 stage cells become  $CD44^+CD25^+$ , in the DN3 stage  $CD44^-CD25^+$  and in the DN4 stage they are  $CD44^-CD25^-$ . These DN4 cells are the immediate progenitors of the  $CD8^+CD4^+$  thymocytes which are called double positive (DP) cells and from which mature single positive  $CD4^+$  or  $CD8^+$  cells arise [153]. During thymic selection at the DP stage, development of thymocytes bearing an  $\alpha\beta$ T cell receptor with low affinity for self peptide-MHC-complexes is promoted whereas thymocytes exhibiting strong affinity are deleted.

During maturation thymocytes migrate through the thymus. In the pre-DP stages this migration takes place in the cortex. It has been shown that DN cells first move outwards and then turn back and migrate towards the medulla and undergo positive and negative selection processes. The exact mechanism that drives this migration is not known [154-156] but it has been determined that DP thymocytes are guided to the medulla via CCR7 ligands where, following selection, they reside for 3 to 14 days before they emigrate to the periphery [157, 158] (Figure 1.5). During this time they sample many self Ags and any auto-reactive thymocytes at this DP stage can be cleared. It has been shown that thymic epithelial cells (TEC) and cross-presenting DCs are essential for this process and that a lack of contact with these cells allows auto-reactive clones to escape the thymus [159, 160]. Consistent with this, it has also been indicated that a disruption of the thymic microenvironment might cause the breakdown in tolerance in autoimmune models [161, 162].

Two major T cell classes arise in the thymus and these can be distinguished according to their T cell receptor (TCR). In the first group the TCR consists of an  $\alpha$  and  $\beta$  chain which are linked by two disulphide bridges, in the second group the TCR is structurally similar but consists of a  $\gamma$  and  $\delta$  chain. The diversification of the TCR occurs by recombination between the V, D and J gene segments with minor variations in detail for each locus. In a mouse  $1-2 \times 10^8$  T cells display about  $2 \times 10^6$  different TCRs [163] and in an adult human the diversity is even higher [164, 165]. The  $\alpha\beta$ T cell lineage can be further divided into  $CD4^+$ T cells that is restricted by MHC II and  $CD8^+$ T cells that recognise peptide MHC I complexes.  $CD4^+$  and  $CD8^+$   $\alpha\beta$ T cells are primarily responsible for Ag-specific cellular immunity whereas  $\gamma\delta$ T cells have often been considered to play a more innate-like role. Although  $\gamma\delta$ T cells are involved in specific primary immune responses, they also play an important role in immunoregulation as well as in tumour surveillance and wound healing [166-169].

#### **1.3.1.1 The influence of DCs on T cell development in the thymus**

As stated above, DCs are very efficient APC and play a key role in inducing and regulating immune responses. It is now generally accepted that they also play a role in mediating negative selection of  $\alpha\beta$ T cells in the medulla of the thymus [170]. During this process DCs present self-Ag in the context of MHC Class I or II to DP T cells and initiate cell death upon binding of the TCR to the peptide/MHC complex. Hence only T cells that do not strongly recognise self-peptides are released from the thymus.

Currently three types of DCs can be found in the thymus, two of them being cDCs, and the other being the pDC. Among the cDCs a major and a minor population can be distinguished. The unique feature of thymic DCs is that the major population is  $CD8^+$  and that there is evidence for its development from early thymocyte precursors (ETPs) [171]. By contrast, the minor  $CD8^-$  population seems to derive from the bloodstream whilst the origin of the thymic pDCs remains unclear. Studies conducted on thymic-generated  $CD8^+$  cDCs have shown that these cells express

low levels of AIRE and can cross-present tissue specific Ags captured from medullary epithelial cells: hence, these studies suggest that such cells could be crucial for negative selection [172, 173]. However it has been proposed that the CD8<sup>+</sup> cDCs which enter the thymus from the bloodstream might have a different role. Since they are capable of carrying Ag from the periphery it has been suggested that these cells might be involved in the generation of regulatory cells [174]. The function of thymic pDCs has not been elucidated but their involvement in negative selection seems to be unlikely since they are very poor presenters of Ag unless subjected to microbial stimulation. However, several studies have suggested the possibility of cross talk between pDCs and cDCs. For example, it was shown that type I IFNs secreted by pDCs upon viral infection could activate immature cDCs and lead to the production of IL12, IL15, IL18 and IL23 which subsequently could induce a T cell mediated response [175-182]. Recent studies also indicate a possible role of pDCs in the development of Tregs [183-186].

However the function of the different DC subtypes in the thymus is not fully understood and their role in T cell development remains to be explored.

#### **1.4 The interaction between T cells and DCs**

After DCs encounter Ag in the periphery they migrate to the secondary lymphoid organs where they form cognate interactions with T cells. Once a T cell has recognised Ag presented by DCs, bidirectional signalling involving co-stimulatory molecule counterstructures and TCR-peptide/MHC complexes takes place. Using two photon microscopy it has recently been shown that prolonged contact *in vivo* is necessary for full activation of T cells [187-189]. Furthermore it has been demonstrated that sustained engagement between these two cell types is necessary to form the immunological synapse which allows transduction of TCR signals [190].



### **1.4.1 The immunologic synapse**

It has been proposed that optimal T cell activation can only be achieved by the formation of an immunological synapse (IS) (Figure 1.6). Such an IS consists of a central core supramolecular activation cluster (cSMAC) containing the TCR - MHC/peptide complexes on T cells and DCs respectively and CD28/CTLA-4 - CD80/CD86 interactions and is surrounded by an outer ring structure consisting of the integrin LFA-1 and ICAM. LFA-1 and ICAM form the so called peripheral supramolecular activation cluster (pSMAC) [191-197] which is believed to stabilise the inner complex. Currently it is thought that upon signalling through the TCR and co-stimulatory receptors, the TCR becomes transported into the centre of the IS and that LFA-1 diffuses to the outside of the synapse where it binds to ICAM-1 on APCs [198]. Interestingly, CD28 seems to play a crucial role in the formation of the inner cluster as blocking the molecule or using CD28 deficient mice inhibits formation of the inner complex of the IS [199, 200].

### **1.4.2 Co-stimulatory molecules**

CD4<sup>+</sup>T cells must receive additional co-stimulatory signals to become fully activated. The best characterised co-stimulatory pathway is the interaction between CD28 on the T cell with CD80/CD86 on the APC. CD28 is a transmembrane protein that belongs to the immunoglobulin superfamily. Signalling through CD28 leads to expression of CTLA-4 (cytotoxic T lymphocyte Ag) which acts as a negative regulator of CD28 and controls the duration of effector immune response [201]. Analyses of the signal transduction pathway revealed that the co-stimulatory function of CD28 is mediated by activation of signal transduction molecules that can associate with the cytoplasmic tail of CD28 following ligation with B7 (CD80/CD86) family molecules. Among these signal transduction molecules is phosphatidylinositol 3-kinase (PI-3K) [202] which has been shown to increase the expression of the anti-apoptotic protein Bcl-xl [203]. Signalling through the PI-3K pathway also regulates entry into the cell cycle by mediating down-regulation of the inhibitor of cell cycle progression p27Kip1 [204]. Furthermore PI-

3K signalling also induces IL2 production possibly through increased nuclear localisation of NFAT [205, 206].

Furthermore in conjunction with PI-3K, CD28 mediates its co-stimulatory effects by associating with the guanine nucleotide exchange factor Vav-1 and then, together with Vav-1, regulating the activity of TEC kinases. These kinases promote T cell activation by regulating phospholipase C $\gamma$ 1 (PLC- $\gamma$ 1) activity [207]. CD28 signalling also supports TCR-mediated signalling to fully activate several other kinases such as the MAPK, ERK and JNK [208, 209].

Apart from its role in effector T cell activation CD28 has been implicated in playing a role in effector T cell differentiation. However, data regarding this topic is controversial as some *in vivo* studies show that the CD28-CD80/CD86 complex is involved in Th2 differentiation by promoting IL4 production [210-213], whereas other studies demonstrate a role in Th1 induction upon production of IL2 and IFN $\gamma$  [214].

Another molecule, inducible co-stimulatory (ICOS), which has homology to CD28, has also been shown to become up-regulated upon T cell activation and play a role regulating T cell differentiation. It does this via interaction with ICOSL on DCs and appears to act to promote Th2 effector development [215]. However several studies have since shown that ICOS does not only play an important role in directing Th2 responses [216] but can also promote Th1 responses [217]. Similarly, members of the TNF family such as CD40 and OX40 have also been implicated in the co-stimulatory process. CD40 is expressed on a variety of cells including T cells and binds to CD154 which can be found on several cells, including DCs and also T cells. It has been shown that initial stimulation of CD40 on DCs promotes production of IL12 and IFN $\gamma$  [218] and hence it was proposed that signalling through this receptor promotes Th1 responses. This proposal was supported by the finding that Hyper IgM immune syndrome (HIGM) patients, which have a defect in either CD40 or CD154, are susceptible to encephalitis during a *Toxoplasma gondii* infection due to impaired CD40-CD154 signalling [219]. Furthermore CD40 or CD154 deficient mice were shown to have a defective Th1 immune reaction and

a high mortality upon *Leishmania* infection [220, 221]. Interestingly, in a study in which DCs were primed with *Schistosoma*-soluble egg Ag to induce a Th2 response it was determined that this response also depended on a CD40-CD154 interaction [222]. Thus it appears that this receptor complex can also induce Th2 as well as Th1 responses.

Similarly, controversial data also exists for OX40 which binds to OX40L on DCs. Thus, whilst *in vitro* work stimulating this receptor demonstrated a role in promoting Th2 responses, *in vivo* studies suggested OX40-OX40L interactions promote autoimmune encephalomyelitis [223] and arthritis in animal models, thus implying a role in Th1 differentiation. However a study in a murine asthma model also indicated a role for this DC-T cell receptor complex in Th2 responses [224].

In conclusion, it appears that most co-stimulatory molecules can initiate Th1 or Th2 responses depending on the immunological context, but the mechanisms underlying such effector cell decisions, however, are as yet not known.

## **1.5 Dendritic cell-mediated T effector cell development**

### **1.5.1 Th1 cell development**

#### **1.5.1.1 Dendritic cell-derived Th1 polarising/pro-inflammatory signals**

Upon contact with a Th1-inducing pathogen, DCs mature and present Ag to T cells. To polarise these T cells towards Th1 effector cell differentiation requires specific DC-derived signals both in terms of secreted cytokines and cell-cell interactions (Figure 1.7).

One of the most important regulators of Th1 responses is IL12 (Figure 1.8). IL12 is a 70kD protein, consisting of a p40 and p35 subunit, which is mainly produced by DCs, monocytes and macrophages [225-229]. Both subunits have to be expressed simultaneously to result in secretion of the bioactive p70 heterodimer which is capable of mediating the biological effects of IL12 [230]. In addition to the bioactive form, the p40 subunit is produced in large excess. It has been shown that p40 can form homodimers and these have been proposed to be natural inhibitors of IL12

p70 signalling [231, 232]. The IL12 receptor consists of two chains the IL12R $\beta$ 1 and the IL12R $\beta$ 2 chain and is primarily expressed on activated T cells and NK cells. The p40 subunit binds to the IL12R $\beta$ 1 chain to recruit Tyk2 whereas the p35 subunit, as well as the bioactive p70 form of IL12, bind to the IL12R $\beta$ 2 chain which recruits Janus kinase (Jak) 2. Signalling through the IL12R complex then initiates phosphorylation, dimerization, and nuclear translocation of the transcription factors STAT1, 3, 4 and 5 and leads to the activation of IL12- responsive genes [229]. The importance of STAT4 has been shown by STAT4 deficient mice where a decreased production of IFN $\gamma$  and defective Th1 cell development could be seen [233-235]. Studies have also shown that individuals lacking a functional IL12 receptor are more prone to infection with salmonella and mycobacteria but not with viruses [236, 237]. One of the main functions of DC derived IL12 is the promotion of a Th1 controlled immune response and hence, the stimulation of IFN $\gamma$  production in naïve T cells [231]. Thus upon activation, IL12 is produced by DCs which in turn stimulates cognate CD4<sup>+</sup>T cells and NK cells to secrete IFN $\gamma$  [238, 239]. Secreted IL12 has also been shown to have an effect on DCs as it enhances further IL12 production through a positive feedback loop and promotes secretion of IFN $\gamma$  by DCs [240, 241] (Figure 1.7).

Recently, two other members of the IL12 family have been identified. These two cytokines are IL23 and IL27 (Figure 1.8). Both are heterodimers, IL23 consisting of the IL12p40 and a p19 subunit and IL27 comprising a p28 subunit and the Epstein-Barr virus-induced gene 3 (EBI3) [242-244]. Activation of the IL23 receptor complex activates almost the same signalling cascade as IL12 does but predominantly activates STAT3/4 heterodimers in contrast to STAT4 homodimers [243]. IL27 signals through Jak1, STAT1 and STAT3 [244, 245]. Both induce IFN $\gamma$  production in T cells and NK cells, though their effects are slightly different. Thus, although IL27 has almost the same functions as IL12, its ability to carry them out seems to be dependent on either IL12 or IL18 expression [231, 244, 245]. Moreover, IL23 is not as efficient at inducing IFN $\gamma$  as IL12 or IL27 but, unlike IL12, it plays an important role in supporting IFN $\gamma$  production and proliferation of memory T cells [246]. Interestingly some studies have indicated a role for IL23, a cytokine crucial for initiating Th17 development, in pathogenesis in autoimmunity models.

For example, p19 deficient mice do not develop any clinical signs of joint or bone pathology in collagen-induced arthritis models [247] and skin graft rejections are accelerated in p40 transgenic mice [248].

IL18 is another strong inducer of IFN $\gamma$ . As a member of the IL1/TLR receptor family it signals through IRAK and leads to the activation of NIK which subsequently results in the nuclear translocation of NF- $\kappa$ B [249, 250]. IL18 is expressed by DCs, monocytes and macrophages as pro-IL18 which is cleaved into the biologically active form by IL1 $\beta$ -converting enzyme upon stimulation with factors such as LPS [249]. On its own, IL18 only induces low levels of IFN $\gamma$  in CD4<sup>+</sup>T cells whereas, in combination with IL12, IFN $\gamma$  production is strongly enhanced [251].

The role of type I IFN such as IFN $\alpha$  and IFN $\beta$  in Th1 polarisation is controversial. All type I IFNs bind a heterodimeric receptor which is composed of the IFN $\alpha$ R1 and R2 chain. Type I IFN receptor binding leads to the transient activation of STAT4 [252, 253] which is associated with Th1 development. Moreover, it has been shown that IFN $\alpha$  has a strong anti-viral effect and hence, until recently it was widely accepted that type I IFNs are involved in Th1 polarisation. However it has also been shown that IFN $\alpha$  can inhibit IL12 production by DCs [254]. By contrast, *in vitro* studies using pDCs which secrete high levels of IFN $\alpha$  and subsequently drive strong Th1 responses upon exposure to viruses or CpG demonstrated that this response was almost entirely dependent on IL12 production [90]. Thus it is not entirely clear what role type I IFNs play in the polarisation of naïve T cells.

DCs also express membrane bound factors that may play a role in selective Th1 or Th2 cell polarisation. For example, ICAM-1 (intracellular adhesion molecule-1) which binds LFA-1 (leukocyte function associated molecule-1) on T cells is one such molecule that DCs up-regulate during their maturation. It has been shown that the ICAM-1/LFA-1 complex is involved in Th1 cell polarisation and that this process involves the MAP-kinases ERK and JNK [255, 256]. Furthermore CD80 and CD86 have been implicated in Th1 cell development and in IFN $\gamma$  production, although the data concerning their role in the latter function is controversial as they have also been implicated in the differentiation of other effector T cells. Interestingly, studies

in this laboratory determined that although both co-stimulatory molecules were expressed at higher levels under Th1 favouring conditions they are required for both IL4 and IFN $\gamma$  production [146].

#### **1.5.1.2 The Th1 response**

Th1 cells promote cell-mediated immunity and provide protection against intracellular protozoa, such as *Leishmania* and *Toxoplasma gondii*, bacteria, viruses and yeast. For example, they secrete IFN $\gamma$  and lymphotoxin (LT) which activate microbicidal activity as well as cytokine production in macrophages [50, 52-54]. Such Th1 responses are usually accompanied by the production of IgG antibodies, in the case of mice IgG2a antibodies, which bind to Fc gamma receptors and complement proteins and hence are involved in the opsonization and phagocytosis of particular microbes [257]. LT and IFN $\gamma$  have also been shown to recruit and activate macrophages and IL2 and IFN $\gamma$  can promote activation of NK cells and CTLs. Studies concerning the expression of chemokine receptors have shown that Th1 cells up-regulate CXCR3 and CCR5 and are therefore able to respond to chemokines such as CXCL9 and CXCL10 which are secreted by macrophages and direct the Th1 cells to sites of inflammation [258].

#### **1.5.1.3 Th1 signalling**

The Th1 signalling pathway starts with the activation of STAT1- and STAT4-associated cytokine receptors. IL12, secreted by mature DCs, binds to the IL12 membrane receptor on T cells which activates the Janus kinase Jak-STAT (signal of transducer and activator of transcription) pathway [231]. Jak2 and Tyk2 undergo phosphorylation and subsequently activate STAT4 to induce the production of IFN $\gamma$  [259]. IFN $\gamma$  from T cells or NK cells, as well as type I IFNs and IL27, initiate STAT1 signalling [244, 260, 261]. STAT1 induces up-regulation of the transcription factor Tbet, the master regulator for Th1 differentiation, which in turn promotes the up-regulation of the IL12 receptor and increases the production of IFN $\gamma$  [262]. (Figure 1.9)

## **1.5.2 Th2 cell development**

### **1.5.2.1 Dendritic cell-derived Th2 polarising signals**

In contrast to Th1 inducing factors, very little is known about the Th2 cell-promoting activities of DCs. Rather, some researchers have suggested that the absence of IL12 and IFN $\gamma$  alone may be enough to result in the stimulation of Th2 effector cells [263]. However a recent study, where schistosoma egg Ag primed DCs were used, has clearly shown that the Th2 polarizing factors OX40L is required for Th2 cell development [147] (Figure 1.7). OX40 ligand (OX40L) on DCs interacts with OX40 on T cells and is thought to play a role in proliferation of CD4<sup>+</sup> cells and their Th2 development. OX40L is a type II transmembrane molecule with a TNF homology domain in the C terminus [264]. Although it is not expressed on resting murine DCs, it is rapidly up-regulated upon CD40 ligation [265, 266]. The expression levels of OX40L depend on the initial priming conditions of the DCs and it has been shown that OX40L is not expressed if DCs are primed with IFN $\gamma$  or poly I:C. However DCs primed with Th2 promoting substances such as PGE2 or schistosomal egg Ag readily express OX40L levels which could be further up-regulated by CD40 ligation [147].

IL4 is widely established to play a pivotal role in polarising naïve Th2 cells. Some studies suggest that murine DCs are able to produce low levels of IL4 in response to certain pathogens [267] but all in all very little data concerning IL4 producing DCs has been obtained. Most likely the main source of IL4 are T cells themselves and it has been demonstrated that T cells from RAG<sup>-/-</sup> mice primed with their cognate Ag can secrete IL4 without addition of IL4 to the culture indicating that IL4 acts in an autocrine manner to drive the appearance of Th2 cells in the absence of IL12 and IFN $\gamma$  [268].

Similarly, monocyte chemoattractant protein 1 (MCP-1) is a chemokine that is rapidly produced by DCs upon TLR activation and binds to the CCR2 receptor on T cells. It attracts monocytes, memory T cells, NK cells and basophils, which can produce IL4 (NK and basophils), and it has been associated with Th2 cell development in murine infectious and allergic disease models. In these models MCP-1 was shown to promote an up-regulation of IL4 by T cells. In humans

however there is no evidence for a role of MCP-1 in the induction of a Th2 immune response.

#### **1.5.2.2 The Th2 response**

Th2 cells secrete IL4, IL5, IL6 and IL13 and are responsible for immunity to extracellular pathogens. Th2 cells have also been shown to stimulate B cells to produce high levels of IgM and IgG isotypes. In the mouse the major Th2 related isotype is IgG1, which is non complement fixing and therefore acts as an excellent neutralising antibody. IL4 is also the major inducer of B-cell switching to IgE production and, at high levels, is therefore a key initiator of IgE dependent, mast-cell-mediated reactions [269]. These reactions include the release of toxic mediators, which are important in establishment of protection against helminths, such as *Heligmosomoides polygyrus* [270] and *Trichuris muris* [271]. Similarly, IL5 has been shown to recruit and activate eosinophils [272] and hence mice lacking this cytokine show a marked defect in eosinophil responses to helminths [273]. Eosinophil recruitment to the sites of immune reactions is mediated by chemokines such as eotaxin [274]. Upon activation, eosinophils release toxic mediators including eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and major basic protein (MBP). These proteins protect against helminth infection but they have also been associated with pathology in allergic disease [275]. Mast cells are also controlled by Th2 cytokines and their activation results in the release of potent immune mediators, such as histamine, leukotrienes, proteases and TNF- $\alpha$ .

#### **1.5.2.3 Th2 signalling**

Th2 differentiation is initiated through binding of IL4 to its receptor which initiates signal transduction involving Jak1 and Jak3. Downstream this leads to the activation of STAT6 which in turn up-regulates the transcription factor GATA3 [276-278]. GATA3 then autoactivates its own expression and also transactivates both the IL4 and IL5 promoter. In addition to its Th2 promoting functions GATA3 has been shown to be a potent inhibitor of Th1 differentiation by down-regulating IFN $\gamma$



production [279]. Furthermore the transcription factor suppresses IL12 receptor expression and STAT4 activity. (Figure 1.10)

### **1.5.3 Th17 cell development**

#### **1.5.3.1 Th17 cell polarising Factors**

Recently a new type of effector T cell has been identified which mainly secretes high amounts of IL17A and thus is called a Th17 cell. Initially it was believed that these Th17 cells shared a common developmental pathway with Th1 cells however data from recent studies indicates that they are likely to develop from distinct lineages [280-282] (Figure 1.7).

During recent years many studies concerning the development of Th17 cells have been carried out and three cytokines, namely IL23, TGF $\beta$  and IL6, which are secreted by DCs and macrophages, that are crucial for Th17 lineage commitment, have been identified. As Th17 cells express the receptor for IL23 and require this cytokine for their effector function, it was initially believed that IL23 was responsible for Th17 development. However recent studies discovered that IL23 was not necessary for development of this T cell subset [283-285] but rather two other cytokines, namely IL6 and TGF $\beta$ , were found to act synergistically to drive the Th17 response [283-285]. It was shown that whereas TGF $\beta$  alone induced a Treg response, addition of IL6 deviated this reaction towards Th17 development [284, 285]. The importance of TGF $\beta$  for Th17 cell development was also demonstrated using TGF $\beta$  knock out mice. In this study it was determined that mice homozygous for TGF $\beta$ 1 deficiency were devoid of Th17 cells whereas hemizygous mice were still capable of producing some Th17 cells [285, 286].

Although it has been discovered that IL23 is not directly involved in Th17 development it has been established that the cytokine functions subsequent to Th17 commitment. IL23, which is an IL12 like cytokine (Figure 1.8), comprising the IL12p40 subunit and the IL12p35 homologue p19, binds a receptor complex consisting of the IL23R and the IL12R $\beta$ 1 receptor chain [243, 246]. Hence, as

IL23R is up-regulated by IL6 and TGF $\beta$  during initial development of Th17 cells, it was suggested that IL23 amplifies or stabilizes this T cell phenotype [286]. Indeed, in a recent study it was demonstrated that IL23 augments production of IL17 from memory but not naïve T cells [287]. In light of this new T helper cell the current model of T cell differentiation has been revised and an adaptation of this model can be found in Figure 1.7.

### **1.5.3.2 The Th17 response**

A role for Th17 cells was first discovered when studying autoimmune disease using mouse models of experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA). Both models had been associated with a Th1 response as studies using IL12p40 blocking antibody resulted in ablation of the disease [288]. However when it was discovered that the p40 subunit was also part of IL23, and that blocking it would also result in a lack of this cytokine, it was no longer clear whether the previously obtained results were due to an inhibition of IL12 or IL23 [246]. Therefore studies investigating this question were carried out and it was revealed that IL23 was the cytokine critically linked to the disease [247, 289]. Thus as it had been known that IL23 induced production of IL17 from effector and memory CD4 T cells [287] the data strongly implicated a role of Th17 cells in autoimmunity. In later studies in CIA models the role of IL17 was confirmed as neutralization of the cytokine led to a decrease of disease severity [290] whereas overexpression exacerbated it [291].

The IL17 family of cytokines comprises six members, namely IL17 (IL17A), IL17B, IL17C, IL17D, IL17E and IL17F [292-294]. IL17E is also known as IL25 and is mainly expressed by Th2 cells. The main cytokines secreted by Th17 cells are IL17 (IL17A), IL17F and IL6. IL17 and IL17F have been shown to have a high amino acid sequence identity and also seem to share similar functions [294, 295]. IL17 is a pro-inflammatory cytokine and has been shown to induce the production of IL6, granulocyte colony stimulating factor (G-CSF) and several chemokines in fibroblasts and epithelial cells [294]. Furthermore, studies revealed that treatment of fibroblasts with IL17 resulted in an up-regulation of matrix metalloproteases and

genes encoding chemokines [282]. IL17 has been shown to be a key mediator of autoimmune disease such as rheumatoid arthritis, psoriasis, inflammatory bowel disease and asthma [290, 291, 294, 296, 297] but it has also been found to be important in host defense against bacterial infection. For example, in a study using mice deficient for the IL17 receptor infected with *Klebsiella pneumonia*, it was explained that the impaired immune reaction was due to fact that G-CSF and CXCL2 production was reduced in the lungs which in turn reduced neutrophil recruitment to that organ [298].

### 1.5.3.3 Th17 signalling

To date five members of the IL17 receptor family have been determined through sequence homology searches [293] but only IL17RA which binds IL17 and IL17F has been well studied [295]. The receptor is expressed throughout the body and exists as a complex prior to ligand binding. Upon binding IL17 or IL17F the receptor complex undergoes a conformational change and the intracellular domains dissociate [299, 300]. Even though many studies concerning the development of IL17 secreting cells have been conducted still very little is known about the signalling cascades that lead to the Th17 phenotype (Figure 1.11). Among the few things that have been discovered, however, are that Tbet, which promotes Th1 cell development, can inhibit development of Th17 cells [301]. Furthermore the cytokines IL4, IL27 and the IFNs have also been found to down-regulate Th17 production [280, 282, 302, 303]. In addition Stat4 and Stat6 which are important for Th1 and Th2 development were reported not to be required for Th17 development [280, 282]. Recently a very interesting discovery was made when the transcription factor ROR $\gamma$ t was identified as the putative key transcription factor for Th17 development and a strong correlation between ROR $\gamma$ t and IL17 expression was found [304]. However as the ligands for ROR $\gamma$ t are unknown it is not clear how the transcription factor initiates or influences IL17 transcription. In a recent paper another interesting finding was made when important roles for Stat3 and Stat4 were identified [305]. It was shown that Stat3, which can bind the IL17A promoter in response to IL23 stimulation [306], is actually bound to it during Th17 differentiation with TGF $\beta$  and IL6 [305]. Furthermore it was discovered that ROR $\gamma$ t

expression is Stat3 dependent. In addition, Stat4 was required for the secretion of IL17 from IL23 primed cultures after restimulation either with anti-CD3 or cytokines indicating that this transcription factor is necessary for the maximal development of Th17 cells [305]. Nevertheless, even though a lot of research has been carried out recently investigating the mechanisms of Th17 development, most of the signalling cascades that lead to the production of IL17 secreting cells remain unknown.

#### **1.5.4 Regulatory T cell development**

##### **1.5.4.1 Dendritic cell-derived signals that drive Regulatory T cell development**

During the past few years two major subsets of regulatory T cells have been distinguished: naturally occurring Tregs which develop in the thymus and adaptive Tregs which originate from peripheral naïve or central memory T cells [307-311]. Currently a lot of evidence suggests that DCs are involved in the development of both types of Tregs and hence, that DCs play a very important role in the development of peripheral tolerance (Figure 1.7). Previously, immature DCs (iDCs) were implicated in the generation of Tregs [312-314] however more recent studies have shown that Tregs expand under the influence of Ag-processing DCs [315, 316] indicating that a certain amount of co-stimulation is necessary for the development of Tregs. This has led to the identification of semimature DCs that express most of the surface maturation markers but are unable to secrete the majority of cytokines produced by fully mature DCs [150, 317]. Recent studies have demonstrated that these DCs can be protective in Th1 models such as experimental autoimmune encephalitis (EAE) [317] and collagen induced arthritis (CIA) [318].

DC-derived cytokines that have been widely associated with Treg development are IL10 and TGF $\beta$  [311, 319, 320]. Thus, it has been shown that IL10 induces anergy in T cells by inhibiting the proliferation and cytokine production of those cells. Furthermore, studies have determined that IL10 inhibits the full maturation of DCs and down-regulates MHCII molecule expression on the cell surface [321, 322].

This in turn has a negative effect on the production of IL12 [321, 322]. Collectively therefore, IL10 treatment of DCs leads to the development of regulatory phenotype DCs which in turn secrete IL10 and promote the development of IL10 secreting Tregs [322, 323]. Studies with bacterial and parasite-derived molecules have also been shown to drive production of IL10 producing regulatory DCs [324, 325].

Apart from IL10 there are several other factors that may contribute to the development of Tregs such as novel members of the B7 family [326] and the notch signalling pathways [327]. For example, recently the DC-specific B7 molecule, PD-L2, also called B7-DC, and its counter-structure PD-L1 have been proposed to have negative regulatory functions in the immune response [328, 329], however a definitive answer has not yet been found.

#### **1.5.4.2 T regulatory cells**

The ability to discriminate self from non self is a crucial function of the immune system and a breakdown in this mechanism leads to the development of autoimmune disease. Even though T cells undergo a complex selection process in the thymus, not all self-reactive T cells are removed and some (5%) can be found even in healthy individuals suggesting that a mechanism of immune suppression exists. The concept of regulatory or “suppressor” T cells emerged over 20 years ago, however, the lack of identification of a phenotype for these cells saw this field fall into disrepute and it was not until the elegant work of Sakaguchi and co-workers that a role for regulatory T cells re-emerged [330]. These studies showed that adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> depleted T cells induced organ-specific autoimmune disease in immunodeficient recipients and that mice thymectomized on day 3 did not exhibit autoimmunity if CD4<sup>+</sup>CD25<sup>+</sup> T cells were replaced indicating a role for CD4<sup>+</sup>CD25<sup>+</sup> T cells in controlling self-tolerance.

Currently two different types of CD4<sup>+</sup>CD25<sup>+</sup> Tregs can be distinguished which differ in their origin, Ag specificity and effector mechanism (Figure 1.12). One of these subsets develops during the normal process of T cell maturation in the thymus and is called the naturally occurring population (nTreg). The other subset also derives

from a thymic precursor but develops in the periphery from the CD4<sup>+</sup>CD25<sup>-</sup> T cell population due to suboptimal Ag exposure and/or co-stimulation [312-316].

#### **1.5.4.2.1 Naturally arising regulatory T-cells (nTreg)**

Several different markers have been associated with Tregs but the most widely used one appears to be CD25. Furthermore nTregs also express CTLA4 and GITR (glucocorticoid-induced TNF receptor) [331]. However care has to be taken when using CD25 or any of the other markers in distinguishing Tregs from the conventional T cell population since all three markers are readily up-regulated upon TCR activation.

In a normal animal, nTregs account for 5-10% of all peripheral CD4<sup>+</sup>Tcells. They possess powerful immunomodulatory functions and it has been shown that their removal leads to the development of autoimmune disease [332]. Currently it is not known whether nTregs derive from their own precursor or if particular circumstances during Ag presentation in the thymus are responsible for their development. Recent studies suggest that nTregs not only prevent the activation of autoreactive T cells, but also modulate the immune response to infectious agents and prevent and down-regulate exuberant inflammatory responses [333].

The signals for generating Tregs in the thymus, as well as in the periphery, are not entirely understood but CD28 has been indicated to have an important function in the development of Tregs [334]. It has been proposed that a strong TCR ligation and co-stimulation might be required for nTregs to develop from their precursors and that CD28 co-stimulation could be necessary to maintain a stable peripheral pool of Tregs by promoting their survival. Moreover, downstream induction of IL2 or Bcl-2 might mediate these two functions as not only it has been shown that a disruption in IL2 signalling leads to a lack of nTregs but also that some studies revealed that IL2 is required during thymic development for the production of nTregs [335, 336]. However, molecules such as CD80 and CD86, CD40, CD40L have also been implicated in the production of Tregs as their absence results in a significant reduction in frequency and function of nTregs [337].

Recent studies have indicated a key role for the transcription factor FoxP3 in the generation of Tregs [338-340]. Thus, Tregs have been shown to express high amounts of FoxP3 and FoxP3-deficient mice are unable to produce CD4<sup>+</sup>CD25<sup>+</sup>Tcells [338]. Furthermore studies revealed that transfer of Tregs into FoxP3 deficient mice suppressed the development of autoimmunity and that over-expression of this gene increased the production of Tregs [338].

The molecular mechanisms underlying T cell mediated immune suppression are not known. However, currently it is believed that activation of Tregs via the TCR, or via TLR pathways, is needed to initiate suppression of CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses [341-346]. In addition, studies investigated whether nTregs had a direct suppressor effect on other T cells or whether they mediated their suppressor function by modulating DCs. One recent *in vitro* study identified that the suppression is indeed T-T contact dependent as it was carried out in the absence of DCs [344]. However a stabilising role of APCs has not been ruled out (Figure 1.13).

The role in regulation of tolerance and disease by cytokines such as IL10 and TGFβ produced by nTregs has been a subject of wide discussion for a long time. Recently, however, it has been shown that nTreg-mediated immune regulation appears to be contact-dependent rather than cytokine mediated as blocking the action of IL10 and TGFβ does not reduce the function of nTregs [341, 342, 347]. Furthermore T cells unresponsive to TGFβ still have normal responses to nTregs [347]. However, studies in *L. major* indicated that even though IL10 was not needed for nTreg response during the acute phase of infection, nTregs depended on it during the chronic stage of disease [348]. This indicated that IL10 might be required for long term survival of Tregs or for the induction of induced Tregs (iTregs). Co-culture of nTregs and iTregs confirmed that nTregs and the cytokines they secrete are important for the differentiation of naïve T cells into iTregs and indeed, that the type of iTreg that is produced is dependent on the cytokine that is secreted by nTregs [349].

#### 1.5.4.2.2 Induced regulatory T-cells

Activation of naïve peripheral CD4<sup>+</sup>CD25<sup>-</sup>T cells can give rise to both regulatory and effector T cells. The decision as to whether iTregs or T effector cells are produced seems to depend on the stimulus the naïve T cell is exposed to. Thus, in *ex vivo* experiments it has been shown that cytokines such as IL10 and TGFβ, vitamin D3, dexamethasone, CD40-CD40L blockage and culture with immature DCs contribute to the process of iTreg production [350-353]. Two different types of iTregs can be distinguished according to their cytokine expression profile, namely those designated as Th3 cells and Tr1 cells. Th3 cells were first described by Weiner in context of oral tolerance [354]. He identified this subgroup of cells because they only secreted TGFβ which made them distinguishable from Th2 cells that also secreted IL4 and IL10. In 1997 Groux *et al* identified the second type of iTregs, namely Tr1 cells [355]. These cells were shown to be produced in response to cognate Ag in the presence of IL10. Tr1 cells produce high levels of IL10 themselves and efficiently suppress Th1 responses.

It is currently not entirely clear how iTregs are induced but it has been demonstrated that IL10 and TGFβ play a major role in the suppression mediated by these cells as blocking these cytokines reverses their action. For example, it has been shown that IL10 inhibits the production of IL12 and TNF from DCs and macrophages whereas TGFβ inhibits Th1 responses through acting on the transcription factor T-bet and IL12R [356-358]. Furthermore IL10 is known to inhibit the up-regulation of MHC II and co-stimulatory molecules on DCs and could therefore also suppress T effector cell development [358, 359] (Figure 1.13).

#### 1.5.4.3 Treg signalling

Very little is known about the intracellular signalling mechanisms that lead to the development of Tregs. It has been established in many different studies that the forkhead-family transcription factor FoxP3 is the key transcription factor associated with natural Treg development [339, 360] and recent studies have also provided evidence for FoxP3 expression by induced Tregs [361, 362]. The importance of



FoxP3 has been highlighted by experiments using mice deficient for scurf, a product of the FoxP3 gene as these mice develop an autoimmune disease which is fatal after a couple of weeks. It has been suggested that the reason for this fatal outcome of the disease is that the mutation of FoxP3 that causes the loss of function of Scurfin also causes a complete loss of Tregs.

In recent years the regulation of FoxP3 expression and the signalling pathways that lead to the development of nTregs and iTregs have been investigated. TCR signalling has been shown to play a critical role in both nTreg and iTreg generation [363-365], however as the TCR downstream pathways are very complex, to date the precise details are still poorly understood. In addition to TCR stimulation, several studies have suggested that signalling through CD28 is critical for the generation of nTregs [366, 367] whereas production of iTregs appears more likely to depend on the engagement of CTLA-4 by B7 molecules [368]. Furthermore the induction of iTregs appears to depend on TGF $\beta$  and it has been demonstrated that this reaction depends on CTLA-4-B7 signalling [365, 368, 369]. Another molecule that has been implicated in development of iTregs is IL2 [370-373] which upon binding to its receptor signals via a Jak3-Stat5 cascade. Consistent with this, it has been shown that IL2 can up-regulate FoxP3 expression in human CD4<sup>+</sup>CD25<sup>+</sup> T cells via a Stat dependent mechanism [374] and that transient activation of Stat5 is sufficient to increase CD4<sup>+</sup>CD25<sup>+</sup> T cell numbers in IL2 deficient mice [375].

At present, however, the molecular mechanisms that lead to the development of Tregs are not fully understood. Even though, as mentioned above, several molecules involved in the regulation of FoxP3 expression have been discovered more studies are needed to identify the signalling cascades that lead to the generation of these two T cell populations.

## **1.6 Filarial nematodes**

Filarial nematodes are arthropod-transmitted parasites which infect vertebrates including humans. Among the eight species known to infect man three are of major medical importance: *Wuchereria bancrofti*, *Brugia malayi*, *Onchocerca volvulus*. In

the tropics more than 150 million people are infected with one or more of these worms and lymphatic filariasis is a major cause of morbidity in these regions [376]. In humans, significant pathology is mainly caused either through obstruction and damage of the lymphatic system by adult parasites, as in case of *W. bancrofti* and *B. malayi*, or through cutaneous and ocular irritation by larval transmission stages of *O. volvulus*.

The parasites are transmitted by mosquitoes or blackflies when they are in the third-stage larval state. In the skin the larvae develop into the fourth stage and start migrating to the lymphatics where they develop to the adult stage. There, the mature adult female starts to produce and release microfilariae (MF) that enter the bloodstream where they circulate and can be taken up by the insect vector. It has been shown that the adult worms of *W. bancrofti* and *B. malayi* can survive in the host for years, apparently evading the immune response [377].

In endemic areas there is a broad spectrum of response to lymphatic filariasis ranging from asymptomatic cases to patients developing severe clinical symptoms [378, 379]. There is a general association between the ability to clear microfilaremia and apparent clinical symptoms. Patients who are amicrofilaraemic may be immune to the adult worm but very often they exhibit chronic pathology such as elephantiasis. In contrast, people who are microfilaraemic often do not show clinical symptoms of infection although subclinical pathological changes in the lymphatic system have been detected. Some individuals have been shown to stay symptom- and MF-free even though they are exposed to infected mosquito bites.

In individuals with active infection and circulating MF, high titres of IgG4 as well as significant eosinophilia have been found. In contrast to uninfected individuals where IgG4 levels comprise about 5% of Abs in the bloodstream, levels in filarial patients can be as high as 95% [380]. Furthermore IgE levels have also been found to be up-regulated in these patients. Interestingly IgE levels increase with the development of chronic stages of the disease and loss of circulating MF [380]. Therefore it has been suggested that IgE might play a protective role in filariasis

and other helminthic disease [381, 382] and that blocking its function could constitute a parasite immune evasion technique. Indeed it has been demonstrated that parasite induced IgG4 is competing with IgE for epitope recognition [383]. For a long time IgE was thought to be responsible for the development of the chronic pathology found in filariasis but recent papers described an up-regulation of IgG2 and IgG3 in addition to IgE which might indicate that these molecules collectively play a role of in the disease process [380].

Even though filarial nematodes clearly stimulate the host immune system and mount an antibody response to infection they can survive in the host for a long time. It is now accepted that this is accomplished by modulating immune responses but the mechanism underlying this immunomodulation is not clear so far. However it is increasingly accepted that excretory-secretory proteins which are synthesised and secreted by nematodes, and can be found in the bloodstream of infected humans and animals, play a major role in this process.

### **1.6.1 Immunomodulation by filarial nematodes**

Filarial nematodes exert profound effects on the immune system of their hosts [384, 385]. Studies have confirmed that individuals with human filariasis who have MF in their bloodstream exhibit a modulation of T cell responses. Indeed, it has been shown that the production of IFN $\gamma$  an inflammatory cytokine playing a crucial role in initiating a Th1 immune response is suppressed [386, 387] in these individuals whereas IL4 and IL10 levels are enhanced. Therefore it has generally been suggested that such parasite infections bias immune responses towards a Th2 phenotype and consistent with this hypothesis IL4 responses have been found to be elevated in people living in endemic areas whereas IFN $\gamma$  production was shown to be reduced in active infection [387, 388]. Several studies have proposed a role for parasite induced IL10 in the down-regulation of immune responses [389, 390] which would have a detrimental effect on the parasite and consistent with this PBMC from patients with circulating MF (relatively asymptomatic) have been shown to secrete strongly increased amounts of this cytokine compared to PBMC from patients with chronic lymphatic pathology [391]. IL10 has been associated

with the development of Tregs [332, 392-396] and it has been shown that neutralising Ab against IL10 and TGF $\beta$  restore the ability of T cells to mount a response against the parasites [397, 398]. Hence it was hypothesised that filarial nematodes induce T reg populations that suppress T effector responses [399, 400]. Thus, although eradication of the pathogen is the desired outcome, initial persistence might be crucial in developing memory T cells which in turn would confer protection to re-infection with the same parasite. However, a recent study revealed that during filarial infection, T regs act in an IL10 independent manner [401].

In recent years the mechanisms by which parasites could modulate the immune response were investigated and as DCs are the main APCs involved in initiating an immune response it was suggested that such parasites might achieve immune hyporesponsiveness by modulating DC function. It is now well established that parasite-derived molecules have an immunomodulatory function and promote parasite survival in the host and it has been shown that exposure to at least one of these products (ES-62) results in a modulation of the DC phenotype and that this consequently has an effect on the T cell response [146, 402-404].

### **1.7 ES-62 is a filarial nematode excretory-secretory molecule with immunomodulatory properties**

ES-62 is an excretory-secretory glyco-protein which has a molecular mass of 62kD and was initially identified in rodents infected with the filarial nematode *Acanthocheilonema viteae* [405]. Later on, database searches revealed that homologues of the parasite product were also secreted by other nematodes [406, 407] and recently ES-62 was cloned from *A. viteae* and *B. malayi* cDNA libraries [408, 409]. A common feature of ES proteins, including ES-62, is the presence of phosphorylcholine (PC) which is attached to the protein via N-linked carbohydrates [410, 411]. PC is a conserved structural component of a variety of prokaryotic and eukaryotic pathogens that has a strong immunomodulatory function and has been suggested to be responsible, at least in part, for the immunomodulatory effects of ES proteins on the immune system [402, 411-421]. Consistent with this, it has

been shown that ES proteins can be found in the bloodstream of infected animals and humans. Indeed, the highest levels of ES products have been detected in the bloodstream of patients with MF who represent the most hyporesponsive category of patients [422]. Furthermore infected rodents show high levels prior to the onset of patency [405]. Thus it seems that parasites release ES products in order to subvert host immune responses and it is likely that the immunological characteristics associated with filarial disease are, at least in part, the result of immunomodulation by these proteins.

Consistent with this ES-62 has been shown to have potent immunomodulatory effects on macrophages, DCs, B cells and the antibody response [146, 420-426]. Thus, in recent *in vitro* and *in vivo* studies it has been shown that ES-62 promotes a DC phenotype that induces Th2 responses [146]. In addition it has been established that ES-62 dampens down the pro-inflammatory cytokine response of DCs and macrophages elicited by LPS. Moreover it has been shown that murine DCs and macrophages derived from bm progenitors exposed to ES-62 *in vivo*, via implanted osmotic pumps, are hyporesponsive to subsequent stimulation with LPS [418]. However, ES-62 itself induces a low but transient production of pro-inflammatory cytokines such as IL12, TNF $\alpha$  and IL6 and thus it is thought that this may possibly subsequently block the substantial production of pro-inflammatory cytokines by inflammatory mediators [425] or alternatively, reflect abortive activation of these pathways. Additional studies have shown that such production of IL12 and TNF $\alpha$  is abrogated in TLR4 and MyD88 deficient mice suggesting that TLR4 and MyD88 are important in the ES-62 signalling pathway and that ES-62 may act to subvert normal pro-inflammatory TLR4-signalling.

Although much has been learned about the structure and function of ES-62 many questions remain unanswered. As the molecule is large and complex and has been shown to have various immunomodulatory functions *in vivo* and *in vitro* many more studies are needed to fully understand how the protein interacts with the immune system and which structures are responsible for the observed functions.

## 1.8 General Aims

Parasitic filarial nematodes are often tolerated in the human hosts for decades with little evidence of pathology due to parasite-induced immune modulation. Consistent with this excretory-secretory molecules such as ES-62, which are produced by a number of filarial nematodes, have been shown to exhibit immunomodulatory activities that are broadly anti-inflammatory in nature. ES-62 does not appear to directly influence T cells but rather to act via modulation of the maturation of dendritic cells (DCs). Dendritic cells are specialised Ag-presenting cells critical for initiating and regulating immune responses as in addition to priming and tolerising T cells they produce cytokines that have important immune regulatory functions. To date more than 6 different DC subtypes have been identified and these appear to arise from at least two different cell lineages. These subtypes are not only phenotypically distinct but are also thought to perform different functions and to be restricted to certain lymphoid organs. It is therefore planned to investigate the effects of ES-62 on the functional phenotype of individual DC subtypes and the potential consequent modulation of Th effector responses, both *in vitro* and *in vivo* disease models. ES-62 is a PC-containing molecule and PC is a molecular pattern associated with pathogen products of a diverse range of organisms. Thus previous studies have investigated the role of TLRs in ES-62-mediated immunomodulation and discovered TLR4 as a putative receptor for the parasite product. However as ES-62 shows homology to other molecules such as PAF and has recently been shown to bind the mannose receptors it was decided to further investigate the functional role of these putative ES-62 co-receptors. Therefore in particular it was planned to

- 🧪 Characterise the effects of ES-62 on spleen- and bone marrow-derived DCs and on their ability to prime and polarize T cell responses
- 🧪 Characterise whether ES-62 had differential effects on distinct spleen-derived DC subtypes and whether ES-62 exposure of such DC subtypes to ES-62 altered their ability to prime and for T cell polarize responses

- ✚ Investigate the effects of exposure of DCs, from murine models of inflammatory disease, CIA and allergic airway inflammation, to ES-62 on DC responses and their consequent ability to prime and/or polarise T cells
- ✚ Further characterize the role of TLR4 in ES-62-mediated immunomodulation
- ✚ Investigate whether the mannose and PAF receptor can act as functional ES-62 receptors or co-receptors

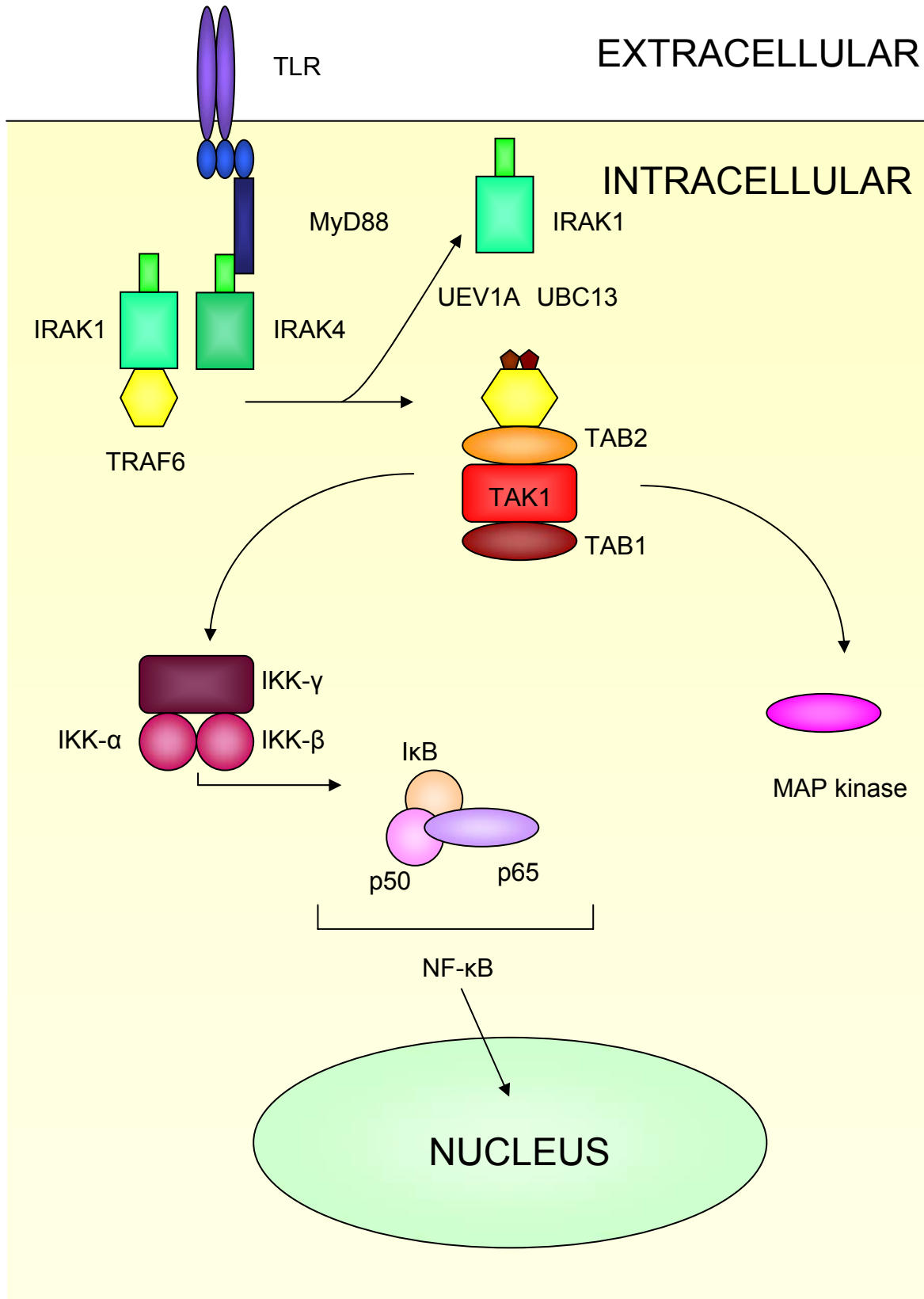
### **Table 1.1 Toll like receptors and their ligands**



Receptor	Ligand	Origin of ligand	Expression
TLR1	Triacyl lipopeptides Soluble factors	Bacteria and mycobacteria <i>Neisseria meningitidis</i>	extracellular
TLR2	LPS Peptidoglycan Lipoteichoic acid Lipoarabinomannan Phenol-soluble modulin Glycoinositolphospholipids Glycolipids Porins Atypical lipopolysacharide Zymosan	Various pathogens Gram-positive bacteria Gram-positive bacteria Mycobacteria <i>Staphylococcus epidermidis</i> <i>Trypanosoma cruzi</i> <i>Treponema maltophilum</i> <i>Neisseria</i> <i>Leptospira interrogans</i> Fungi	extracellular
TLR3	dsRNA	Viruses	intracellular
TLR4	Lipopolysaccharide Taxol Fusion protein Envelope protein Endogenous ligands such as heat-shock proteins	Gram-negative bacteria Plants Respiratory syncytial virus Mouse mammary tumor virus Host	extracellular
TLR5	Flagellin	Bacteria	extracellular
TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan	<i>Mycoplasma</i> Gram-positive bacteria Fungi	extracellular
TLR7	ssRNA Imidazoquinoline Loxoribine Bropirimine	Viruses Synthetic compounds Synthetic compounds Synthetic compounds	intracellular
TLR8	ssRNA Imidazoquinoline	Viruses Synthetic compounds	intracellular
TLR9	CpG-containing DNA	Bacteria and viruses	intracellular
TLR10	not determined	not determined	not determined
TLR11	not determined	Uropathogenic bacteria	not determined

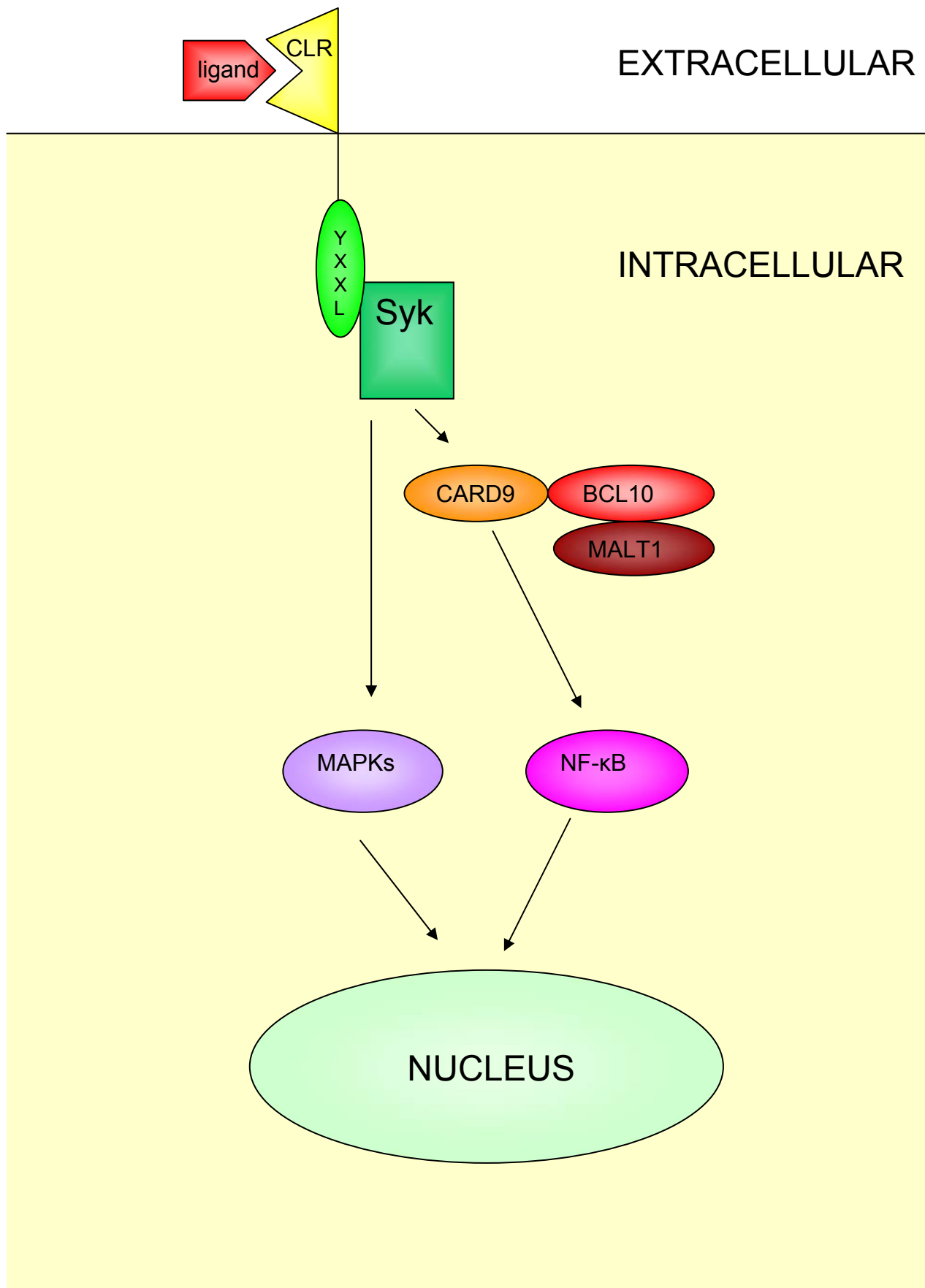
## Figure 1.1 TLR4-mediated signal transduction

Upon pathogen-derived TLR ligand binding, the TIR domain of the TLR dimerises and associates with the signalling molecule myeloid differentiation primary-response protein 88 (MyD88). MyD88 recruits IL-1R associated kinase 4 (IRAK4) which in turn associates with IRAK1 and initiates its phosphorylation. Tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) binds to the phosphorylated IRAK1, dissociates from the receptor and forms a complex with transforming-growth-factor- $\beta$ -activated-kinase (TAK1), TAK1-binding protein (TAB1), and TAB2. IRAK then dissociates from TRAF6 and the remaining complex translocates to the cytosol where it associates with ubiquitin-conjugating enzyme 13 (UBC13) and UEV1A which induce the ubiquitinylation of TRAF6 and leads to the activation of TAK1. TAK1 phosphorylates MAP kinases and the inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase (IKK) complex which initiates processes that subsequently lead to the degradation of I $\kappa$ B and the translocation of NF- $\kappa$ B to the nucleus.



**Figure 1.2 Brief description of the signalling cascades after Dectin-1 stimulation**

Upon ligand binding, the tyrosine kinase Syk becomes activated. This in turn triggers either the activation of mitogen-activated protein kinases (MAPKs) or NF- $\kappa$ B via the adaptor CARD9 which forms a complex with the adaptor proteins Bcl10 and MALT1. Signalling via MAPKs and NF $\kappa$ B then results in the transcription of immune response genes in the nucleus.



**Table 1.2 DC subtypes and their characterisation in terms of surface marker expression**

	Conventional DCs					plasmacytoid dendritic cell precursor	IKDC
	"lymphoid"	"myeloid"			"langerhans"		
Distribution	CD4 <sup>-</sup> CD8 <sup>+</sup> CD205 <sup>+</sup> CD11b <sup>-</sup>	CD4 <sup>+</sup> CD8 <sup>-</sup> CD205 <sup>-</sup> CD11b <sup>+</sup>	CD4 <sup>-</sup> CD8 <sup>-</sup> CD205 <sup>-</sup> CD11b <sup>+</sup>	CD4 <sup>-</sup> CD8 <sup>-</sup> CD205 <sup>+</sup> CD11b <sup>+</sup>	CD4 <sup>-</sup> CD8 <sup>lo</sup> CD205 <sup>hi</sup> CD11b <sup>+</sup>	CD11c <sup>medium</sup> CD45RA <sup>hi</sup> MHC II <sup>lo</sup>	CD11c <sup>medium</sup> CD45RA <sup>hi</sup> CD11b <sup>+</sup> NK1.1, NKG2D, Ly 49
Spleen	23	56	19	<4	<1	not determined	detected
Thymus	70	Small population that is CD8 <sup>-</sup> and Sirpα <sup>+</sup>			unknown	Small, Ly6C <sup>+</sup>	detected
MLN	19	4	37	26	<4	not determined	detected
Skin draining LN	17	4	17	20	33	not determined	detected

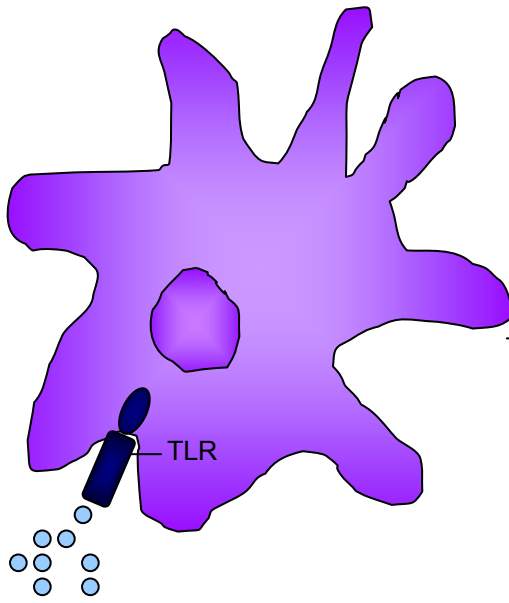
Table adapted from Shortman, K. et. al., Nat. Rev. Immunol. 2002, 2(3), 151-61

### **Figure 1.3 Figure 1.3 Dendritic cell maturation**

Following exposure to pathogen products that signal via TLRs, immature DCs initiate a maturation programme and undergo a series of phenotypical changes and lose their capacity to take up Ag. During this process DCs up-regulate MHC II complexes, co-stimulatory molecules such as CD40, CD80/CD86 and adhesion molecules such as CD54 which are essential for the activation of naïve T cells. Depending on the initial stimulus DCs secrete specific cytokines and chemokines which are important for polarising T cell responses for example, either in a Th1 or Th2 direction. In addition cell-cell contact can lead or contribute to DC maturation.

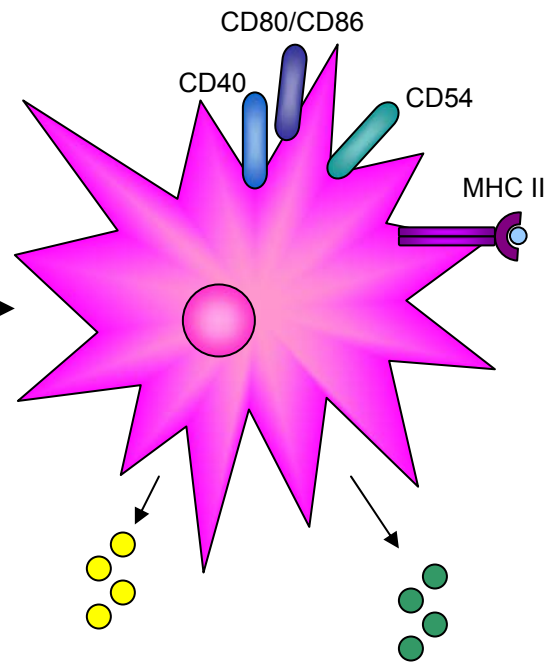


Immature DC



Pathogen products

Mature DC



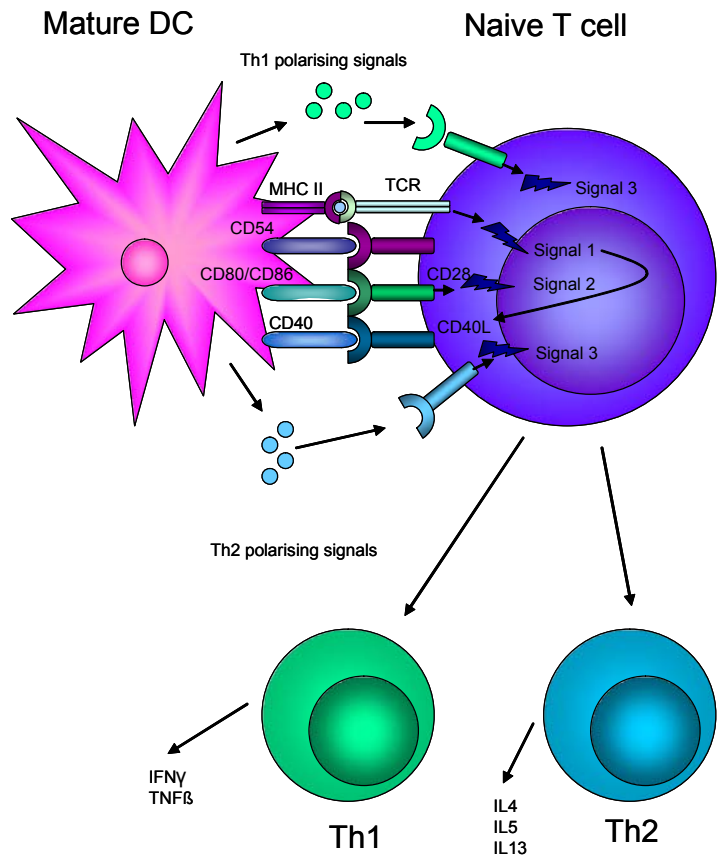
Th1 polarising signals

Th2 polarising signals

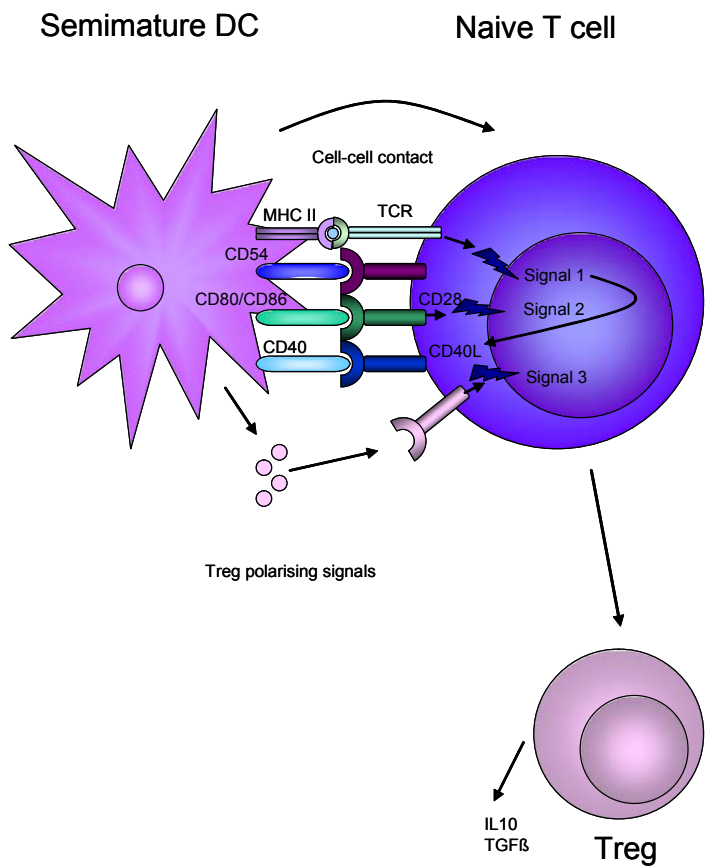
### **Figure 1.4 Signals involved in T cell polarisation**

DCs use three kinds of signals to (referred to as signal 1, 2, 3) to initiate a T cell response. Signal 1 is provided by the expression of cognate Ag in the context of MHC II complexes on the DC surface which is recognised by T cell receptors (TCR) on Ag-specific T cells. DCs also express up-regulated levels of co-stimulatory molecules such as CD80 and CD86 upon maturation (signal 2). Taken together, signal 1 and signal 2 initiate T cell priming and lead to the up-regulation of CD40L expression, rendering the T cell sensitive to CD40 mediated signals. Signal 3 is the polarising signal that is mediated by various soluble or membrane bound factors and promotes the development of Th1 or Th2 (a) cells respectively. If DCs are semi-mature due to insufficient activation they are unable to provide all three signals to the same extent as activated mature DCs and are believed to preferentially prime Treg (b) cells.

a

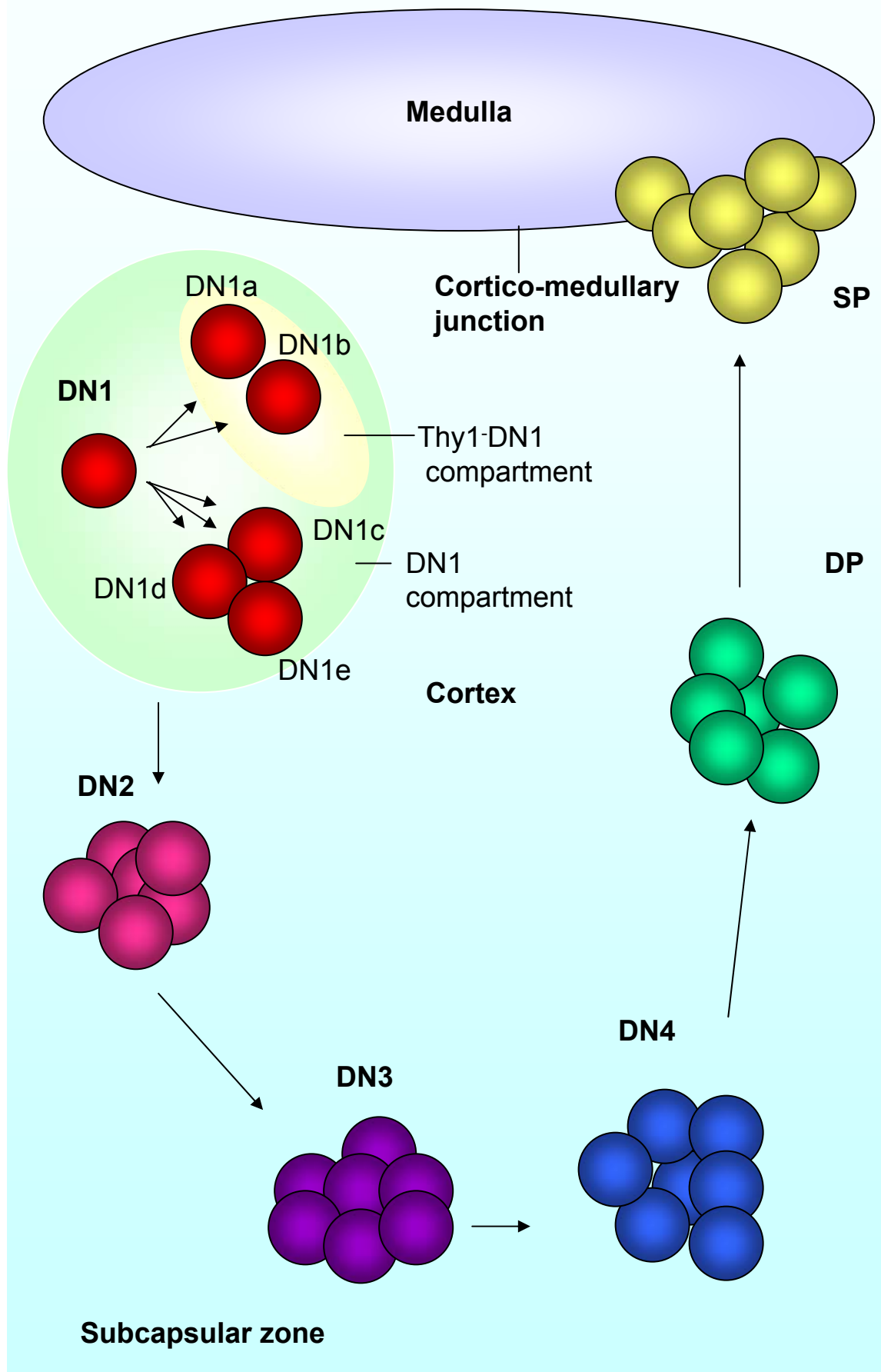


b



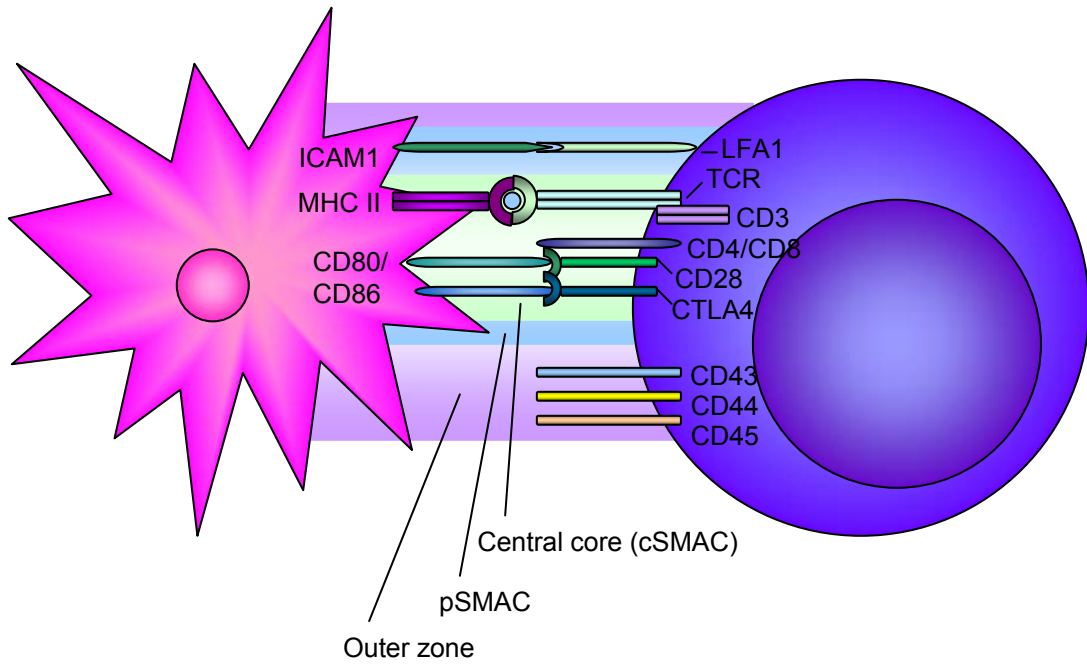
### **Figure 1.5 T cell positive and negative selection in the thymus**

The first thymocytes are called double negative 1 (DN1) due to their surface marker expression profile and can be found in the DN1 compartment of the thymus. They can further be divided on the basis of CD24 and c-Kit into two fractions that are c-Kit high (DN1a and b), and three fractions (DN1c, d and e) that are c-Kit low. The DN1a and b subgroups can only be found in the Thy1<sup>-</sup>DN1 compartment. As these cells mature they migrate through the thymus and progress through three more stages the DN2 stage, the DN3 stage and the DN4 stage. The DN4 cells are the immediate progenitors of the CD8<sup>+</sup>CD4<sup>+</sup> thymocytes which are also called double positive (DP) cells and from which single positive CD4<sup>+</sup> or CD8<sup>+</sup> cells arise. In the pre DP stages all cell migration takes place in the cortex. DN cells migrate towards the medulla and undergo positive and negative selection processes.



### **Figure 1.6 Overview of the immunologic synapse**

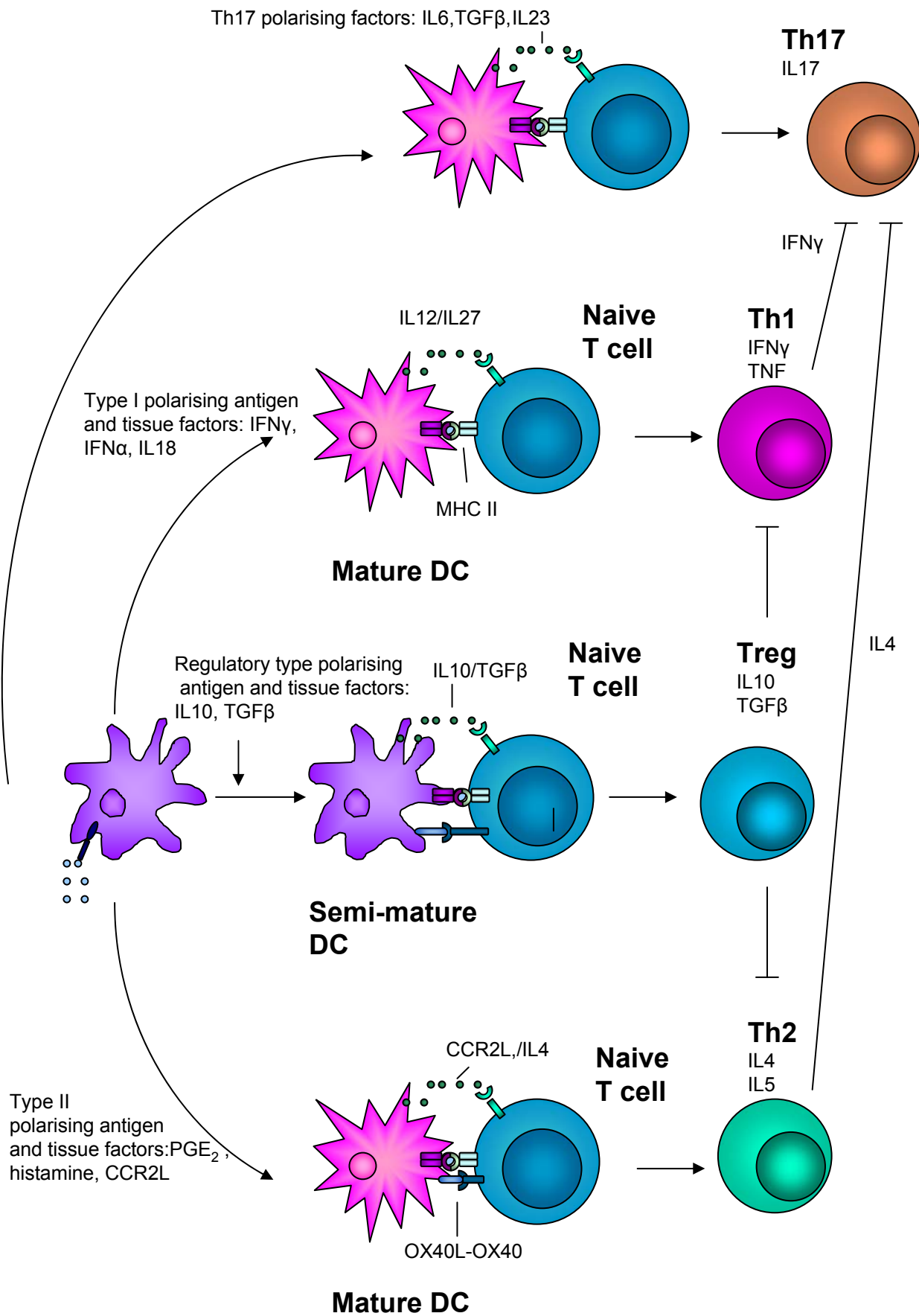
Optimal T cell activation can only be achieved by the formation of an immunological synapse (IS). Such an IS consists of a central core of TCR/CD3/CD28-MHC complex the so called central supramolecular activation cluster (cSMAC) and is surrounded by an outer ring structure consisting of the integrin LFA-1 and ICAM. LFA-1 and ICAM form the peripheral supramolecular activation cluster (pSMAC) which is believed to stabilise the inner complex. Surrounding the pSMAC is an outer zone in which molecules such as CD43, CD44 and CD45 can be found.



### **Figure 1.7 The influence of DCs on T cell polarisation**

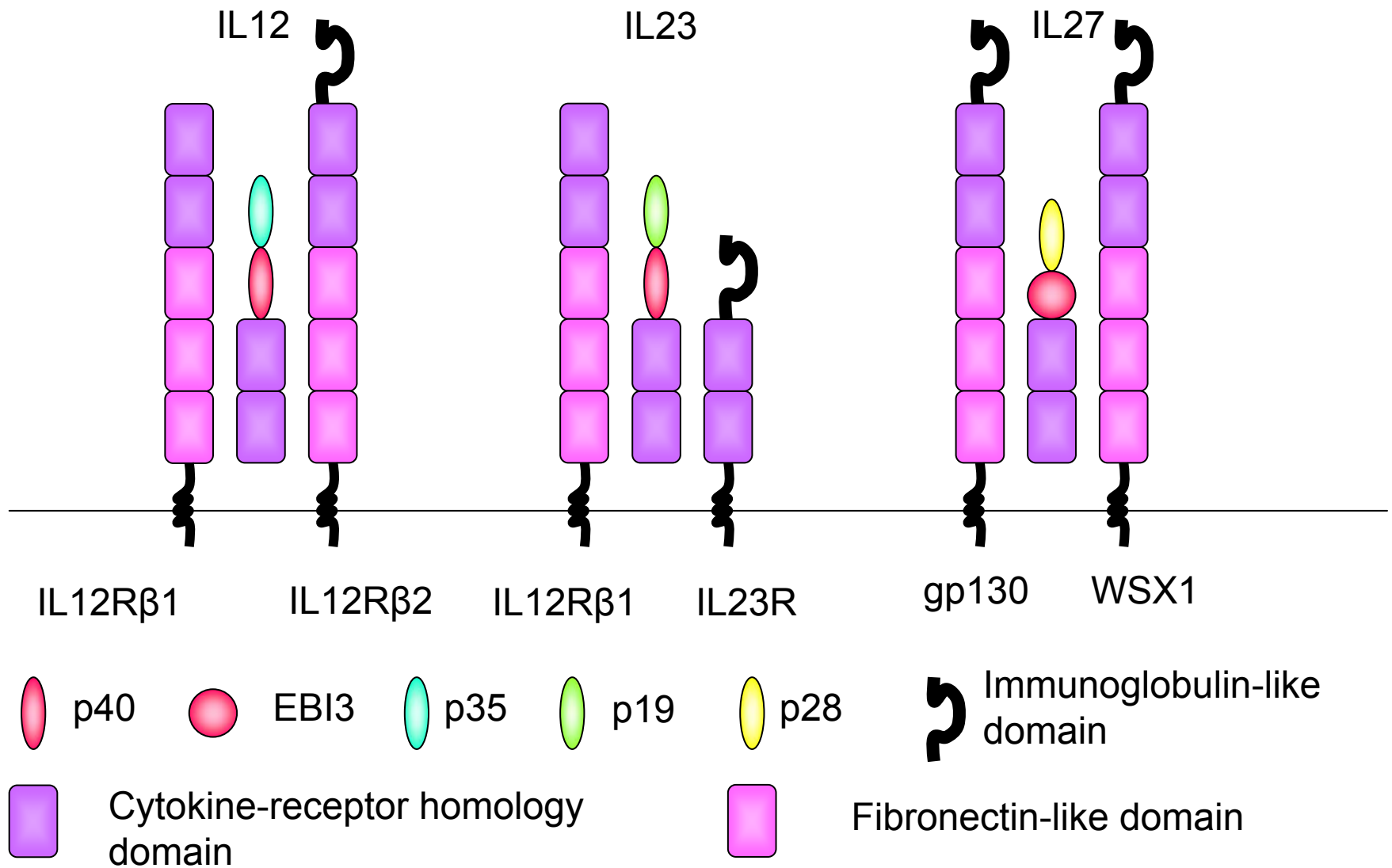
DCs can be polarized to become fully activated mature DCs in the presence of type 1 or type 2 promoting Ags and tissue factors. However in the presence of regulatory-type Ags and tissue factors this full DC maturation is inhibited resulting in the development of semi-mature DCs. Depending on the initial stimulation DCs also start secreting different sets of cytokines and chemokines and promote the development of different T helper cells. Mature DCs are capable of inducing Th1, Th2 and Th17 cells whereas semimature DCs are thought to induce the development of T regulatory cells. T regulatory cells have been shown to inhibit Th1 and Th2 effects by secreting IL10 and TGF $\beta$ . Th17 cell development has been shown to be suppressed by the Th1 cytokine IFN $\gamma$  and the Th2 cytokine IL4.





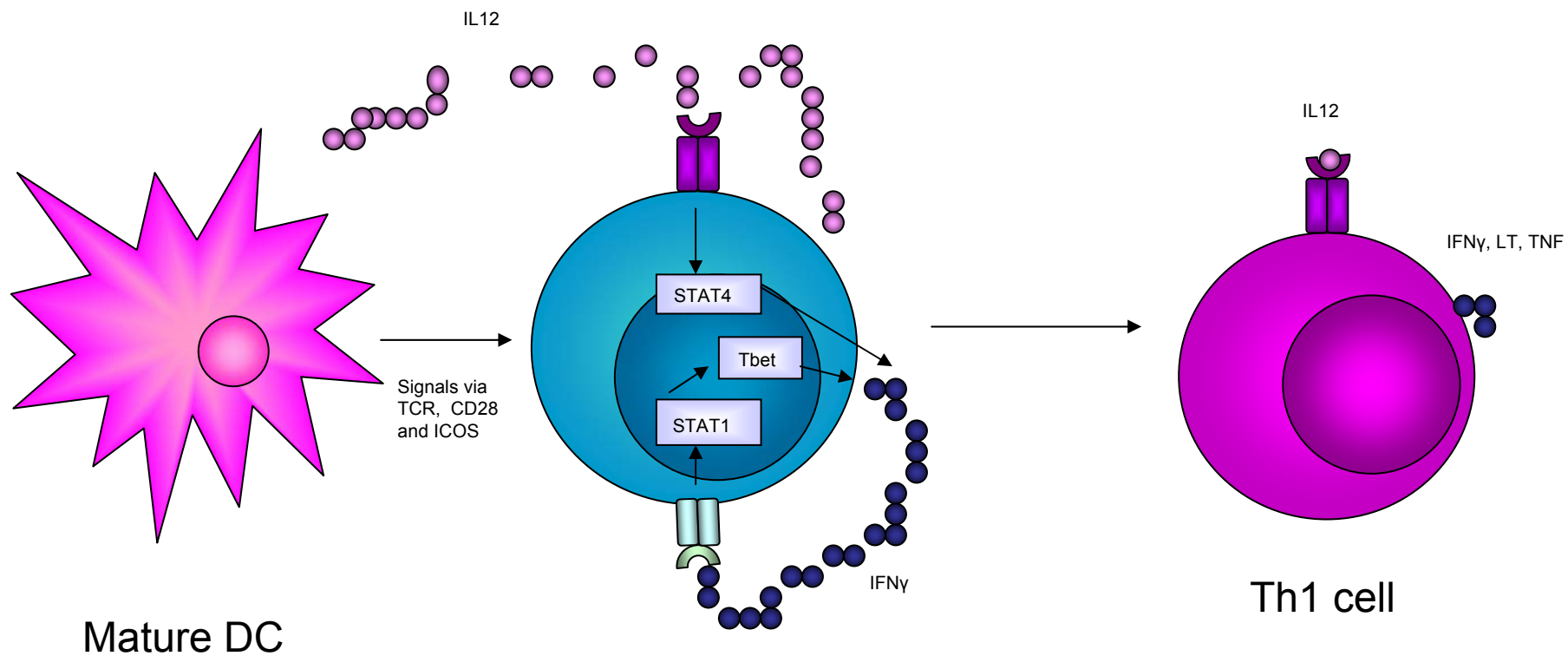
**Figure 1.8 The interleukin 12 family of cytokines and their receptors.**

IL12p70 is composed of two subunits, namely p35 and p40 and binds to the IL12 receptor which comprises of the IL12R $\beta$ 1 and the IL12R $\beta$ 2 component. The p40 chain of IL12 can also dimerise with the p19 chain to form the cytokine IL23. Also the IL23 receptor is a heterodimer which contains the IL12R $\beta$ 1 component of the IL12 receptor and the IL23 R chain. IL27 and its receptor show a close evolutionary relationship with IL12 and IL23 and their receptors. The cytokine itself is made up of the Epstein-Barr virus (EBV) induced molecule 3 (EBI3) which is a homologue to p40 and the p28 subunit. IL27 binds a receptor composed of gp130 and WSX1.



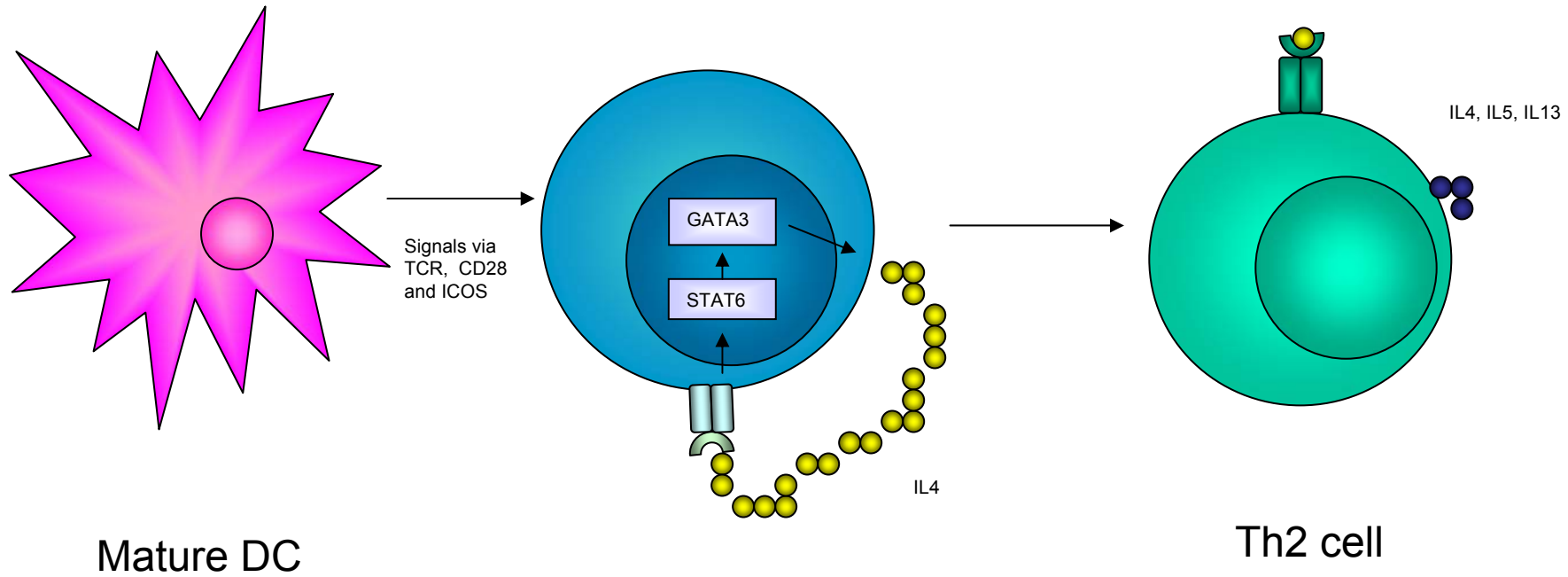
### **Figure 1.9 Th1 signalling cascades**

Upon activation of naïve T cells by signals through the TCR, CD28 and ICOS the IL12 receptor is up-regulated at a low level. IL12 from DCs, macrophages or T cells can bind the receptor and initiate signalling via STAT4. This leads to the production and secretion of IFN $\gamma$  which in turn can bind to its receptor and initiates activates STAT1. STAT1 signalling increases the expression of Tbet which further potentiates the production of IFN $\gamma$  and promotes full development of the Th1 phenotype



### **Figure 1.10 Th2 signalling cascade**

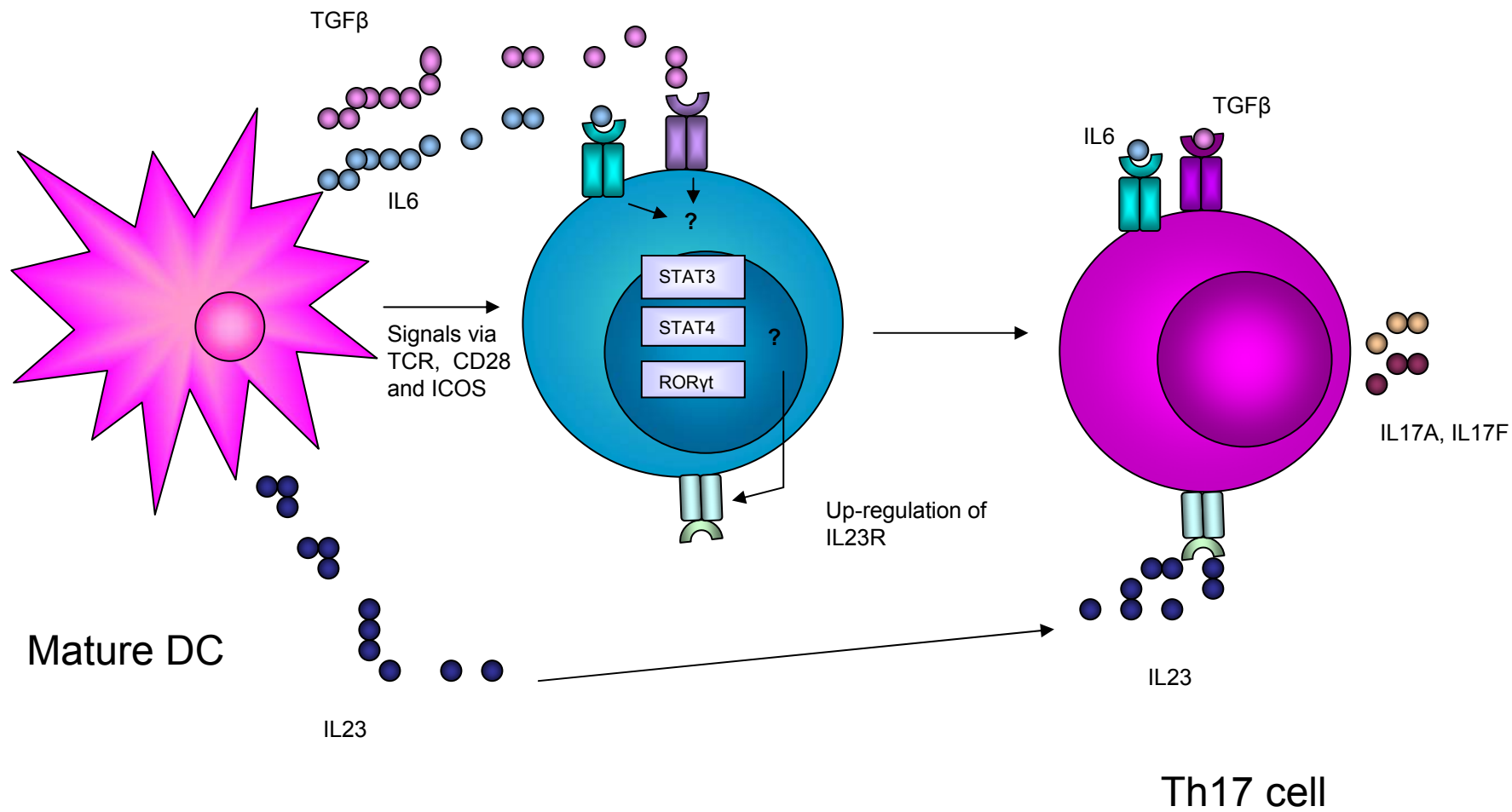
Upon activation of naïve T cells by signals through the TCR, CD28 and ICOS the IL4 receptor is up-regulated at a low level. IL4 which is most likely produced by T cells themselves can bind the receptor and initiate signalling via STAT6. STAT6 signalling increases the expression of GATA3 which further potentiates the production of IL4 and promotes full development of the Th2 phenotype



### **Figure 1.11 Th17 signalling cascades**

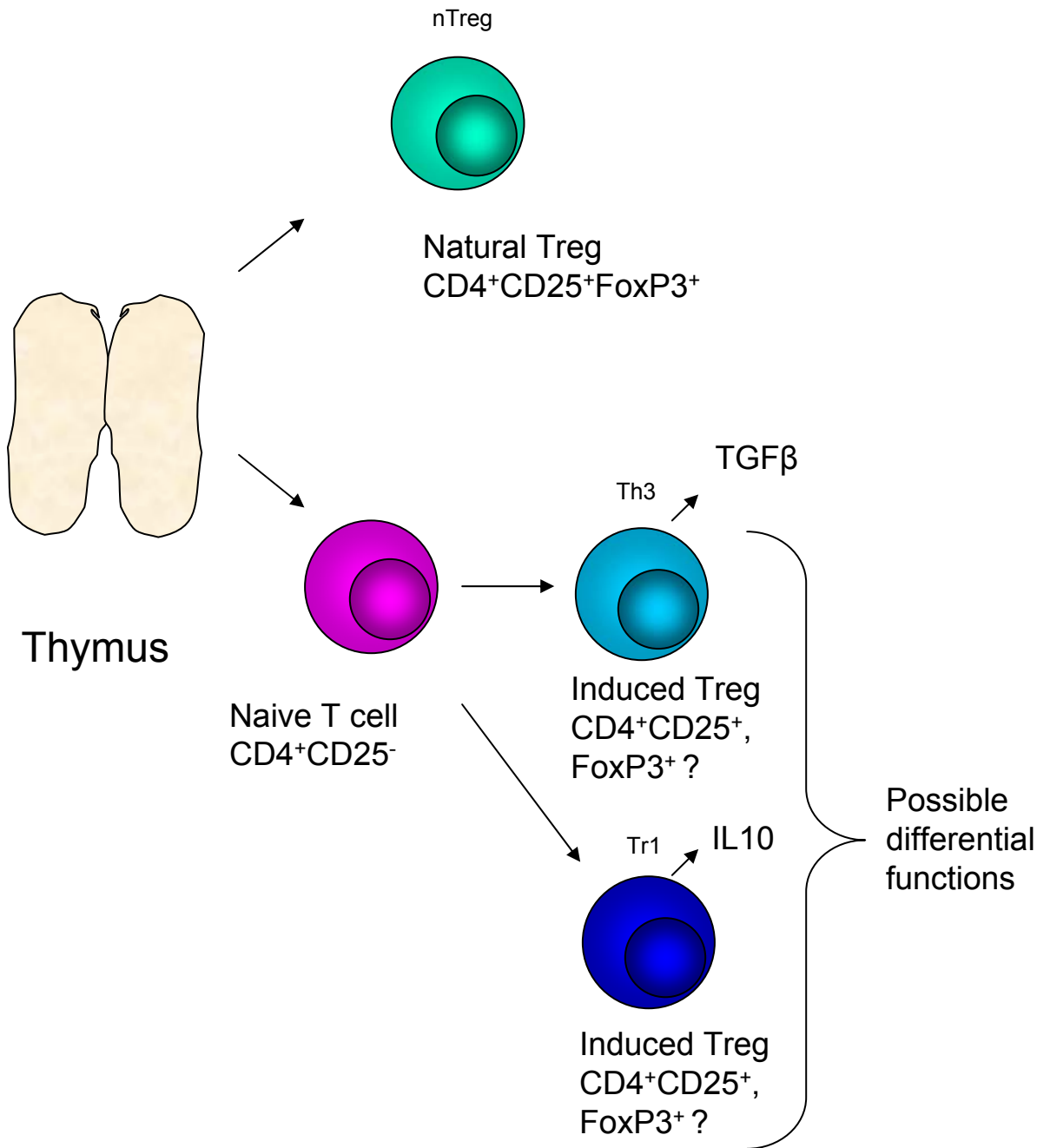
Upon activation of naïve T cells by signals through CD28 and ICOS the IL6 and TGF $\beta$  receptors are up-regulated and respond to IL6 and TGF $\beta$  secreted by DCs to initiate signalling. The signalling cascade is currently unknown but there is evidence that STAT3, STAT4 and the transcription factor, ROR $\gamma$ t play important roles in promoting Th17 development. During this process the IL23 receptor becomes up-regulated and binds IL23 produced by DCs. This leads to the production and secretion of IL17 A and IL17F and with it to the full development of the Th17 phenotype.





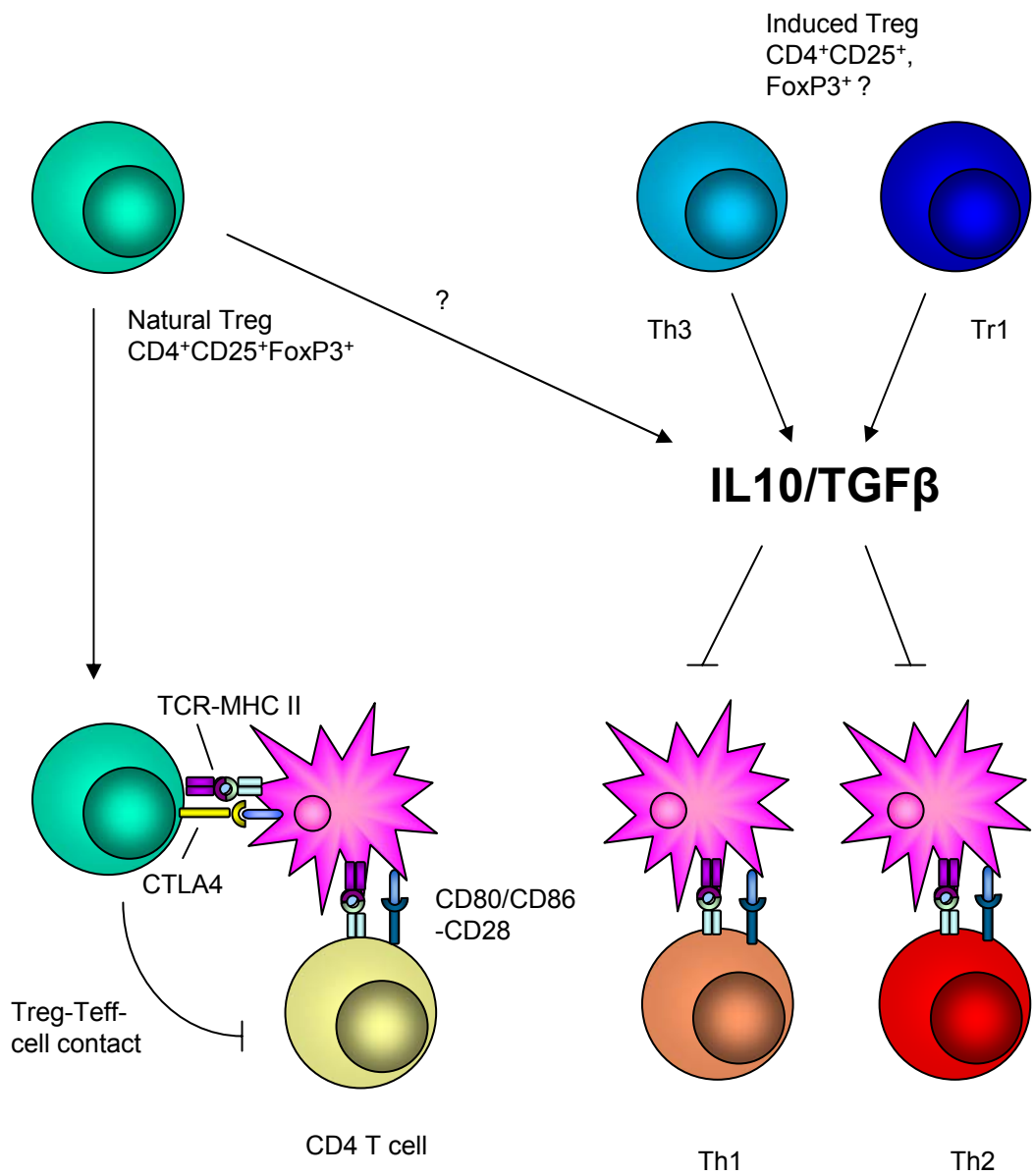
### **Figure 1.12 T regulatory subsets**

There are two major types of regulatory cells, the natural regulatory cells and induced regulatory cells. Natural regulatory cells develop in the thymus and express the surface marker CD25 and the transcriptional repressor FoxP3. By contrast, induced regulatory cells are induced from naïve T cells in the periphery and can be distinguished into Tr1 and Th3 cells according to their cytokine expression profile.



### **Figure 1.13 T cell mediated immunosuppression**

The mechanisms of T cell mediated immune suppression seem to be multifactorial. It is currently believed that natural Tregs mediate their function via direct Treg-T effector cell contact, however a stabilising role of DCs has not been ruled out in this process. Tr1 and Th3 cells secrete immunosuppressive cytokines IL10 and TGF $\beta$  which are believed to inhibit proliferation of, and cytokine production by, effector T cells. There is also some evidence that natural Tregs secrete these cytokines and can deliver a negative signal for T cell activation this way.



## **Chapter 2**

### **Materials and Methods**

## 2.1 Materials

All antibodies and ELISA kits used are commercially available and are listed in Table 2.1 and Table 2.2. Primers and probes were custom made or purchased as pre-prepared kits and are listed in Table 2.3. All other materials used were of the highest grade available and are described in the following sections.

## 2.2 Animals

Male 6-8 week old BALB/c, DBA/1, C57BL/6, C3H/HeN, C3H/HeJ and TLR2 knock-out (KO), TLR4 KO, MyD88 KO (all on the C57BL/6 background) mice were used to generate bone marrow (bm)-derived and spleen (sp)-derived dendritic cells (DCs). BALB/c, DBA/1, C57BL/6, C3H/HeN and C3H/HeJ mice were purchased from Harlan Olac (Bicester, U.K.). TLR2, TLR4, MyD88 KO mice and their wild type C57BL/6 littermates, were maintained at the Universities of Manchester and Cambridge and were a generous gift from Prof. S. Akira, University of Osaka, Osaka, Japan. ST2 KO mice (on a BALB/c background) were maintained at the University of Glasgow and were a generous gift from Dr. A. McKenzie, MRC Laboratory of Molecular Biology, Cambridge, U.K.

For co-culture experiments, mice homozygous for the transgenic TCR which is specific for OVA peptide<sub>323-339</sub> in the context of I-A<sup>d</sup> (DO.11.10 on a BALB/c background) [427], were used as T cell donors. The tg TCR was detected by flow cytometry using the clonotypic monoclonal Ab KJ1.26. For co-culture experiments using DCs from the CIA model, T cells were harvested from DBA/1 mice in which collagen-specific disease had been induced. For co-culture experiments containing DCs from TLR KO mice, T cells from Ova-specific OTII TCR transgenic mice were used (C57BL/6 background).

All animals were maintained under standard specified pathogen-free animal house conditions with free access to both water and standard rodent pellets at the University of Glasgow Central Research Facilities in accordance with local and Home Office regulations.

## **2.3 Murine models of inflammation**

### **2.3.1 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 [426] or by Mr D. Asquith University of Glasgow (Glasgow, U.K.). Male DBA/1 mice received 200 µg of bovine type II collagen (CII; Sigma-Aldrich or Chondrex) in CFA (Difco, Detroit, MI) by intradermal injection (d 0). Collagen (200 µg in PBS) was given again on d 21 by i.p. injection. Control mice received phosphate buffered saline (PBS) alone at the same timepoints or were given dexamethasone (200 µg/kg) treatment to suppress the onset of arthritis. 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 or measurement of paw swelling with callipers.

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

At the end of each model protocol, spleens, draining and peripheral LNs and MLNs were harvested and used for further *ex vivo* culture experiments or sections were prepared for staining (Figure 2.1).

### **2.3.2 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 [428], 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 d 0 and 14. On d 14 mice were additionally



challenged intranasally with 10 µg aerosolised OVA in PBS (after anaesthesia was induced with avertin (1,1,1-tribromoethanol) dissolved in amyl alcohol and diluted 1/40). On d 25, 26 and 27 mice were anaesthetised and re-challenged with 10 µg aerosolised OVA in PBS, administered intranasally. Control mice received PBS in place of OVA. Mice were euthanized on d 28 by lethal intraperitoneal injection of anaesthetic.

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. ES-62 and asthma+ES-62 mice received 2 µg ES-62 in PBS, by subcutaneous injection in the scruff, on d -2 (2 d before d 0), 12, 25 and 27. This concentration of ES-62 has been shown to be a physiologically relevant dose, similar to that secreted during a filarial nematode infection [429]. Mice in the control and asthma groups received PBS on these d. (Figure 2.2)

## 2.4 Preparation of ES-62

ES-62 is a major secreted glycoprotein of the rodent filarial nematode *Acanthoceilonema viteae* and homologue (77% with *Brugia malayi*) [430] of molecules found in filarial nematodes that parasitize humans [419]. The molecule consists of a tetramer of identical monomers of 62 kDa that contain PC attached to N-type glycans [431]. ES-62 was purified to homogeneity from spent culture medium of adult *A. viteae* using endotoxin-free reagents by Dr. KM Houston and Dr. C Watson at the Department of Immunology, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, Glasgow, UK essentially as described previously [423]. In detail, ES-62 was prepared from 500 ml of spent medium (endotoxin-free RPMI 1640; Invitrogen Life Technologies, Paisley, UK, with added endotoxin-free glutamine (2 mM), endotoxin-free penicillin (100 U/ml), and endotoxin-free streptomycin (100 µg/ml) following culture of adult *A. viteae*. To remove larval forms (microfilariae) released by the adult female worms, the medium was passed through a 0.22 µm filter (Sigma, Poole, UK). It was then

transferred to a stirred ultracentrifugation unit containing a YM10 membrane (Amicon, Stonehouse, UK). 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, Berkshire, UK), it was further concentrated to 200-500  $\mu$ l using Centricon microconcentrators with a 30 kDa cut-off membrane (Amicon). The sample was applied to a 30 x 1 cm Superose 6 column (HR 10/30; Pharmacia, Milton Keynes, UK) fitted to an isocratic fast protein liquid chromatography system (Pharmacia) 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 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 ES-62 sample was confirmed using an Endosafe kit (Charles River Laboratories, Kent, UK). ES-62 is used at a working concentration that has an endotoxin reading of <0.003 endotoxin units/ml.

## **2.5 Generation of bone marrow-derived dendritic cells (bmDCs) and treatment with immunomodulators**

Bone marrow was isolated from mouse femurs and single cell suspensions were prepared and treated to remove red cells by incubation with 0.168 M  $\text{NH}_4\text{Cl}$  (pH 7.2) on ice for 7 min. Cells were then filtered through cotton wool to remove dead cells prior to culture. To prepare bmDCs, bone marrow cells were cultured in RPMI-1640 complete medium containing, 2 mM glutamine, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 10% FCS (Invitrogen) supplemented with 10% conditioned medium generated by the GM-CSF-secreting X63 myeloma cell line and 50  $\mu$ M 2-Me (2-Mercapto-ethanol) for 6 d at 37°C/5%  $\text{CO}_2$ , with fresh medium supplied on d 4. On d 6, the loosely adherent immature bmDC were harvested and used as a source of DC. Where indicated, in some experiments d 6 bmDC were further purified prior to maturation using magnetic anti-CD11c beads according to the manufacturer's instructions (Miltenyi Biotec, Surrey, UK).

Following isolation, bmDC or, where indicated, CD11c<sup>+</sup> bmDC were plated at 2x10<sup>6</sup> cells/well in 6 well plates in the presence or absence of 2 µg/ml ES-62 for 24 h. On d 7, the bmDCs were further cultured for 24 h in the presence or absence of 1 µg/ml LPS (*Escherichia coli* serotype 055:B5, Sigma) or 40 µg/ml CpG-ODN 1826 (5'-TCCATGACGTTTCCTGACGTT-3', Autogen Bioclear). On d 8, culture supernatant was removed for cytokine analysis and cells were either used for investigation of co-stimulatory molecule and surface marker expression or pulsed with Ag and co-cultured with Ag-specific T cells from DO11.10 mice.

## **2.6 Generation of spleen-derived dendritic cells and treatment with immunomodulators**

Spleens were harvested from mice, injected with collagenase type 3 (Worthington; 2 mg/ml in PBS), cut into small pieces and then incubated in collagenase for 20 minutes at 37<sup>0</sup>C. A single cell suspension was prepared by pressing spleens through nytex mesh (Cadisch Precision Meshes Ltd, London, U.K.) and splenic red blood cells were removed by hypotonic lysis using 0.168 M NH<sub>4</sub>Cl (pH 7.2). Cells were then filtered through cotton wool to remove dead cells prior to culture and then cultured in RPMI-1640 complete medium containing 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-Me, 10% FCS (Invitrogen) and supplemented with 10% conditioned medium generated by the GM-CSF-secreting X63 myeloma cell line at 37<sup>0</sup>C/5% CO<sub>2</sub>. spDC were purified prior to culturing using anti-CD11c beads according to the manufacturer's instructions (Miltenyi Biotec, Surrey, UK).

CD11c<sup>+</sup> spDC were plated at 2x10<sup>6</sup> cells/well in 6 well plates in the presence or absence of 2 µg/ml ES-62 for 24 h. On the following d, spDCs were further cultured for 24 h in the presence or absence of 1 µg/ml LPS (*Escherichia coli* serotype 055:B5, Sigma). After a further 24 h culture supernatants were removed for cytokine analysis and cells were pulsed with Ag and co-cultured with DO11.10 T cells.

## **2.7 Generation of spleen-derived dendritic cell subsets and treatment with immunomodulators**

Splenic single cell suspensions (obtained as described in section 2.6) were incubated with antibodies raised against surface markers specific for DC subtypes to isolate CD4<sup>+</sup>DCs, CD8<sup>+</sup>DCs, DN DCs and pDCs by high speed cell sorting (Table 2.4). The stained cells were then sorted into 24 well plates at  $2.5 \times 10^4$  cells/well or where indicated in 48 well plates at  $1.25 \times 10^4$  cells/well using the FACSAria cell sorter (BD) (Figure 2.3-2.5; see section 2.11 for FACS Aria staining protocol).

The sorted DC subsets were incubated in RPMI-1640 complete medium containing 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-Me, 10% FCS (Invitrogen) and supplemented with 10% conditioned medium generated by the GM-CSF-secreting X63 myeloma cell line at 37°C/5% CO<sub>2</sub> in the presence or absence of 2 µg/ml ES-62 for 24 h. On the following d, the spDC subtypes were further cultured for 24 h in the presence or absence of 1 µg/ml LPS (*Escherichia coli* serotype 055:B5, Sigma) or 40 µg/ml CpG-ODN 1826. On d 3 culture supernatant was removed for cytokine analysis and cells were pulsed with Ag and used for co-cultures with T cells from DO11.10 mice.

## **2.8 DO11.10 transgenic T cells**

T cells from DO.11.10 mice contain, in their germline DNA, rearranged TCR-V $\alpha$  and TCR-V $\beta$  genes that encode a TCR specific for the chicken ovalbumin peptide 323-399 bound to I-A<sup>d</sup> MHC class II molecule [427], which can be detected by the anti-clonotypic KJ1.26 monoclonal antibody (mAb) [432]. The presence of these transgenes inhibits rearrangement of endogeneous TCR genes and since the transgenic TCR is MHC class II restricted, a large fraction of the T cells from these mice are positively selected in the thymus to become CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells. Importantly, approximately 90% of these CD4<sup>+</sup>T cells exhibit a naïve phenotype

(CD45RB<sup>high</sup>CD62L<sup>high</sup>) allowing clear examination of T cell activation and differentiation [433].

## **2.9 Purification of CD4<sup>+</sup>CD62L<sup>high</sup> T cells using microbeads**

Purified naïve CD4<sup>+</sup>CD62L<sup>high</sup> T cells from DO.11.10/BALB/c or OT II/C57BL/6 mice were used as a source of responder T cells for bmDCs and spDCs derived from BALB/c or C57BL/6 mice, respectively. Peripheral lymph nodes (axillary, brachial, inguinal, cervical; PLN), mesenteric lymph nodes and spleens were pooled and single cell suspensions of lymphocytes were prepared by forcing cells through a nytex mesh (Cadisch Precision Meshes, London, U.K.) using a syringe plunger. CD4<sup>+</sup> T cells were purified using a CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec) and this population was subsequently enriched for naïve CD62L<sup>high</sup> cells using anti-CD62L microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, lymphocytes were resuspended in MACS buffer (PBS pH7.2, supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA.) and incubated with a biotin-antibody cocktail which contains biotin-conjugated antibodies against CD8α (Ly-2), CD45R (B220), DX5, CD11b (MAC-1) and Ter-119. Cells were then incubated with anti-biotin microbeads and after washing, the CD4<sup>+</sup> T cell population was obtained by negative selection using MACS separation. This enriched CD4<sup>+</sup> T cell population was then incubated with anti-CD62L microbeads and the CD4<sup>+</sup>CD62L<sup>high</sup> population was obtained using positive MACS selection. The number of KJ1.26<sup>+</sup>CD4<sup>+</sup> T cells was calculated by staining a small aliquot of the CD4<sup>+</sup>CD62L<sup>high</sup> population with anti-CD4 FITC and biotinylated-KJ1.26/PE and subsequently analysed by flow cytometry (Figure 2.6).

## **2.10 Purification of KJ1.26<sup>+</sup>CD4<sup>+</sup>CD62L<sup>high</sup> T cells and CD4<sup>+</sup> T cells using the FACS Aria cell sorter**

FACSAria purified naïve KJ1.26<sup>+</sup>CD4<sup>+</sup>CD62L<sup>high</sup> T cells from DO.11.10/BALB/c mice (Figure 2.7) were used as a source of responder T cells for spDC subtypes derived from BALB/c mice and CD4<sup>+</sup> T cells from DBA/1 (CIA-model) mice (Figure 2.8) were used as a source of responder T cells for spDC subtypes derived from

DBA/1 mice from the CIA model. Peripheral lymph nodes (axillary, brachial, inguinal, cervical; PLN), mesenteric lymph nodes and spleens from DO.11.10 BALB/c mice or DBA/1 mice respectively were pooled and single cell suspensions of lymphocytes were prepared by forcing cells through a nytex mesh (Cadisch Precision Meshes, London, U.K.) using a syringe plunger. After preparing a single cell suspension, cells were stained (see section 2.11) for CD62L and KJ1.26 expression (DO.11.10 BALB/c mice) or for CD3 and CD4 expression (DBA/1 mice) using specific antibodies (Table 2.1). The stained cells were then washed twice, transferred to a FACS tube by pipetting them through a nytex mesh and sorted into either 24 well plates at  $2.5 \times 10^5$  cells/well, or where indicated in 48 well plates (stated in appropriate sections) at  $1.25 \times 10^5$  cells/well, using the FACS Aria cell sorter (BD). The sorting purity of all FACS area cell sorts was greater than 99%.

## **2.11 FACS Aria staining protocol**

Single cell suspensions were washed twice (in 5 ml FACS tubes; Becton Dickinson, UK) with cold sterile PBS containing 0.5% BSA at 400 x g for 5 min at 4°C. Cells were incubated with primary antibodies (Table 2.1), at a concentration previously determined by titration of optimum binding, for 30 min at 4°C. Cells were washed with 3 ml cold sterile PBS containing 0.5% BSA (400 x g, 5 min, 4°C) and then resuspended in 4ml cold sterile PBS containing 0.5% BSA following staining with antibodies directly conjugated to fluorochromes. Following incubation with biotinylated antibodies, the cells were incubated with an appropriate fluorochrome-streptavidin conjugate (BD Pharmingen, Oxford, UK conc). Immediately prior to sorting, cells were pipetted through a nytex mesh to to guarantee a single cell suspension and transferred to a FACS tube. Cells were kept at 4°C under sterile conditions at all times. Labeled beads were used to enable compensation and isotype controls were used to set up gates for positive stains.

## **2.12 *In vitro* DC and T cell co-cultures**

bmDCs and spDCs were prepared and matured in the presence and absence of ES-62 and/or LPS as described in sections 2.5, 2.6 and 2.7. bmDCs, spDCs and

spDC subtypes were then co-cultured ( $2.5 \times 10^4$  cells) with  $CD4^+KJ1.26^+CD62L^{\text{high}}$  ( $2 \times 10^5$  cells) (prepared as described in section 2.9 and 2.10) in a total volume of 1ml RPMI 1640 medium at  $1 \times 10^6$  cells/ml supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100  $\mu\text{g/ml}$ ), L-glutamine (2 mM), 2-mercaptoethanol (50  $\mu\text{M}$ ), 1% non-essential amino acids and sodium pyruvate (1 mM) (all from Gibco Life Technologies, Paisley, U.K.) in 24 well plates for 72 h at  $37^\circ\text{C}$  and 5%/CO<sub>2</sub>. At this point, cell supernatants were then removed and frozen for future cytokine analysis and cellular RNA was extracted using the RNeasy micro kit (Quiagen) and stored at  $-70^\circ\text{C}$  for mRNA analysis at a later time. In some experiments however, following removal of aliquots of the cell supernatants, fresh medium containing 50 ng PMA (Sigma) and 500 ng ionomycin (Sigma) was added and the cells cultured for a further 24 h.

### **2.13 *Ex vivo* cultures of LN cells and splenocytes from murine asthma model**

LNs and spleens from the ovalbumin-induced asthma model were harvested on d 28 of the treatment protocol. Single-cell suspensions were obtained by pressing lymph nodes and spleens through nytex mesh (Cadisch Precision Meshes Ltd, London, U.K.) and red blood cells were removed by hypotonic lysis. Cells were cultured at  $1 \times 10^6$  cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100  $\mu\text{g/ml}$ ), L-glutamine (2 mM), 2-mercaptoethanol (50  $\mu\text{M}$ ), 1% non-essential amino acids and sodium pyruvate (1 mM) (all from Gibco Life Technologies, Paisley, U.K.) in the presence and absence of Ag (whole OVA at 500  $\mu\text{g/ml}$ ).

### **2.14 *In vitro* spDC subtype and T cell co-cultures from the CIA model**

Spleens from CIA model mice (described in section 2.3.1) were harvested on d 41 of the treatment protocol. spDC subtypes were isolated and restimulated in the presence or absence of type II collagen (10 or 50  $\mu\text{g/ml}$ ) for 3 h. Such DCs ( $1.25 \times 10^4$  cells/well) were then pre-treated in the presence or absence of 2 $\mu\text{g/ml}$  ES-62, 1 $\mu\text{g/ml}$  E. coli 055:B5 LPS or 40  $\mu\text{g/ml}$  CpG-ODN 1826 (Autogen Bioclear) for 2 h

before co-culturing them with FACS Aria purified CD4<sup>+</sup> activated T cells from DBA/1 mice ( $1.25 \times 10^5$  cells/well) in RPMI 1640 medium supplemented with 20% 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.) in a total volume of 1ml in 48 well plates for 72 h at 37<sup>0</sup>C and 5%/CO<sub>2</sub>. At the end of the culture period cell supernatants were then removed and frozen for future cytokine analysis and cellular RNA was extracted using the RNeasy micro kit (Qiagen) and stored at -70<sup>0</sup>C.

### **2.15 Preparation of RNA extracts**

Cells were washed twice with ice-cold PBS before transferring them to microcentrifuge tubes. From there on the RNeasy micro kit protocol for extracting RNA from animal cells was followed including the step of DNase treatment. All buffers and wash solutions are provided in the kit or were prepared as recommended in the protocol. Briefly, cells were lysed and cell lysates were transferred to the columns. RNA was bound to the columns and residual DNA was digested with DNase I. In the final step of the protocol RNA was eluted in DEPC water and stored at -70<sup>0</sup>C until use.

### **2.16 Taqman real-time PCR**

RNA was reverse transcribed using 100 U Superscript II RT (Life Technologies, Gaithersburg, USA) at 42<sup>0</sup>C for 50 min in the presence of 50 nM Tris-HCl (pH8.3) containing 75 mM KCl, 3 mM MgCl<sub>2</sub>, 5 mM DTT, 0.5 mM dNTPs and 5 µM Oligo(dT)<sub>16</sub> (Gibco BRL, Paisley, UK) or using the high capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's protocol (RNA extraction from T cells from CIA model co-cultures). Primers and fluorogenic probes (Table 2.3) were designed using the PrimerExpress<sup>TM</sup> v3.0 program and purchased from VH Bio Ltd. or Applied Biosystems (ABI). The fluorogenic probes contained a reporter dye (FAM) covalently attached at the 5' end and a quencher dye (TAMRA) covalently attached at the 3' end, and were HPLC purified. Extension



from the 3' end was blocked by attachment of a 3' phosphate group.

PCR reactions were either performed in the ABI-prism 7700 Sequence Detector or in the 7900 HT Sequence Detector (ABI). PCR amplifications were performed in a total volume of either 10, 15 or 25  $\mu$ l, containing the qPCR mastermix (Eurogentec) and 0.5-1 $\mu$ l cDNA sample, 900 nM of each primer and 200nM of each probe. Each PCR amplification was performed using the following conditions: 2 min at 50<sup>0</sup>C and 10 min at 94<sup>0</sup>C, followed by a total of 50 two-temperature cycles (15 s at 94<sup>0</sup>C and 1 minute at 60<sup>0</sup>C). Data analysis was performed using the Sequence Detection software (ABI) and samples were normalised by their reference reporter hypoxanthine-guanine phosphoribosyltransferase (HPRT) or 18s ribosomal RNA, as indicated.

## **2.17 Analysis of cell surface marker expression by flow cytometry**

Cells were washed twice (in 5 ml tubes; Becton Dickinson, UK) with 200  $\mu$ l cold FACS buffer (PBS containing 0.5% BSA, 0.1% sodium azide) at 400 x g for 5 min, 4<sup>0</sup>C. Cells were then incubated in 50  $\mu$ l of primary antibody (Table 2.1), at a concentration previously determined by titration of optimum binding, for 30 min at 4<sup>0</sup>C. Cells were washed with 1ml FACS buffer (400 x g, 5 min, 4<sup>0</sup>C) and then resuspended in 300  $\mu$ l FACS buffer following incubation with antibodies directly conjugated to fluorochromes. Following incubation with biotinylated antibodies, cells were stained by addition of the appropriate fluorochrome-streptavidin conjugate (BD Pharmingen, Oxford, UK, 1/100 dilution of commercial stock). Immediately prior to data acquisition 50  $\mu$ g/ml propidium iodide (Calbiochem) was added to each sample to enable exclusion of dead cells from the analyses. Cellular fluorescence data was acquired using a Becton Dickinson FACSCalibur<sup>TM</sup> flow cytometer and analysed using Cell Quest software (Becton Dickinson) or FlowJo software (Tree Star Inc, OR, USA) (Figure 2.9)

### 2.17.1 Intracellular staining

Intracellular analysis of FoxP3, GATA3 and Tbet expression was carried out on T cells from *in vitro* DC/T cell co-culture experiments. The cells were centrifuged at 1500 rpm for 5 min and washed 3 times with FACS buffer. They were then stained with FITC-labelled KJ1.26 anti-TCR antibody for 20 min at 4<sup>0</sup>C and washed 3 times with FACS buffer. The cells were then resuspended in 100 µl Cytofix/Cytoperm solution (BD Pharmingen) for 20 min at 4<sup>0</sup>C and washed twice with Perm wash (BD Pharmingen). Permeabilised cells were subsequently stained with the relevant anti-transcription factor antibodies (Table 2.1) for 30 min at 4<sup>0</sup>C. This was followed by two washing steps and another staining step under the same conditions as before with the appropriate fluorochrome-labelled secondary antibodies. Control cells were incubated with relevant isotype control antibodies. Cells were finally washed 3 times with FACS buffer, resuspended and analysed for intracellular transcription factors by flow cytometry. (Figure 2.10)

### 2.18 Cytokine ELISA

IL2, IL4, IL5, IL6, IL10, IL12p40, IL12p70, IFN- $\gamma$ , IFN $\alpha$ , IFN $\beta$ , TGF $\beta$ , IL17A, IL17E and TNF- $\alpha$  levels were analysed using OPTeia Mouse ELISA kits (BD Pharmingen), Cytoset ELISA kits (BIOSOURCE), DuoSet ELISA kits (R&D) or antibody pairs (BD Pharmingen) according to the suppliers' recommendations. (Table 2.2). Briefly, Nunc-immuno Maxisorp plates were coated overnight at 4<sup>0</sup>C in the buffer recommended in the manufacturer's protocol. Plates were then blocked for 2 h with 10% FCS in PBS at 37<sup>0</sup>C and samples and standard were diluted in culture medium and incubated for 1 h at 37<sup>0</sup>C or overnight at 4<sup>0</sup>C. Detection antibodies and avidin or streptavidin (supplied with the kits) were diluted in blocking buffer and incubated for 1 h each at 37<sup>0</sup>C. Plates were washed at least five times between stages. Finally, plates were developed using TMB substrate and absorbances at 630 nm determined. ELISAs were performed in triplicate for each individual sample (samples within each group were not pooled prior to ELISA).

## **2.19 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 required.

Lymph node and spleen sections were stained using various similar protocols (Figure 2.11). In general, tissue sections were incubated in acetone for 20 min, air dried and rehydrated with PBS containing 1% goat serum before being incubated in blocking reagent containing 6% BSA, 10% goat serum and 1% FC-block. For intracellular staining 0.1% TritonX (Sigma) was added to all buffers. 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 Tris buffered solution (TBS; Sigma) after each treatment. Prepared sections were stained for expression of surface markers and intracellular cytokines and transcription factors as follows.

Primary antibodies and isotype controls were added to the slides and sections were stained either for 1 h at room temperature (surface marker) or over night at 4°C (intracellular staining). After three washing steps with TBS, secondary antibodies conjugated to Q-dots or streptavidin conjugates were added to the sections for another h. Sections were washed with TBS then incubated for 15 minutes with Hoechst 33342 (Invitrogen), washed again and slides were then mounted using Vectashield mounting medium (Vector) or Cytoseal60 (Richard-Allen Scientific). Photographs of sections were taken at x20 magnification using a Hammamatsu camera and analysed using Openlab imaging software (Improvision, Coventry, U.K.).

## **2.20 Analysis of tissue sections by Laser scanning cytometry**

The laser scanning cytometer (LSC) is a microscope based cytofluorometer that combines the advantages of flow cytometry and image analysis [434]. Fluorescence of individual cells is measured rapidly, with high sensitivity and accuracy comparable to that of flow cytometry. Cell analysis on slides eliminates cell loss, which generally occurs during repeated centrifugation in sample preparation for flow cytometry. The data generated by LSC are equivalent to data generated by flow cytometers, however, LSC can measure more than just total fluorescence per cell. As LSC measurements are slide based, it is possible to record the position of each cell on the slide as a feature so that the cells can be relocated after measurements are completed and re-examined visually or by image analysis. To distinguish cells from background, contours are drawn round each event by the LSC software using a threshold set by the user, and a number of parameters for each event are recorded. Some key parameters recorded by the LSC are the integral (the sum of pixel values in the data contour), max pixel (the maximum pixel value within the data contour) and area (the area of the thresholding contour).

Standard cell contours were set to determine individual FoxP3 positive T cells. For B220<sup>+</sup> B cells, evenly spaced Phantom contours were applied to the whole tissue section, since cells are so densely packed within follicles it is impossible to set contours for individual cells. Tissue maps were generated from this data and the number and percentage of FoxP3 positive T cells located within the T cell area was determined using Wincyte software (Compucyte Corp, Essex, UK).

## **2.21 Statistics**

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

**Table 2.1 Antibodies and conjugated fluorochromes used for flow cytometry or immunofluorescence**

Antibody Specificity	Isotype	Conjugate	Company
B220	Rat IgG2a $\kappa$	FITC, APC	BD
CD3	Hamster IgG1 $\kappa$	Biotin, unconjugated, PE	BD
CD4	Rat IgG2a $\kappa$	PerCP, PE, FITC	BD
CD8	Rat IgG2a $\kappa$	FITC, PE, APC	BD
CD11b	Rat IgG2b $\kappa$	FITC, PE	BD
CD11c	Hamster IgG1 $\lambda$	Biotin, FITC, PE	BD
CD19	Rat IgG2a $\kappa$	FITC	BD
CD16/32		Unconjugated	BD
CD62L	Rat IgG2a $\kappa$	PE, FITC	BD
TLR4	Rat IgG2a $\kappa$	PE	BD
MHCII	Mouse IgG2b $\kappa$	FITC	BD
CD40	Rat IgM $\kappa$	FITC	BD
CD54	Hamster IgG1 $\lambda$	Biotin	BD
CD80	Hamster IgG2 $\kappa$	FITC	BD
CD86	Rat IgG2a $\kappa$	FITC	BD
mPDCA-1		PE	Miltenyi
TCR KJ1.26	Mouse IgG2a	Biotin	
IL17A		Biotin	Biosource
FoxP3	Rat serum	PE	eBioscience
Tbet	Mouse IgG1	PE	eBioscience
GATA3	Mouse IgG1	Unconjugated	Santa Cruz
anti-mouse IgG (H+L)		PE, Biotin, Q-Dot-565	BD, Invitrogen
anti-rat IgG (H+L)		PE, Biotin, Q-Dot-605, 655	BD, Invitrogen
		Streptavidin-APC, PE, PerCP, FITC	BD
		Strptavidin-Q-Dot-525, 565, 585, 605, 655, 705	Invitrogen

**Table 2.2 ELISA kits or antibody pairs used to establish the amount of cytokines found in cell culture supernatants**

<b>Cytokine</b>	<b>Company</b>	<b>ELISA kit name</b>
IL2, IL4, IL5, IL6, IL10, IL12p40, IL12p70, TNF $\alpha$ , IFN $\gamma$	BD Pharmingen	OptEIA Set
TGF $\beta$	BD Pharmingen	Paired antibodies
IFN $\alpha$ and $\beta$	PBL Biomedical laboratories	Paired antibodies
IL17A, IL25	R&D	Duaset ELISA kit
IL6, IL10, IFN $\gamma$	Biosource	Cytoset ELISA kit



### **Table 2.3 Primers and probes used for quantitative PCR**

All primers and probes were purchased from VH Bio Ltd. All probes contained the reporter dye 5'-6-carboxy-fluorescein (FAM) and a quencher dye 3'-6-carboxy-tetramethyl rhodamine (TAMRA) and were HPLC purified.

Name	Forward primer	Reverse primer	Probe
FoxP3	GCAGGGCAGCTAGGTA CTTGTAG	TCGGAGATCCCCTTTGT CTTATC	TCCGAACAGCATCATCC TTCTTAGCATCC
GATA3	GGTGGACGTACTTTTGA ACATCGA	CGTAGCCCTGACGGAG TTTC	TCAAGGCAACCACGTCC CGTCCTAC
Tbet	GCCAGGGAACCGCTTA TATG	AACTTCCTGGCGCATCC A	ACCCAGACTCCCCAAC ACCGG
IL17A	GAAGCTCAGTGCCGCC A	TTCATGTGGTGGTCCAG CTTT	CAGCGCTGTGTCAATGC GGAGG
IFN $\gamma$	CGGCAGATCGTGGATG CT	TGTTCACTCGAAACA CCAGAA	CTGCGTGCACCCGCTG CAA
IL10	TGCAGCAGCTCAGAGG GTT	TGGCCACAGTTTTCAGG GAT	CCTACTGTCATCCCCA GCCGCT
IL6	CTGCAAGAGACTTCCAT CCAGTT	GAAGTAGGGAAGGCCG TGG	CCTTCTTGGGACTGATG CTGGTGACA
HPRT	CTTCCTCCTCAGACCGC TTTT	AACCTGGTTCATCATCG CTAATC	AGCCGACCGGTCCCGT CATG
TLR4	GCCAAGTTTCTCTGGAC TAACAAGTTTAGA	GCCAAGTTTCTCTGGAC TAACAAGTTTAGA	GCCAAGTTTCTCTGGAC TAACAAGTTTAGA
TLR2	AAGATGCGCTTCCTGAA TTTG	TCCAGCGTCTGAGGAAT GC	CGTTTTTACCACCCGGA TCCCTGTACTG
TLR3	GGAACATTTCTCCCCAA TGGA	AGGACGCCTGCTTCAAA GTC	AACAAGACCAATCTCTC AAATTTTGCC
TLR9	AGGCTGTCAATGGCTCT CA	TGAACGATTTCCAGTGG TA	TGCCGCTGACTAATCTG CA
TLR5	GCACGAGGCTTCTGCTT CA	GCATCCAGGTGTTTGAG CAA	CATTCTGTGCCATTCA AAGTCTTTGCTG

## 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 (d 0). Collagen (200 µg in PBS) was given again on d 21 by *i.p.* injection.

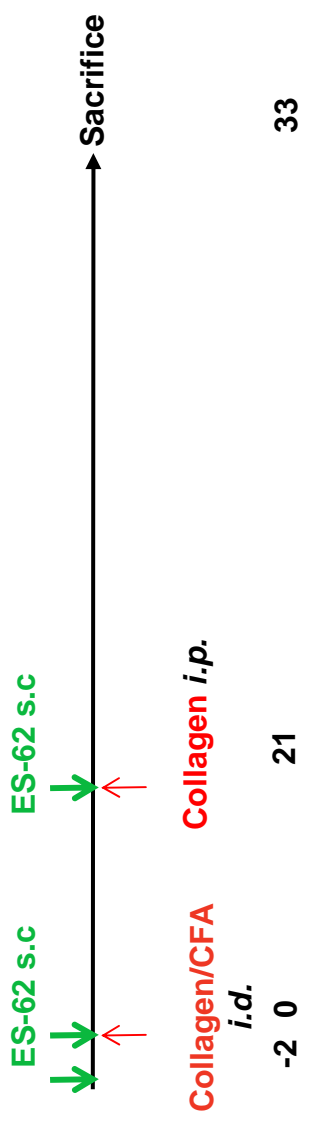
Prophylactic treatment protocol: Mice were treated with 2 µg ES-62 (*s.c.*) on d -2, d 0, and d 21.

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

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

Prophylactic ES-62 treatment protocol

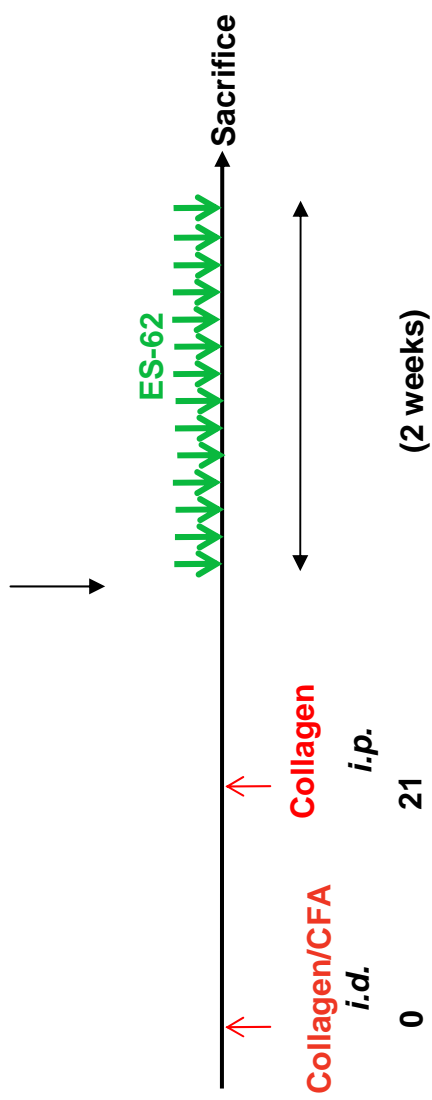
Collagen-induced arthritis protocol



Onset of  
clinically  
detectable  
arthritis

Therapeutic ES-62 treatment protocol

Collagen-induced Arthritis protocol



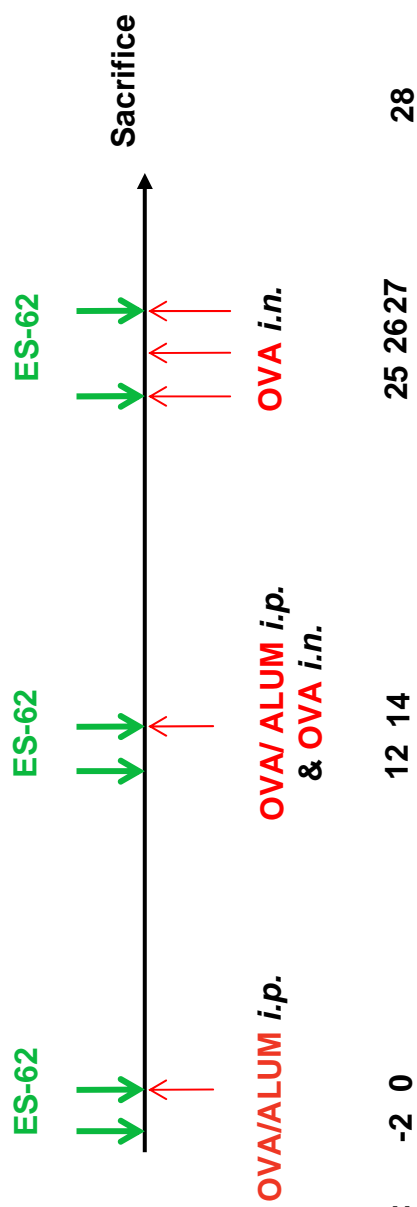
## Figure 2.2 Ovalbumin-induced airway inflammation protocol

BALB/c mice were sensitised with an ovalbumin (100 µg)/aluminium hydroxide (2mg) emulsion intraperitoneally (OVA/ALUM *i.p.*) on d 0 and 14 and challenged with OVA (diluted in PBS) intranasally (OVA *i.n.*) on d 14 (10 µg), 25 (10 µg), 26 (10 µg) and 27 (10 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 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 d before and on the d of OVA administration, i.e. on d –2, 0, 12, 14, 25, 26 and 27. Mice in control treatment groups were administered with PBS in place of OVA/ALUM, OVA or ES-62. On d 28 of the protocol mice were sacrificed by lethal injection of anaesthetic.

Prophylactic ES-62 treatment protocol

OVA-induced airway inflammation protocol



**Table 2.4 FACS Aria cell sorting of DC subtypes on the basis of their differential surface markers**

All cDCs were stained for CD11c, CD11b, CD4 and CD8 expression. pDCs were either stained for mPDCA and CD11c expression or for CD11c, CD11b and CD45R. To divide the cDC cell population into subtypes, cells were sorted according to previously defined differential combinations of surface markers. To eliminate certain cell populations some gates were set on surface marker-negative populations (surface marker for negative selection)

spDC subtype (Name)	Surface marker	Surface marker for positive selection	Surface marker for negative selection
CD4 <sup>+</sup>	CD11c CD11b CD4	CD11c-PE CD11b-FITC CD4-PerCP	CD8-APC
CD8 <sup>+</sup>	CD11c CD8	CD11c-PE CD8-APC	CD4-PerCP CD11b-FITC
DN	CD11c CD11b	CD11c-PE CD11b-FITC	CD4-PerCP CD8-APC
pDC	CD11c CD11b CD45R	CD11c-PE CD45R-APC	CD11b-FITC
pDC	mPDCA CD11c	mPDCA-PE CD11c-FITC	



### **Figure 2.3 FACS Aria sorting of spDC subtypes**

**a** Flow diagram displaying an overview of the sorting pathways leading to the purification of CD4<sup>+</sup>DCs, CD8<sup>+</sup>DCs and DN DCs

**b** 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 (P1) indicates cells of the correct size and granularity associated with dendritic cells

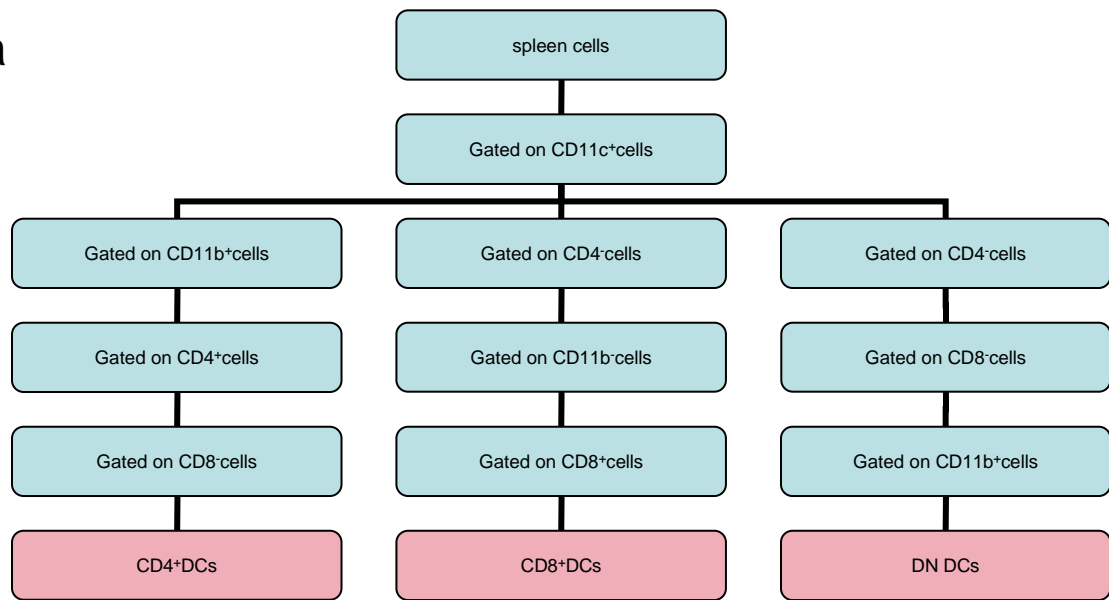
**c** Histogram displaying all cells within the dendritic cell gate. The gate (P9) indicates all CD11c-PE positive cells according to the isotype control.

**d** Histogram displaying all cells within the CD11c-PE positive cell population. The gate (P12) indicates all CD11b-FITC positive cell according to the isotype control.

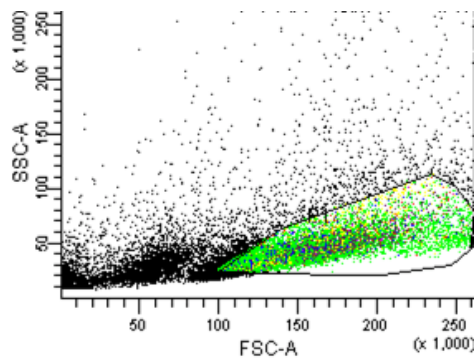
**e** Histogram displaying the CD11c<sup>+</sup>CD11b<sup>+</sup> positive cell population. The gate (P10) indicates all CD4-PerCP positive cells according to the isotype control.

**f** Histogram displaying the CD11c<sup>+</sup>CD11b<sup>+</sup>CD4<sup>+</sup> cell population. The gate (P13) indicates all CD8-APC negative cells according to the isotype control. Cells within this gate were positive for the surface markers CD11c, CD11b and CD4 but negative for CD8 and were hence sorted as the CD4<sup>+</sup>DC population.

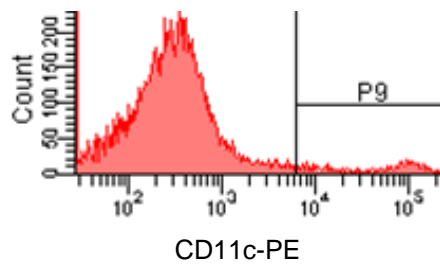
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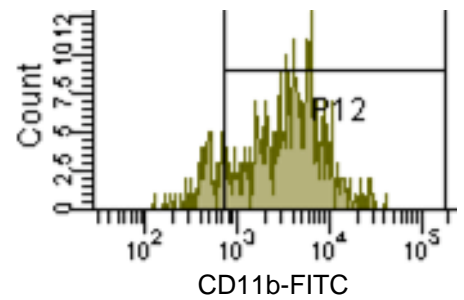
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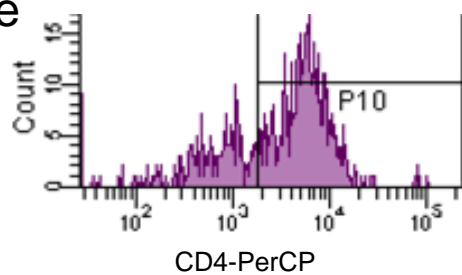
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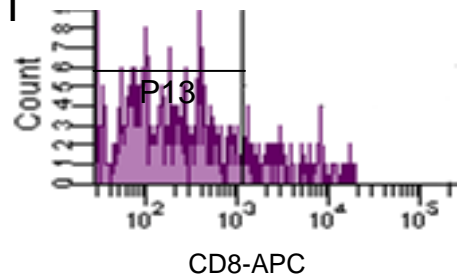
d



e



f



## **Figure 2.4 FACS Aria sorting of pDCs with mPDCA antibody**

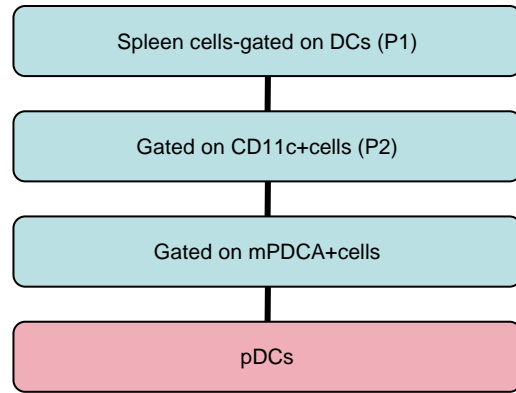
**a** Flow diagram displaying the sorting pathway for pDCs

**b** 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 (P1) indicates cells of the correct size and granularity associated with dendritic cells

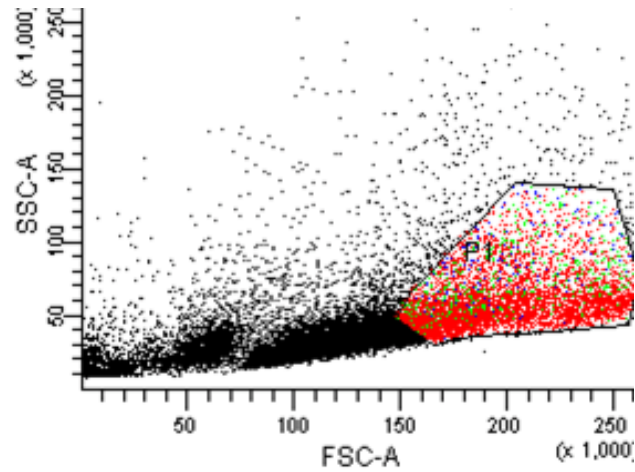
**c** Histogram displaying all cells within the dendritic cell gate. The gate (P2) indicates all CD11c-FITC positive cells according to the isotype control.

**d** Histogram displaying the CD11c<sup>+</sup> cell population. The gate (P3) indicates all mPDCA positive cells according to the isotype control and cells within the gate were sorted on the FACS Aria as pDC population

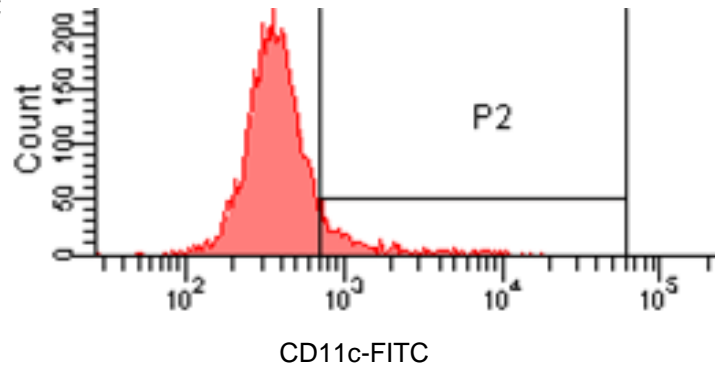
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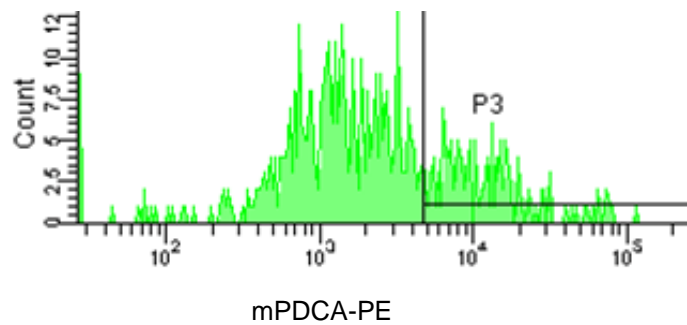
b



c



d



## **Figure 2.5 FACS Aria sorting of pDCs according to their surface markers**

**a** Flow diagram displaying the sorting pathway for pDCs

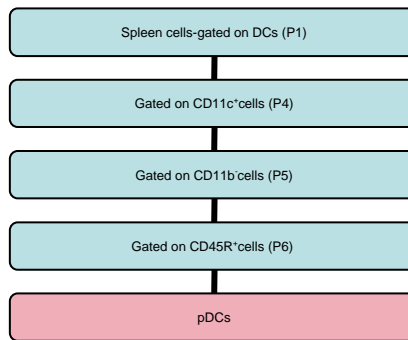
**b** Dot plot of forward scatter versus side scatter, showing the population of cells deemed healthy by their position on this plot. Forward scatter gives a measure of the size of cells, whilst side scatter gives a measure of their granularity. The gate (P1) indicates cells of the correct size and granularity associated with dendritic cells

**c** Histogram displaying all cells within the dendritic cell gate. The gate (P4) indicates all CD11c-PE positive cells according to the isotype control.

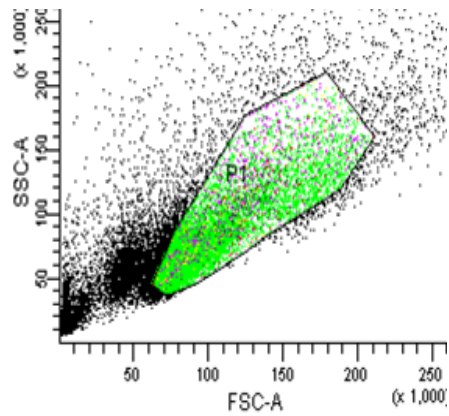
**d** Histogram displaying the CD11c positive cell population. The gate (P5) indicates all CD11b-FITC negative cells according to the isotype control. This negative selection was done to eliminate CD4<sup>+</sup>DCs and DN DCs

**e** Histogram displaying the CD11c<sup>+</sup>CD11b<sup>-</sup> cell population. The gate (P6) indicates all CD45R-APC positive cells according to the isotype control. Cells within this gate were positive for the surface markers CD11c and CD45R and were sorted as the pDC population.

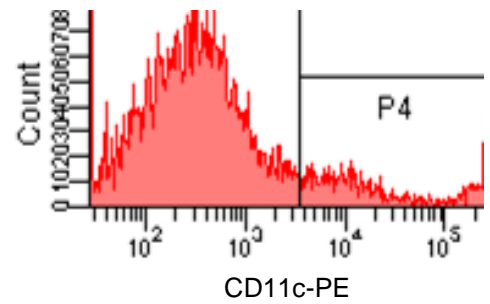
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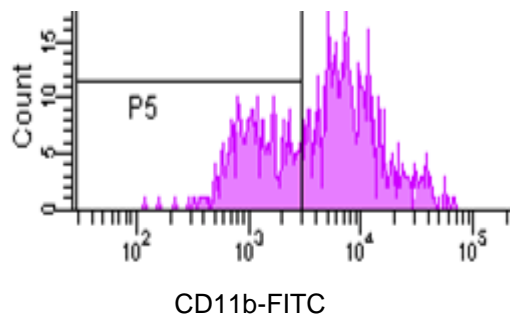
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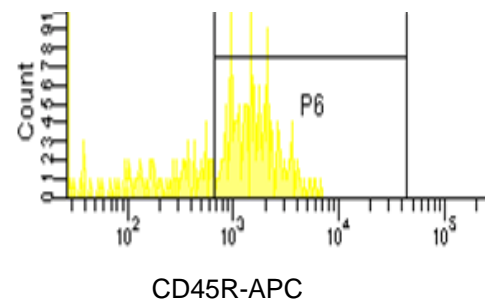
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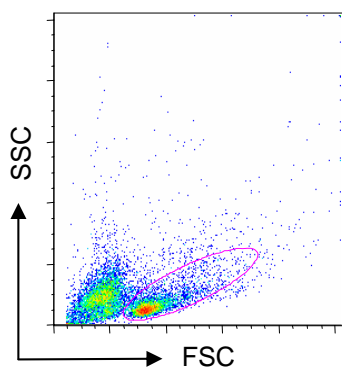
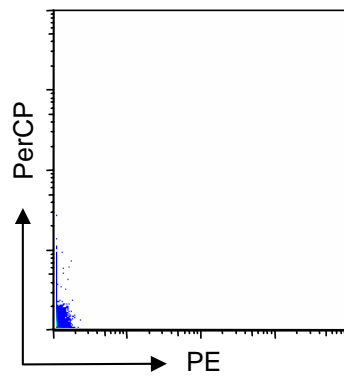
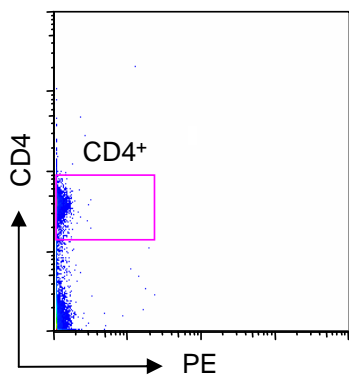
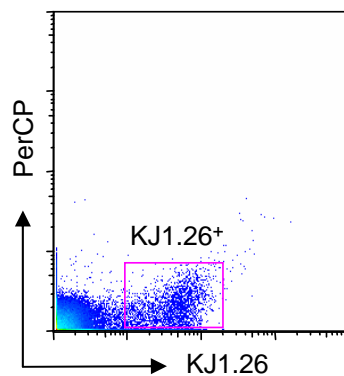
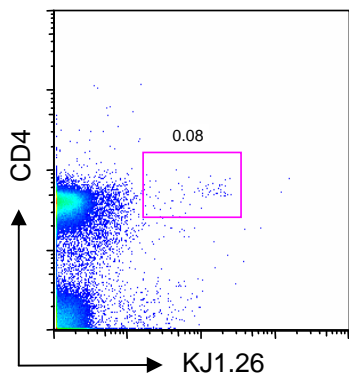
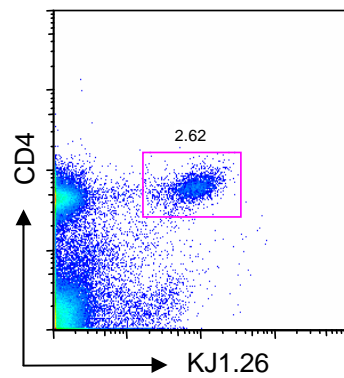
**Figure 2.6 FACS analysis of CD4<sup>+</sup> transgenic OVA-specific TCR T cells from DO.11.10 BALB/c mice**

**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<sup>+</sup> T lymphocytes (**c**) Dot plot of lymphocytes single stained with the anti-clonotypic KJ1.26-biotin antibody and streptavidin-PerCP conjugate. The antibody binds the TCR expressed by CD4<sup>+</sup> 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<sup>+</sup>KJ1.26<sup>+</sup> T cells from an unimmunised mouse with a gate for double positive CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells.

**f** Dot plot showing the population of transgenic CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells from an immunised mouse. From this plot the population of CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells can be clearly identified and gated, thus allowing the percentage of CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells from the total lymphocyte population to be calculated.

**a****b****c****d****e****f**



**Figure 2.7 FACS Aria sorting of CD4<sup>+</sup> transgenic OVA-specific TCR T cells from DO.11.10 BALB/c mice**

**a** Flow-diagram displaying the sorting pathway used to purify CD62L<sup>+</sup>KJ1.26<sup>+</sup>T cells.

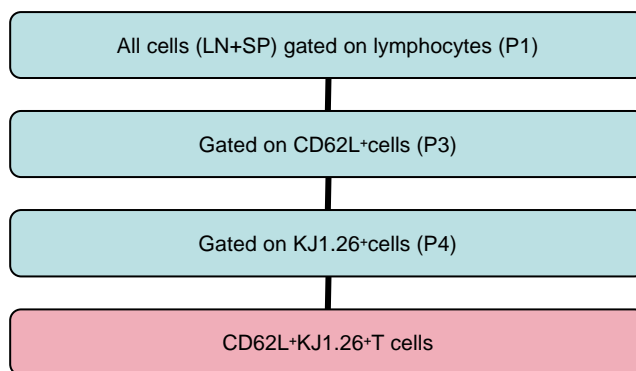
**b** Dot plot of forward scatter versus side scatter, showing the population of cells deemed healthy by their position on this plot. Forward scatter gives a measure of the size of cells, whilst side scatter gives a measure of their granularity. The gate (P1) indicates cells of the correct size and granularity associated with lymphocytes.

**c** Histogram displaying all cells within the lymphocyte population. The gate (P3) indicates all CD62L positive cells according to the isotype control.

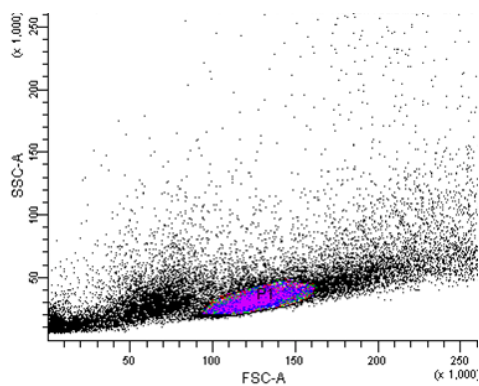
**d** Histogram displaying the CD62L positive cell population. The gate (P4) indicates all KJ1.26 positive cells according to the isotype control.

**e** Dot plot of lymphocytes double stained with the anti-clonotypic KJ1.26 antibody and CD62L antibody. The purple coloured population is positive for both stains and cells within it were sorted as CD62L<sup>+</sup>KJ1.26<sup>+</sup>Tcells

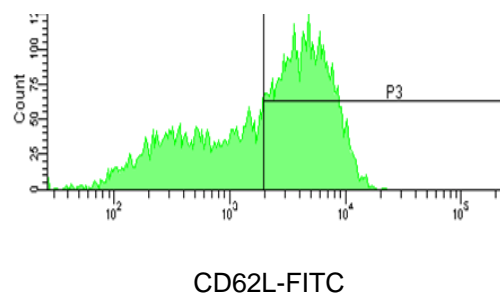
a



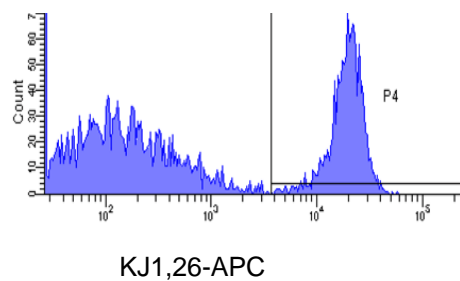
b



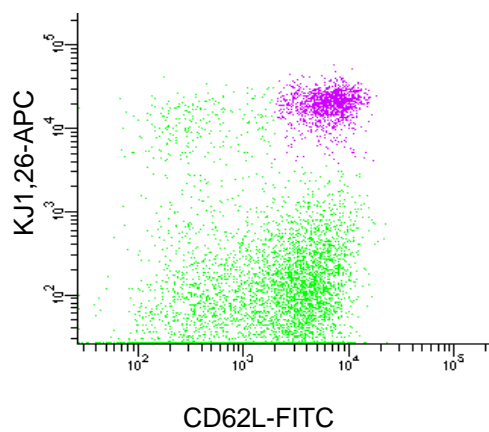
c



d



e



## **Figure 2.8 FACS Aria sorting of CD4<sup>+</sup> T cells**

**a** Flow-diagram displaying the sorting pathway used to purify CD3<sup>+</sup>CD4<sup>+</sup>T cells

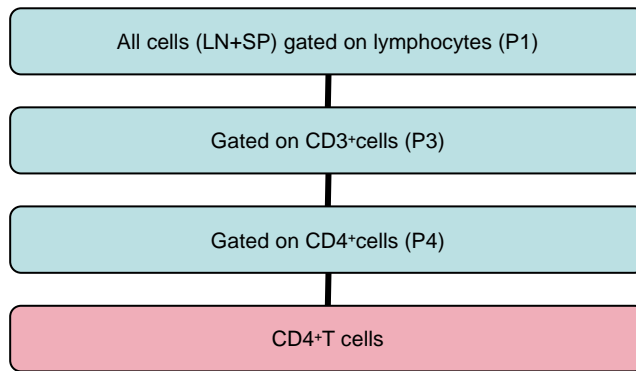
**b** Dot plot of forward scatter versus side scatter, showing the population of cells deemed healthy by their position on this plot. Forward scatter gives a measure of the size of cells, whilst side scatter gives a measure of their granularity. The gate (P1) indicates cells of the correct size and granularity associated with lymphocytes.

**c** Histogram displaying all cells within the lymphocyte population. The gate (P3) indicates all CD3 positive cells according to the isotype control.

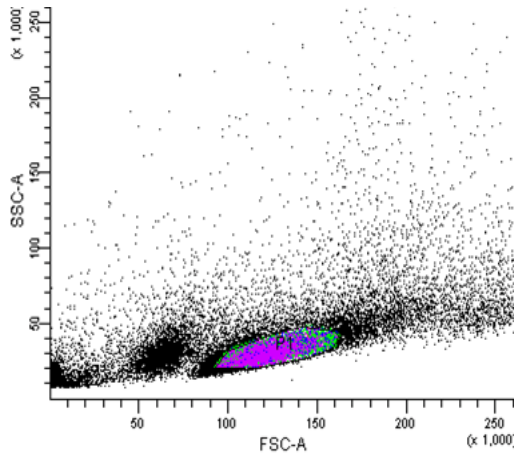
**c** Histogram displaying the CD3 positive cell population. The gate (P4) indicates all CD4 positive cells according to the isotype control.

**d** Dot plot of lymphocytes double stained with the CD3 and CD4 antibody. The purple coloured population is positive for both stains and cells within it were sorted as CD4<sup>+</sup>T cells

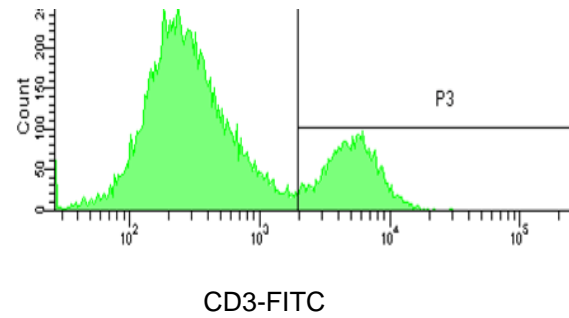
a



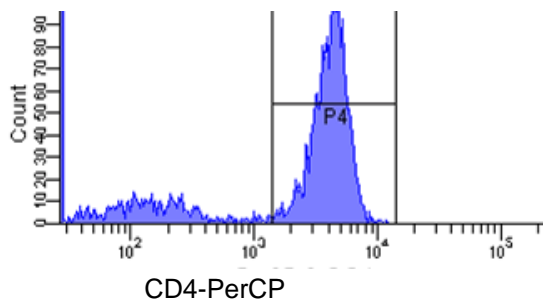
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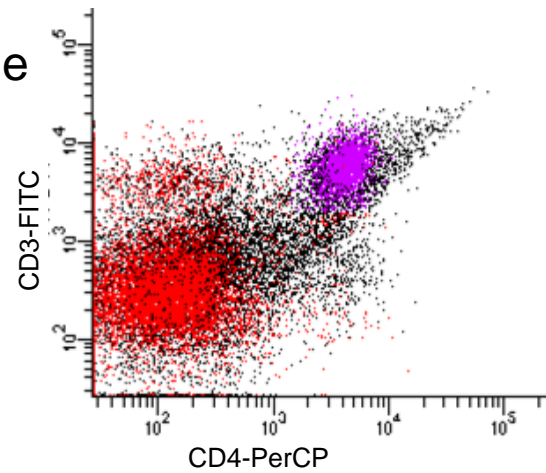
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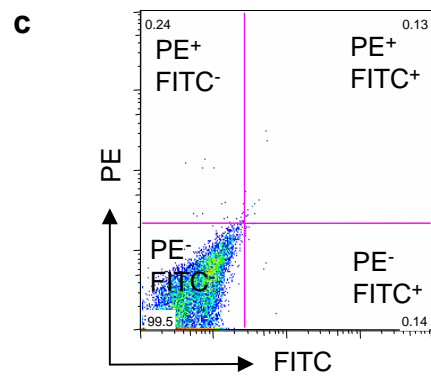
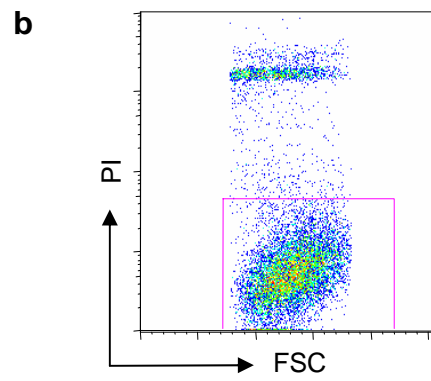
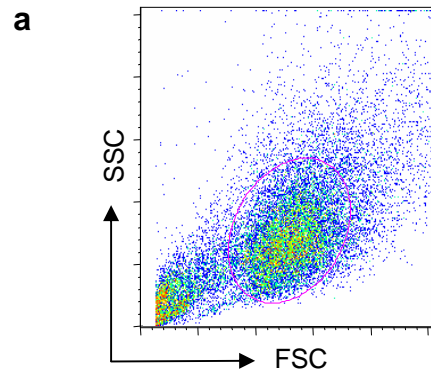


## **Figure 2.9 FACS analysis of costimulatory molecule expression by dendritic cells**

**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  $\mu\text{g/ml}$ ) 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 and by staining with the relevant isotype control antibodies for the co-stimulatory molecule being analysed.



## **Figure 2.10 FACS analyses of intracellular cytokine staining**

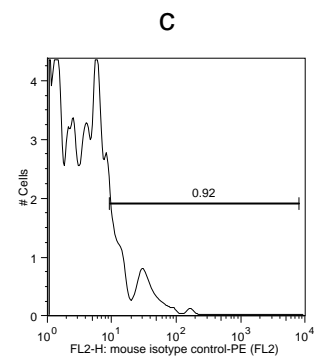
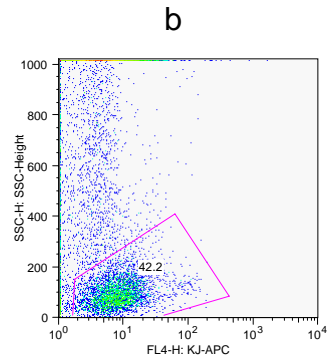
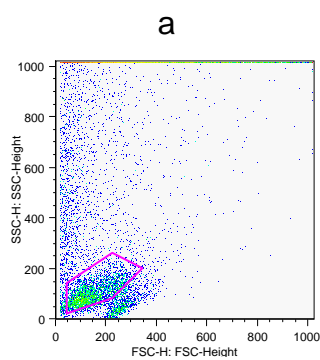
**a** Dot plot of forward scatter versus side scatter, showing the population of cells deemed healthy by their position on these plots. Forward scatter gives a measure of the size of cells, whilst side scatter gives a measure of their granularity. The gate indicates cells of the correct size and granularity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

**b** Dot plot of side scatter versus KJ1.26 staining, showing the population of transgenic T cells.

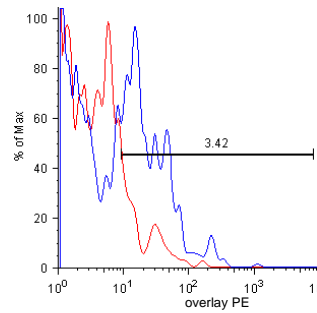
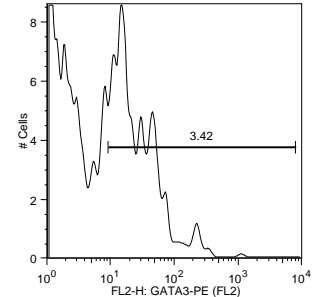
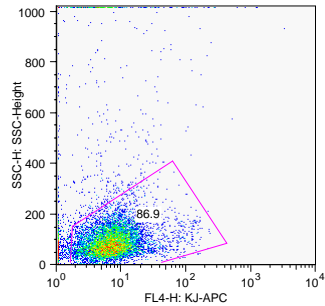
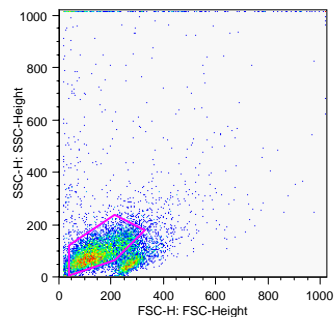
**c** Histogram of KJ1.26 positive cells displaying the transcription factor. Gates are set using the isotype control.

**d** Overlay of histograms displaying the isotype control staining and GATA3 or Tbet staining.

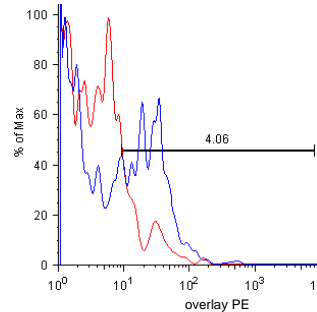
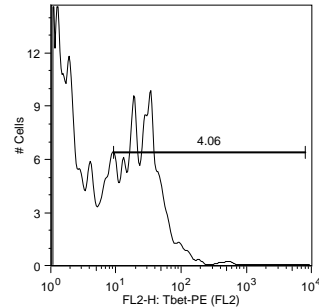
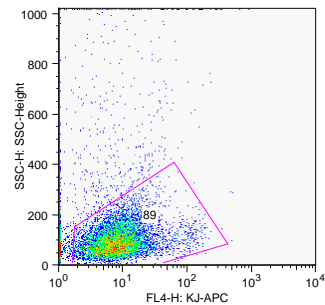
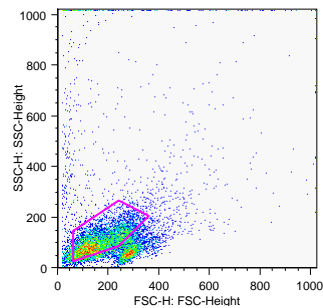
isotype



GATA3



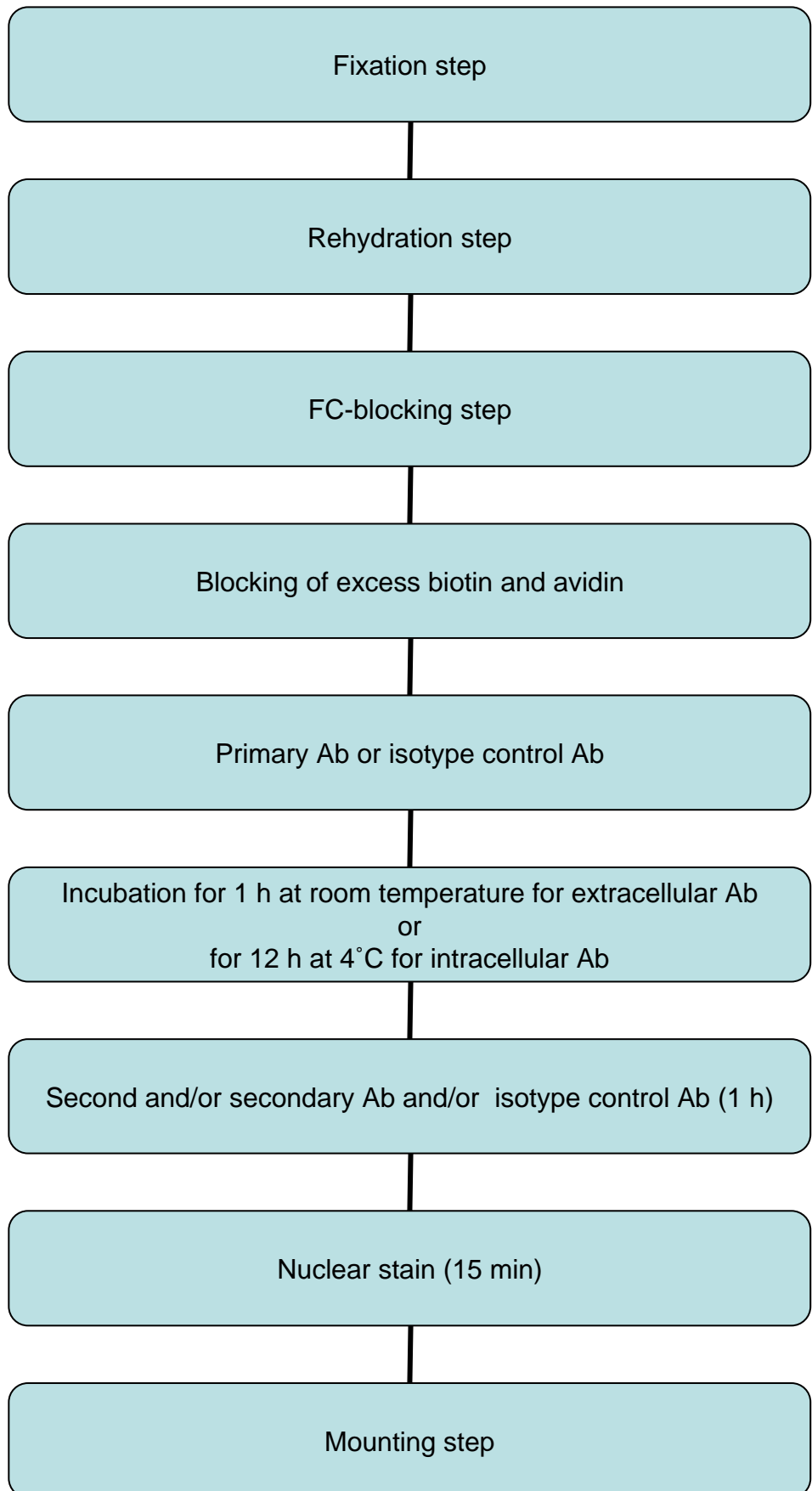
Tbet





**Figure 2.11 Flow diagram displaying a standard staining protocol**

The diagram displays the protocol generally followed when staining LN sections for surface marker and intracellular transcription factors



## **Chapter 3**

# **ES-62-mediated modulation of DC subtype phenotype and subsequent priming of Ag-specific T cell responses**

### 3.1 Introduction

As stated in section 1.2.1, there are several well characterised subtypes of DCs. Initially, it was believed that DCs were of myeloid origin as they could be generated from monocytes under the influence of GM-CSF [435], until it was shown that lymphoid-restricted precursor cells from the thymus could also generate DCs [62], leading to the assumption that whilst CD8<sup>-</sup>cDCs were of myeloid-lineage, CD8<sup>+</sup>cDCs were of lymphoid-lineage origin [62, 435, 436]. Moreover, analyses of immunoglobulin heavy chain rearrangement as a marker of lymphoid origin, revealed that whilst about 50% of thymic cDCs and pDCs exhibit these gene rearrangements, only a few cDCs in spleen and LNs do so [437] supporting the view that most cDCs in peripheral lymphoid organs are derived from myeloid precursors whereas thymic cDCs are generally generated from lymphoid precursors. However, studies using murine precursors, which were transferred into irradiated recipients, demonstrated that all cDC subtypes could be generated from either precursor lineage [438-440].

Consistent with this, an early DC progenitor common to both myeloid and lymphoid lineages has been identified according to its expression of the marker, FLT3 [441]. FLT3 ligand has been shown to be of crucial importance in all DC development and mice lacking its expression exhibit strongly reduced numbers of DCs [442]. Furthermore, it has been demonstrated that administration of FLT3 ligand dramatically increases the number of DCs produced [443]. In addition, intermediate precursors lacking lineage-specific markers (lin<sup>-</sup>), but still expressing some haematopoietic precursor markers and not yet expressing DC markers, have also been identified. Moreover late precursor cells, already expressing the DC marker CD11c but lacking expression of other DC surface markers such as MHCII, that give rise to DCs and macrophages but not monocytes and granulocytes have been detected. Such later DC precursor cells are capable of differentiating into all of the cDC, but not pDC subtypes, upon transfer into the spleen of irradiated recipients, suggesting that pDC development branches off at an earlier developmental stage [444]. Interestingly, whilst cells that expressed CD45R in addition to CD11c were able to produce pDCs as well as cDCs, those which were CD45R negative

appeared to be cDC-restricted [445]. Furthermore, it has been shown that the bone marrow contains fully developed pDCs but only very few fully developed cDCs indicating that cDCs need to undergo further differentiation in the periphery [446].

By contrast, the early stages of DC development in the spleen are not very well understood, although currently it is believed that early DC precursor cells from the bone marrow arrive in spleen through the blood stream [447, 448]. Recently a late murine cDC precursor has been identified in murine spleen which has a weak phagocytic activity, does not express monocyte surface markers and is unable to develop into macrophages upon stimulation with GM-CSF [449]. By contrast this precursor cell is capable of producing all three major splenic DCs subtypes (Figure 3.1) *in vivo*, although it cannot generate pDCs. At present, no late or early splenic precursor of pDCs has been detected.

Interestingly, upon infection with microbes, or as a consequence of inflammation, an additional late DC precursor and resultant mature DC subtypes can be detected. For example, one of the best described of these so called “inflammatory” DCs appears to be derived from CCR2<sup>+</sup> monocytes [449, 450] as these monocytes are able to differentiate into DCs upon transfer into mice subjected to vigorous inflammatory responses. Furthermore, using mice deficient for the GM-CSF receptor, it has been demonstrated that this transformation requires GM-CSF [124, 451, 452] and that these DCs can be generated *in vitro* by culturing monocytes in the presence of GM-CSF [124]. Phenotypically, such “inflammatory” DCs can be distinguished from “steady state” DCs by their high expression of CD11b and MAC3 and their lack of CD4 and CD8 expression [449]. Recent studies have revealed that these monocyte-derived “inflammatory” DCs induce protective Th1 responses in *Leishmania* infected mice [453] and they have also been found in other infection, inflammation and autoimmune models [454].

### **3.1.1 Splenic cDC subtypes**

In the spleen, 3 major subtypes of cDC comprising some 80% of all splenic DCs as well as a pDC (20%) population have been described [66]. About 60% of this cDC

population are CD11c<sup>+</sup>CD11b<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>CD205<sup>-</sup> DCs which from now on will be referred to as the CD4<sup>+</sup> DC subtype. The other two cDC subtypes, which each account for about 20 % of splenic cDC, are the CD11c<sup>+</sup>CD11b<sup>-</sup>CD4<sup>-</sup>CD8<sup>+</sup>CD205<sup>+</sup> DCs and the CD11c<sup>+</sup>CD11b<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD205<sup>-</sup> DCs which from now on will be termed CD8<sup>+</sup> and double negative (DN) DCs respectively. Recently, however, a new minor splenic subtype of cDCs has been discovered, namely interferon killer DCs (IKDCs), which are defined by their expression of CD45RA, CD11c, CD122 and NK1.1. By contrast, pDCs in the spleen express the surface markers CD11c<sup>lo</sup>CD45RA<sup>hi</sup>CD11b<sup>-</sup>CD205<sup>-</sup>CD4<sup>±</sup>CD8<sup>±</sup>.

Under normal healthy conditions (steady state), murine cDCs and pDCs have been shown to localize in different compartments of the spleen. Thus, the CD4<sup>+</sup> and the DN DCs are mostly found in the marginal zones whereas the CD8<sup>+</sup> DCs are located in the T cell zones [455-457]. pDCs are also mainly found in the T cell-rich area and they appear to have migrated there through the marginal zone areas of the spleen [458]. However, this DC distribution profile changes if such DCs are exposed to maturational stimuli. For example, it has been shown that following exposure to *Toxoplasma gondii* extracts or LPS, CD4<sup>+</sup> and DN DCs migrate to the T cell areas of the spleen, possibly to interact with CD8<sup>+</sup> DCs and T cells, whilst pDCs seem to have the capacity to be recruited to any site of inflammation [69]. Interestingly, in addition to localizing to different areas of the spleen, DC subtypes also exhibit significant differences in lifespan. Thus, whilst in the steady state, all splenic cDCs have a very rapid turnover time of not more than 4 d [76], the CD8<sup>+</sup> cDC subpopulation has the shortest lifespan of all and is usually replaced within 3 d. By contrast, pDCs are the most long lived DCs and can remain in the spleen for about two weeks [69].

Even though all splenic DCs share the same basic functions of taking up Ag and presenting it to naïve T cells, the different subtypes also selectively exhibit more specialised functions. For example it has been shown that CD8<sup>+</sup> DCs are the only DC subtype which can cross-present cell-associated Ags on MHC class I molecules and this has been attributed to their ability to capture dead cells [459-461]. By contrast, soluble or immunocomplexed Ags, or Ags associated with

bacteria, have been reported to be cross-presented by all cDCs [459, 460]. However a recent study suggested that whilst all cDCs can indeed take up soluble Ag, only CD8<sup>+</sup> DCs are efficient at cross-presentation indicating that they alone might possess specialized machinery required for this function [462]. Consistent with this proposal, only CD8<sup>+</sup> DCs can process cellular, soluble and latex bead-associated Ags by cross-presentation, although all DCs are able to capture and present such Ags using the MHC class II pathway [463].

Another interesting difference between DC subtypes is their capacity to differentially produce certain cytokines. For example, it has been reported that although all DCs are able to produce IL12p70 upon microbial stimulation together with CD40 ligation [464], CD8<sup>+</sup> DCs are reported to be very efficient producers of this cytokine upon stimulation with pathogen-derived products alone [59, 465, 466]. Therefore, activated CD8<sup>+</sup> DCs have been suggested to be extremely good Th1 inducers [59, 61, 467]. At first sight, it is difficult to reconcile these findings with the reported ability of these cDCs to induce CD8<sup>+</sup> T cell deletion or CD8<sup>+</sup> T cell unresponsiveness. Nevertheless, a possible mechanism for this latter property could lie in their ability to take up apoptotic cells via the lectin receptor CD205, as Ag processing via this receptor has previously been reported to induce tolerance [468].

Similarly, another important cytokine, IFN $\alpha$  has been widely reported to specifically and rapidly secreted by pDCs [69, 71] following stimulation of TLR7 or TLR9 upon virus infection [100, 106, 107]. Recently, however, some studies have also suggested that IKDCs might produce this cytokine [85, 86]. Moreover, there is considerable controversy regarding which DC subsets are responsible for the production of IFN $\gamma$  as CD8<sup>+</sup> DCs [241], DN DCs [466] or even both of these DC subtypes [469] have been found to produce this cytokine. In addition, precursors of the CD8<sup>+</sup> DC subtype, as well as the Ly6C<sup>-</sup> form of pDCs, (both of which are routinely found in DC subset preparations), can induce IFN $\gamma$  production [470]. Moreover, when it was proposed that IKDCs comprised a separate subtype, these cells were recognised as being capable of secreting IFN $\gamma$ . Indeed, a recent study

has now demonstrated that IKDCs are the most efficient producers of this cytokine [470].

As all of these studies show, there are many differences in the phenotype, the location and the cytokine profile of individual DC subsets and thus, it is likely that these DCs may mediate specific roles in the immune response.

### **3.1.2 cDC subsets in other secondary lymphoid organs**

Generally, the DC subtypes characterised in the spleen can also be found in LNs and in the thymus, although the relative proportions of the subtypes can be quite different in these secondary lymphoid organs. Thus, in LNs there are relatively far fewer CD4<sup>+</sup> DCs than in the spleen [67] whilst in the thymus, CD8<sup>+</sup> DCs are the major cDC population, accounting for up to 90% of all cDCs [66]. Another major difference between LN and splenic DC populations is that LNs also contain migratory DCs [78, 471, 472]. These migratory DCs arrive via the lymph and their number increases drastically during infection and inflammation [473-475]. For example mesenteric LNs (MLNs) contain an additional DC subtype which is not found in significant numbers in the spleen. This subtype, which expresses the surface markers CD8<sup>low</sup>CD205<sup>+</sup>CD11b<sup>+</sup>, is thought to have migrated to the MLNs from the gut via Peyer's patches [74]. Similarly, skin draining LNs contain migratory DCs which correspond to Langerhans cells, a DC characterised by the expression of the lectin langerin, and DCs derived from the dermis which show similar properties to migratory DCs in the MLNs [67, 471, 476]. Draining LNs from the lung, liver and kidney contain a subtype which is distinct from all other migratory DCs as it expresses the unique surface marker set CD8<sup>-</sup>CD11b<sup>-</sup>CD205<sup>+</sup> [68].

### **3.1.3 Plasmacytoid DCs**

pDCs can be found in all secondary lymphoid organs and in the bone marrow [71, 477]. In addition to their possible role in viral defense (via the production of IFN $\alpha$ ), many other functions have been determined for pDCs. For example it has been shown that pDCs, matured in response to either viruses or CpG-



oligodeoxynucleotides (CpG-ODN), induce the proliferation of T cells that produce both IFN $\gamma$  and IL10 [478], whereas stimulation of pDCs with IL3 and CD40L primes T cells to secrete IL4, IL5 and IL10 [175, 479, 480]. Some studies also investigated the ability of pDCs to prime memory T cells and found that influenza infection could induce pDCs that indeed not only prime virus-specific primary responses but also memory T cell responses [481]. Perhaps surprisingly therefore, pDCs have also been shown to be involved in Treg induction as they can prime naïve T cells to differentiate into IL10-producing Tregs [184, 186] and consistent with this, it has also been shown that CpG-ODN stimulation of pDCs can induce a Treg phenotype [183]. Furthermore, two investigations, one in an asthma model [185] the other in a *Leishmania major* infection model [482], suggested that pDCs can play an important role in immune suppression.

Interestingly, it has been shown that pDCs can interact with, and influence cDC, NK and B cell function. For example, during HIV infection pDCs can induce maturation of cDCs by producing IFN $\alpha$  [483]. Consistent with this, it has also been shown that, in the absence of type 1 IFN receptors, cDCs do not undergo maturation upon viral infection or in response to double stranded RNA [181, 182]. Thus, pDCs have been shown to play various roles in the immune system and, via interaction with various cell types, they can induce Th1, Th2, T memory and Treg responses although, it is still unclear whether there are different pDC subtypes which may account for such differential responses.

### 3.2 Aims

It has been previously shown by this laboratory that treatment with ES-62, *in vitro*, induces a partial maturation of immature bm-derived CD11c<sup>+</sup> DCs, as evidenced by a slight up-regulation of co-stimulatory molecules (CD40 and CD86) and the secretion of cytokines (IL12p40 and TNF $\alpha$ ). That such maturation is only partial is indicated by comparison with the expression levels of co-stimulatory molecules CD40, CD54, CD80 and CD86 and cytokine production achieved following maturation with LPS [146, 403]. Interestingly, whilst LPS-matured DC induced priming of OVA-specific Th1 (IFN $\gamma$ )-polarised responses, such ES-62-matured DCs primed Th2-biased responses [146]. Similarly, when bmDCs were generated from mice in which the parasite product was administered continuously *in vivo* at physiologically relevant levels, using subcutaneously implanted osmotic pumps to mimic a naturally occurring filarial nematode infection, comparison of the expression of co-stimulatory molecules on DCs exposed to ES-62 *in vitro* and *in vivo* showed very similar phenotypes. However, an increase in CD86 was detected when progenitor cells were pre-exposed to ES-62 *in vivo* and subsequently cultured *in vitro* with LPS. As CD86 has been implicated in promoting Th2 immune responses it was suggested that up-regulation of this co-stimulatory molecule might provide a mechanism for ES-62 to counteract a Th1 response [146].

Although it was postulated that the semi-mature phenotype elicited by ES-62 was likely to be responsible for the Th2-skewing of the T cell, investigation of the roles of CD80/86 was inconclusive, as blocking CD80/86 suppressed both LPS-induced Th1 and ES-62-elicited Th2 responses. Further studies investigating the role of OX40-L and ICOS-L, two molecules proposed to promote Th2 effector responses, [484-488] revealed that ES-62-treated DCs displayed a slightly increased expression of OX40-L but a decreased expression of ICOS-L in comparison to immature (GM-CSF) DCs. Moreover, induction of OX40L by ES-62 was less than that resulting from LPS maturation [403] perhaps therefore suggesting that it may be the overall immature phenotype of ES-62-DC that causes such immunomodulation. However, and perhaps arguing against this proposal, an interesting discovery was made when bmDCs were matured with ES-62 to drive

OVA-specific Th2 responses from OTII (C57BL/6) mice [403], a genetic background that is known to be more disposed to generating Th1 responses [489, 490] than the Th2 prone BALB/c mice [491, 492]. Following exposure of such bmDCs from C57BL/6 mice to ES-62, all co-stimulatory molecules were found to be up-regulated to a higher level than observed with bmDCs-derived from BALB/c mice, albeit still to a lesser extent than that observed in response to LPS [403]. These data suggested that, in a Th1 prone system, ES-62 may have to further activate DCs to counteract the “default” Th1 response effectively.

In addition, studies investigating the effect of ES-62 on DC maturation as indicated by cytokine production were carried out. It was shown that in contrast to LPS, which strongly induced the production of IL12p40 and TNF $\alpha$  by bmDCs [493-495], ES-62 only promoted low level production of these cytokines [146, 425]. Moreover, whilst LPS-maturation induced high mRNA levels for IL12p40, IL18 and IFN $\beta$ , such induction was suppressed in bmDCs which had been pre-exposed to ES-62. Interestingly, when IL10 mRNA levels were measured, an increase in ES-62-treated DCs, and in particular in ES-62 + LPS exposed DCs, was found, whilst little or no IL10 mRNA was detected in DCs cultured only with LPS [403].

These preliminary studies focused on the effects of ES-62 on GM-CSF-elicited, BM-derived CD11c<sup>+</sup> DCs. However, it is emerged in the intervening time period that there are many different subtypes of DC and that there are maturational and functional differences between bm- and tissue (eg spleen) DCs. Thus, it was therefore planned to:

1. extend the initial studies to determine whether ES-62 exerts differential effects on the maturation status of bm- and spleen-derived DCs as evidenced by surface molecule expression on heterogeneous and purified DC populations from the bone marrow and spleen
2. determine whether pre-exposure to ES-62 modulates the maturational phenotype of DCs associated with the induction of Th1 responses such as DC matured with TLR ligands e.g. LPS.

3. investigate whether ES-62 differentially modulates the cytokine responses of heterogeneous and purified CD11c<sup>+</sup> DC populations from bone marrow and spleen and how pre-exposure to ES-62 alters the LPS-mediated cytokine profile of these cells
4. characterise the effects of ES-62-exposed DC populations from bone marrow and spleen on T cell responses and to determine how ES-62 pre-treatment influences the ability of LPS-matured DCs to promote a Th1 cell response.
5. determine whether the putative TLR4-ligand, ES-62 can modulate maturation and effector function of pDCs that have been reported not to express TLR4
6. define whether ES-62 can elicit different effector T cell subsets (e.g. Th1, Th2, Th17, Treg) by differentially modulating the maturation of individual cDC and pDC subtypes.

### 3.3 Results

#### 3.3.1 Characterisation of the effects of pre-exposure to ES-62 on co-stimulatory molecule expression of LPS-matured bmDCs

Whilst ES-62 appears to drive a Th2/anti-inflammatory response via the induction of a semi-mature phenotype of bm-DC, LPS promotes full maturation of such DCs with consequent Th1 polarisation of T cell responses. It was therefore investigated whether exposure to ES-62 could modulate LPS-driven maturation of DCs and Th1 responses as a first step to investigating how ES-62 suppresses Th1 responses *in vivo*.

To examine the effects of pre-exposure to ES-62 on LPS-driven DC maturation, bone marrow from BALB/c mice was harvested and cultured in the presence of GM-CSF for 6 d. Cells were then matured with ES-62 for 24 h and, where indicated, with LPS for further 24 h. As reported in previous studies LPS induced an up-regulation of all co-stimulatory molecules (CD40, CD54, CD80, CD86 (Figure 3.2)) resulting in fully matured DCs secreting large amounts of pro-inflammatory cytokines IL12p40 and TNF $\alpha$  (Figure 3.3). By contrast, ES-62 treatment only slightly increased expression of CD40 and CD86 resulting in a semi-mature phenotype. Furthermore IL12p40 and TNF $\alpha$  levels in culture supernatants from ES-62 exposed DCs were only slightly increased compared to PBS control cultures further supporting the theory that ES-62 maturation leads to the development of a semi-mature DC. Such FACS analysis showed that pre-exposure to ES-62 could not inhibit the subsequent LPS-mediated up-regulation of CD40, CD54, CD80 and CD86 (Figure 3.2). To determine whether ES-62 was able to inhibit LPS induced cytokine production IL12p40 and TNF $\alpha$  levels in culture supernatants were measured and indeed both were reduced compared to those found in LPS-treated cultures (Figure 3.3). This indicated that although ES-62 had little effect on the LPS-induced surface marker expression it could render such DCs refractory to producing cytokines.

Following the above analysis of maturation markers, it was decided to take a closer look at surface markers such as CD205, CD11b and CD45R which are used to

discriminate DC subsets to address whether ES-62 might promote development or survival of one particular subtype. CD205 and CD11b are expressed on most cDC subtypes whereas CD45R, which is also known as B220, is only found on pDCs [66]. As a first step, CD11c<sup>+</sup> DC responses in whole bm cultures were examined as it was possible that ES-62 might elicit its effects on CD11c<sup>+</sup> DCs via indirect actions on other cells within the population and/or by modulating the differentiation of DC progenitors. Analyses of the surface marker expression of CD11c<sup>+</sup> DCs within such bm cultures revealed a substantial increase in the % of cells expressing CD11b in response to all maturational stimuli but only marginal rises in the % of CD11c<sup>+</sup> cells expressing CD45R or CD205 in response to ES-62 and/or LPS compared to control bm cultures. Nevertheless, it appeared that a slight up-regulation of CD45R was elicited preferentially by ES-62. By contrast, in cultures containing purified CD11c<sup>+</sup> bmDCs, the % of cells expressing CD11b was reduced by maturation by either ES-62 or LPS (Figure 3.4).

As these initial studies were carried out with bmDCs, which have to be cultured *in vitro* to fully differentiate into DCs, it was decided to repeat the experiments using DCs harvested from the spleen (spDC). Nevertheless, in order to have a similar environment and also because it is known to be involved in the production of “inflammatory” DCs, it was decided to add GM-CSF to all culture media. Interestingly and in contrast to the bmDC cultures, the majority of the CD11c<sup>+</sup> cells in the spDC cultures were CD45R positive following exposure to GM-CSF (expressed by 20% of all freshly isolated spDCs) and the % of cells expressing this marker could be further slightly increased by culture with LPS. Furthermore the % of CD11b<sup>+</sup> CD11c<sup>+</sup> cells was found to be extremely low (expressed by 60-65 % in freshly isolated spDCs) and further reduced by ES-62 and/or LPS maturation. Similarly, the % of purified CD11c<sup>+</sup> DCs expressing CD205 (expressed by 15-20% of all freshly isolated spDCs) was found to be reduced following LPS treatment (Figure 3.4). Thus this data suggests that bmDCs and spDCs not only change their phenotype in response to ES-62 and LPS maturation but comparison of different surface marker expression levels of purified CD11c<sup>+</sup> and heterogeneous bone marrow cell cultures (including DC progenitors) revealed the possibility for indirect effects of ES-62 and LPS on DC maturation. Furthermore, the unexpected high

number of CD11c<sup>+</sup>CD45R<sup>+</sup> spDCs might be explained by the fact that GM-CSF was added to the splenic cultures and hence an inflammatory environment was created which is also known to alter the DC phenotype.

### **3.3.2 The effects of ES-62 and/or LPS on the cytokine expression profile of bm-derived and spDCs**

One mechanism that has been proposed to contribute to the polarisation of effector T cell responses is the spectrum of cytokines produced by DCs in response to pathogen products/"danger signals". Hence changing the DC phenotype or interfering with internal DC signalling pathways could modulate the secretion profile of these cells which in effect could result in a differently polarised T cell.

As found previously, whilst IL12p40, TNF $\alpha$  and IL6 production, was highest by LPS-matured DCs, it was slightly elevated in ES-62-exposed DCs relative to control immature DCs and this was seen for purified CD11c<sup>+</sup> bmDCs (Figure 3.5), whole bmDC cultures (Figure 3.6) and purified CD11c<sup>+</sup> spDCs (Figure 3.7). Furthermore pre-treatment with ES-62 rendered purified CD11c<sup>+</sup> bmDCs refractory to such LPS induced TNF $\alpha$  and IL12p40 production whereas IL6 was significantly increased compared to the LPS treatment group. By contrast, pre-exposure to ES-62 was not able to significantly inhibit subsequent LPS-induced responses from either whole bmDC cultures (Figure 3.6) or purified CD11c<sup>+</sup> spDC cultures (Figure 3.7).

The biologically active form of IL12 is IL12p70 which comprises the IL12p40 subunit in combination with the IL12p35 subunit [228, 229]. IL12p40 is secreted in excess and is known to form homodimers, which have been proposed to antagonise IL12p70 action [231, 232]. Furthermore IL12p40 is also contained in other cytokines such as IL23 [243] and therefore measuring the p40 subunit does not necessarily simply represent Th1-promoting IL12 bioactivity [496]. Hence it was decided to measure levels of IL12p70. Interestingly, in contrast to that observed with IL12p40, IL12p70 was produced in comparable amounts by ES-62 and LPS-

matured DCs but these levels were not substantially higher than those observed with immature control DCs. Collectively the data suggest that ES-62 does not promote a pro-inflammatory DC response and is quite effective at inhibiting production of pro-inflammatory cytokines induced by other pathogen products from CD11c<sup>+</sup> bmDCs and hence may use this mechanism to suppress Th1 responses. However, ES-62 does not inhibit LPS-driven pro-inflammatory cytokine production from spleen-derived CD11c<sup>+</sup> DCs. Another potential mechanism to counteract Th1 responses is the generation of anti-inflammatory cytokines such as IL10 which can promote the production of Tregs. Hence it was decided to investigate whether ES-62 could also act to antagonise Th1 responses by modulation of LPS-driven IL10 responses. In contrast to previously obtained mRNA data, however, IL10 cytokine levels were found to be lower in ES-62 than in LPS-treated bm DC cultures. Moreover, analysis of both bm and sp-derived, purified CD11c<sup>+</sup> DC populations indicated that a strong increase in IL10 secretion was obtained from LPS but not ES-62-treated DCs (Figure 3.5-3.7) and this LPS-response was not significantly modulated by prior exposure to ES-62 in spleen cell cultures. By contrast, bone marrow cultures pre-exposed to all maturational stimuli, but to ES-62 in particular, displayed a significant decrease in IL10 levels (Figure 3.6).

### **3.3.3 Exposure of bone marrow and splenic DCs to ES-62 can modulate the T cell response in consequent DC-CD4<sup>+</sup> T cell cultures**

The results presented so far have demonstrated that ES-62 can promote the development of bm- and sp-derived DCs that have an immature phenotype that reflects altered surface marker expression and low levels of cytokine production. It has previously been demonstrated in a DC-T cell co-culture system using T cells from DO.11.10 transgenic mice, which express the T cell receptor (TCR) KJ1.26 specific for OVA peptide 323-339 [427], that ES-62 exposed bm-derived CD11c<sup>+</sup> DCs promoted a more Th2 like response (increased IL4, decreased IFN $\gamma$ ), whereas LPS-matured bmDCs drove a strong Th1 reaction [146].

It was decided to repeat and to extend these studies by comparing the responses of CD11c<sup>+</sup> cells purified from the bone marrow and the spleen. Furthermore, it was



planned to determine whether ES-62 could modulate DC-priming of T cell responses by acting indirectly via other cells in bmDC cultures or perhaps indeed, by modulating DC progenitors in such fractions. Moreover, it was investigated whether prior exposure to ES-62 could modulate LPS-matured DC-driven induction of Th1 cell responses.

#### **3.3.3.1 The effect of ES-62-modulation of bmDC maturation on pro-inflammatory cytokine production by DC-T cell co-cultures**

Consistent with previous data [403, 494] showing that LPS-matured bm-derived CD11c<sup>+</sup> DCs drive Th1 responses, IL12p40, IL12p70, IL6 and TNF $\alpha$  responses were found to be generated in LPS CD11c<sup>+</sup> DC-T cell co-cultures in an Ag-specific, concentration-dependent manner (Figure 3.8). Although, it has not been formally demonstrated which cells are secreting these cytokines, IL12 and TNF $\alpha$  are not generally considered to be produced by T cells, suggesting that such production reflects ongoing bi-directional signalling between DCs and T cells to promote a Th1-biased environment. By contrast, ES-62-matured CD11c<sup>+</sup> DCs induced lower levels of IL12p40, IL12p70 and IL6 and no significant production of TNF $\alpha$  in such cultures. Interestingly, whilst prior exposure to ES-62 has little, if any, inhibitory effect on LPS DC-induced IL12p40, TNF $\alpha$  or IL6 responses it appears to ablate the IL12p70 response suggesting that it could potentially suppress Th1 responses in this manner (Figure 3.8). By contrast, whilst in whole bm DC cultures IL6 and IL12p70 production was highest in ES-62 + LPS-treated cultures and increased in an Ag-dependent manner (Figure 3.9), IL12p40 was lowest in these cells.

#### **3.3.3.2 The effects of ES-62-modulation of bmDC maturation on Th1 and Th2 cytokine production by bmDC-T cell co-cultures**

The key cytokine secreted by Th1-polarised T cells is IFN $\gamma$ . As expected, LPS-, but not ES-62-matured bm DCs induced an Ag-dependent rise in IFN $\gamma$  production by whole bmDC-T cell cultures. Furthermore the LPS DC-induced increase in IFN $\gamma$  levels was abolished when these bmDCs were pre-exposed to ES-62 (Figure 3.9). A similar situation was found when purified CD11c<sup>+</sup> DCs were used to prime T

cells. Thus, for example, LPS-matured CD11c<sup>+</sup> DCs induced the highest level of IFN $\gamma$  of all treatment groups, responses being maximal at 30 nM OVA. By contrast, ES-62-CD11c<sup>+</sup> DC induced little IFN $\gamma$  production at this Ag concentration and furthermore, pre-exposure of CD11c<sup>+</sup> DC to ES-62 appeared to ablate their ability to induce Ag-specific IFN $\gamma$  production by T cells (Figure 3.8).

As it was previously reported that such ES-62-modulation of Th1 priming reflected induction of a DC phenotype that was likely to promote Th2 development, the key Th2 cytokines IL4 and IL5 were also measured in these co-culture systems. Indeed, and confirming previous data, co-cultures containing ES-62-treated CD11c<sup>+</sup> bmDCs expressed significantly higher levels of IL4 and IL5 than any other treatment group. However, pre-exposure of ES-62 and subsequent maturation with LPS did not induce production of these cytokines (Figure 3.8). In co-cultures containing whole bmDCs, the highest levels of IL4 were found in those containing ES-62-matured cells, although this was not the case for IL5 (Figure 3.9).

### **3.3.3.3 The effects of ES-62-modulation of bmDC maturation on Treg-associated cytokine production by bmDC-T cell co-cultures**

It has previously been reported that IL10 production was enhanced in an Ag-dependent manner in co-cultures containing ES-62-matured DCs suggesting that the parasite protein could not only bias responses towards a Th2 but might also drive differentiation towards a T regulatory phenotype. Consistent with these findings, only CD11c<sup>+</sup> DCs exposed to ES-62 were able to induce IL-10 in an Ag-dependent manner when co-cultured with T cells. Hence it was decided to investigate the expression of FoxP3, a transcription factor which has recently been shown to be essential for the development of T regulatory cells [338-340, 360], to determine whether its expression was up-regulated in T cells primed by ES-62-matured DCs.

#### **3.3.3.4 The effects of ES-62-modulation of bmDC maturation on the expression of the key Th cell transcription factors Tbet, GATA3 and FoxP3 by Ag-specific T cells in bmDC-T cell co-cultures**

To determine whether the above observed differences between treatment groups reflected modulation of the expression of FoxP3, Tbet and GATA3, the master transcriptional regulators of Treg, Th1 and Th2 development, respectively, [497] [276, 278] responses were measured by intracellular staining of T cells followed by flow cytometric analyses of the % of OVA transgenic T cells expressing high levels of each transcription factor. The obtained data showed that only a few cells expressed any of the transcription factors at high levels, making analyses rather difficult. Nevertheless, such analysis revealed that the reduced IFN $\gamma$  responses (co-cultures containing whole or CD11c<sup>+</sup> DCs) obtained in response to ES-62 modulation of DC phenotype did not reflect a decrease in the % of T cells expressing Tbet nor an increase in GATA3-expressing cells that correlated with the observed changes in cytokine production, indicating that ES-62 treatment did not simply drive a Th1/Th2 switch (Figure 3.10). Moreover, FoxP3 expression was not up-regulated in T cells primed by DCs exposed to ES-62 or ES-62/LPS (Figure 3.10). To determine whether there was a relative increase of one transcription factor to another the ratios of GATA3/Tbet and FoxP3 and of FoxP3/Tbet were calculated (Figure 3.11). Interestingly, this data showed that ES-62 and ES-62 + LPS exposed cultures in general had ratios of GATA3 versus Tbet or FoxP3 which were in favour of GATA3 which is consistent with the proposal that ES-62 promotes Th2 polarisation. By contrast, LPS exposed cultures induced relatively more Tbet than GATA3 production and the ratios of FoxP3 versus Tbet were similar for all groups.

As these analyses were very difficult due to the weak staining it was decided to measure the mRNA expression of the transcription factors in all T cells (60-70% TCR transgenic) from co-cultures containing CD11c<sup>+</sup> bmDC by quantitative PCR (Figure 3.12). Consistent with their Th2-biased cytokine profile, T cells from ES-62-treated co-cultures induced highest levels of GATA3, whilst LPS- and ES-62 + LPS-treated cultures displayed an Ag-dependent decrease in the expression of this

transcription factor. Interestingly, although LPS-matured DCs induced higher expression of T-bet in co-cultured T cells, ES-62 was also able to promote an increase in this transcription factor but at higher Ag concentrations. However, consistent with the data obtained from intracellular staining, no FoxP3 production could be detected. Furthermore, the ratios of the transcription factors were compared and consistent with the data described above also here a relative increase of GATA3/Tbet and FoxP3 in cultures exposed to ES-62 and ES-62 + LPS could be seen (Figure 3.13).

Thus collectively, these data suggest that the observed decrease in IFN $\gamma$  and increase in IL4/IL5/IL10 production observed when DCs are exposed to ES-62 prior to LPS maturation might be due to the ratio of GATA3 versus Tbet being in favour of GATA3. Thus these data demonstrate that pre-exposure to ES-62 can switch LPS-CD11c<sup>+</sup> DCs from inducing Th1 (high IFN, low IL4, no IL10) to an anti-inflammatory Th population (low/no IFN $\gamma$ , low IL4/IL5 and IL10) by an as yet unidentified signalling mechanism. Similarly, when whole bmDC cultures are used to prime Ag-specific T cells, Th1 responses are suppressed possibly due to IL10 production which may be produced by T cell populations which may include a mix of Th2 and/or of Tregs as IL4 and IL5, but not IFN $\gamma$ , are also produced under these conditions.

#### **3.3.3.5 The effects of ES-62-modulation of spDC maturation on pro-inflammatory cytokine production by spDC-T cell co-cultures**

Similarly to the CD11c<sup>+</sup> bmDC co-cultures TNF $\alpha$ , IL12p40 and p70 responses generated in response to LPS-CD11c<sup>+</sup> spDC were increased in an Ag-specific, concentration-dependent manner. By contrast, ES-62-matured purified CD11c<sup>+</sup> spDCs induce lower levels of TNF $\alpha$ , IL12p40, IL12p70 and IL6 in such cultures. Furthermore prior exposure to ES-62 has little, if any, inhibitory effect on LPS DC-induced IL12p40, TNF or IL6 responses and it appears to increase the IL12p70 response (Figure 3.14).

### **3.3.3.6 The effects of ES-62 modulation of spDC maturation on Th1 and Th2 cytokine production by spDC-T cell co-cultures**

IFN $\gamma$  is the key cytokine of a Th1 response. Investigations of the production of this cytokine by Ag-specific T cell co-cultures primed by spleen-derived CD11c<sup>+</sup> DCs provided some rather surprising results. Thus, T cells cultured with ES-62-matured CD11c<sup>+</sup> purified DCs produced the highest levels of IFN $\gamma$ , although this decreased in an Ag-specific manner. However, as observed with bm-derived DCs, exposure of purified CD11c<sup>+</sup> DCs to ES-62 prior to culture with LPS resulted in DCs with the lowest capacity to drive IFN $\gamma$  production, whilst LPS treatment on its own resulted in moderate IFN $\gamma$  production which only showed a marginal trend to increase in an Ag-dependent manner (Figure 3.14). Consistent with this, Tbet levels did not increase in this treatment group (Figure 3.15) until the highest concentration of Ag (10 nM OVA) tested. Thus, to confirm that LPS was capable of inducing Th1 cell development, responses were tested at higher OVA peptide concentrations and results of the LPS cultures were compared to ES-62 cultures. Indeed already at the 30 nM OVA peptide concentration, LPS induced an increase in IFN $\gamma$  and at the 300 nM OVA peptide concentration, levels of the cytokine were two-fold higher in LPS cultures than in ES-62 cultures (Figure 3.16).

The ability of spleen-derived DCs to induce a Th2 response was investigated by examining production of the cytokines IL4 and IL5. Only ES-62-matured CD11c<sup>+</sup> DCs were able to induce IL4 production, and this response was Ag-dependent (Figure 3.14). Consistent with this, analysis of GATA3 expression revealed that this was highest in cells primed by ES-62-treated DCs and (Figure 3.15) and followed a similar profile to IL4 secretion. In addition also the ratio of GATA3/Tbet was in favour of GATA3 in ES-62-treated cultures (Figure 3.17). However, such ES-62-matured CD11c<sup>+</sup> DCs did not induce Ag-specific IL5 production under these conditions. Thus to confirm that ES-62 was capable of driving Th2 development, priming at even higher concentrations of Ag was examined. Indeed these results showed that IL4/IL5 production was highest in ES-62-treated cultures although at the highest OVA peptide concentration tested (300 nM), LPS-matured DCs could induce a similar production of IL4 and IL5 to that seen in ES-62-treated cultures

(Figure 3.16). Thus these data suggested that, as seen with bm-derived CD11c<sup>+</sup> DCs, whilst ES-62-maturation tended to polarise Ag-specific T cells towards a Th2 phenotype, LPS tended to promote Th1 development of such cells.

#### **3.3.3.7 The effects of ES-62-modulation of spDC maturation on Treg cytokine production by spDC-T cell co-cultures**

Consistent with the bias towards Th1 and Th2-like responses observed following co-cultures with LPS- and ES-62-matured splenic DCs, respectively, measurement of IL10 levels did not reveal any up-regulation (Figure 3.14). Nevertheless, FoxP3 levels were measured to determine whether development of a non IL10-secreting regulatory phenotype was being promoted at least at the transcriptional level. Contrary to the data obtained from bmDC-T cell cultures (Figure 3.10) which did not show any difference in FoxP3 expression between the culture groups, although still very low, the highest levels of this transcription factor were found in T cells cultured with ES-62-pre-exposed LPS-matured DCs (Figure 3.15) suggesting that such cells, that did not produce IL4, might rather suppress IFN $\gamma$  production by promoting regulatory cell development. However, when the ratios of Tbet/FoxP3 or GATA3/FoxP3 were measured no relative up-regulation of FoxP3 could be detected (Figure 3.17). It was therefore interesting that although IL10 secretion was hardly detectable even in these cultures, the highest levels of secretion were observed at Ag concentrations that elicited the strongest FoxP3 expression. Although it is possible that this simply reflects that the cytokine had been taken up by cells undergoing a Treg differentiation, it is also possible that such DC maturation elicits a regulatory phenotype that does not act via IL10 secretion. Alternatively, it is possible that such cells had been tolerised being rendered anergic (hyporesponsiveness) in terms of both Th1 and Th2 responses.

#### **3.3.3.8 The effects of ES-62-modulation of spDC maturation on cytokine production by reactivated DC-T cell co-cultures**

Thus, to determine whether these T cells were tolerised, cultures were re-stimulated with PMA and ionomycin to mimic re-challenge with Ag and co-

stimulation, as during the maintenance phase of anergy, tolerised cells are hyporesponsive in terms of cytokine production [498]. Interestingly, the addition of PMA and ionomycin stimulated the production of in general, higher cytokine levels from all treatment groups, with an accompanying loss of polarised responses. In detail, levels of pro-inflammatory/Th1 cytokines (TNF $\alpha$ , IL12p40, IL12p70, IFN $\gamma$ ) were strongly increased with few significant differences between the LPS and ES-62 treatment groups. Similarly, IL4 and IL10 levels were also generally comparable in all culture groups except for IL10 responses generated by cultures containing splenic CD11c<sup>+</sup> DCs. In this case, as with primary Ag challenge, only DCs matured with ES62 + LPS were capable of eliciting IL10 production in response to PMA and ionomycin (Figure 3.18). However, there did appear to be a trend towards lower pro-inflammatory/Th1 cytokine responses elicited by the ES-62 + LPS bmDC groups suggesting that although these cells do not appear to be inducing tolerance they were refractory to priming Ag-specific Th1 responses.

### **3.3.4 Investigation of the influence of ES-62 on the cytokine production profile of cDC subtypes**

It has been shown in various studies that different DC subsets are capable of differential cytokine production [69, 71, 466, 467, 499]. To investigate whether the ability of ES-62 to modulate sp CD11c<sup>+</sup> DC responses reflected masking of effects on one or more DC subtypes, the effects of ES-62 on immature and LPS-matured DC subtype responses was investigated.

#### **3.3.4.1 CD8<sup>+</sup> cDCs (CD11c<sup>+</sup> CD11b<sup>-</sup> CD4<sup>-</sup> CD8<sup>+</sup> CD205<sup>+</sup>)**

This subtype of cDC has been proposed to function mainly in priming Th1 responses. Thus, it was decided to investigate whether ES-62 could mediate its anti-inflammatory action via subversion of CD8<sup>+</sup> DC responses. In addition the effect of ES-62 on CD8<sup>+</sup> DC responses was compared with that of a classical pro-inflammatory Th1-producing pathogen product, LPS and indeed the potential for ES-62 to modulate the responses induced by LPS was also investigated. Thus, the

effect of ES-62 on the production of TNF $\alpha$ , IL12p40 and p70, IL6 and IFNs was examined (Figure 3.19).

Analyses of TNF $\alpha$  production by purified CD8<sup>+</sup> DCs revealed that the control (GM-CSF), ES-62 and LPS treatment groups secreted comparable levels of this cytokine but that pre-exposure to ES-62, followed by LPS maturation, if anything, tended to induce higher levels of TNF $\alpha$  production. Furthermore, and as previously described in literature [499] the CD8<sup>+</sup> cDC population was found to produce high levels of IL12p70. Again no significant difference between any of the treatment groups could be detected suggesting that these cells produce this cytokine spontaneously. In addition, similar IL12p40 levels were observed in groups treated with ES-62 and PBS, however, CD8<sup>+</sup> cDCs exposed to LPS or ES-62 + LPS tended to show reduced expression of this subunit perhaps suggesting that whilst control GM-CSF- and ES-62-treated cells could also promote production of IL23 to drive a Th17 response LPS-maturation induced a DC phenotype that simply promoted the development of Th1 cells. To further investigate whether GM-CSF and/or ES-62-matured CD8<sup>+</sup> DCs were capable of inducing a Th17 response, two other cytokines, namely IL6 and TGF $\beta$ , which have recently been implicated in the development of IL17-secreting cells [283-285], were also analysed. Similar levels of both cytokines were found in ES-62 and LPS-treated cultures, however ES-62-pre-treatment followed by LPS-maturation induced a slight, but not significant, decrease in TGF $\beta$ . Thus it is perhaps unlikely that CD8<sup>+</sup> DCs are involved in inducing IL17-secreting cells upon ES-62 stimulation although they might be involved in amplifying such a reaction through the production of IL23 (Figure 3.19).

As ES-62 failed to induce TGF $\beta$ , indeed there was hardly any difference in TGF $\beta$  production between the treatment groups, it was also unlikely that ES-62 was modulating CD8<sup>+</sup> DCs towards a DCreg phenotype. Nevertheless, it was decided to investigate the production of IL10, and indeed a significant increase in IL10 levels could be detected in ES-62-but not in LPS-exposed CD8<sup>+</sup> DC cultures. However this ability to increase IL10 production was suppressed somewhat if ES-62 pre-treated DCs were subsequently exposed to LPS, although an increase in comparison to the LPS cultures could still be seen (Figure 3.19).



Finally the potential of ES-62 to modulate inflammatory/Th1 responses by mediating the production of IFN $\gamma$  and IFN $\alpha$  was investigated. The production of these cytokines by DC subsets has been controversial [241, 466, 469, 470, 500] with IFN $\alpha$  being mainly regarded as a cytokine secreted by pDCs upon infection with viruses or stimulation with TLR9 ligands such as CpG [71, 98, 470]. Interestingly the level of IFN $\gamma$  secretion was found to be almost equal by all treatment groups indicating that the CD8<sup>+</sup> DC subtype spontaneously produces low levels of the cytokine and that its secretion did not appear to be influenced by further maturation stimuli such as ES-62 or LPS. Comparison of the levels of IFN $\alpha$  production by these DC groups showed that, whilst not significant, LPS tended to induced higher levels of this cytokine than ES-62 and that exposure to both substances appeared to possibly have a synergistic effect on the production of IFN $\alpha$  (Figure 3.19).

#### **3.3.4.2 CD4<sup>+</sup> cDCs (CD11c<sup>+</sup> CD11b<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup> CD205<sup>-</sup>)**

CD4<sup>+</sup> cDCs account for about 60% of all cDCs in the spleen. Thus it was decided to investigate whether ES-62 could mediate its anti-inflammatory immunomodulatory functions via subverting production of pro-inflammatory/Th1-promoting cytokines and/or by inducing regulatory cytokines by these cells.

Perhaps consistent with this proposal, maturation of CD4<sup>+</sup> cDCs with ES-62 did not induce production of TNF $\alpha$  whereas exposure to LPS significantly increased TNF $\alpha$  levels in culture supernatants. However pre-exposure with ES-62 did not inhibit TNF $\alpha$  production consequent to LPS maturation. As observed with CD8<sup>+</sup> DCs, analyses of IL12p70 did not show any significant variation in production of the cytokine between the treatment groups and moreover, the p40 subunit displayed a very similar production profile to active IL12 (Figure 3.20). Similarly, low but comparable levels of IFN $\gamma$  were produced by all groups although no IFN $\alpha$  production by this DC subtype could be detected. Interestingly, and in contrast to that observed with CD8<sup>+</sup> cDCs, culture with GM-CSF alone did not induce significant spontaneous production of TNF $\alpha$ , IL6 or IL12p40.

To determine whether ES-62 could exert its anti-inflammatory properties via this cDC subtype by promoting the production of regulatory cytokines, IL10 and TGF $\beta$  levels were measured in the culture supernatant. No significant differences in the levels of IL10 production amongst the groups could be detected. By contrast, although low, levels of TGF $\beta$  and IL6 production were found to be significantly reduced or completely abrogated, respectively, in the ES-62 group. This perhaps suggests that rather than acting to promote a regulatory phenotype, ES-62 could act on CD4<sup>+</sup> DCs to dampen down production of cytokines involved in promoting Th17 responses (Figure 3.20).

#### **3.3.4.3 DN cDCs (CD11c<sup>+</sup> CD11b<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> CD205<sup>-</sup>)**

As this cDC subtype has a very similar surface phenotype and similar functional properties as CD4<sup>+</sup> DCs, it was initially believed that the DN DC population was simply a more mature form of the CD4<sup>+</sup> DC population and therefore, in most studies, these DC subtypes have not been analysed separately. However several studies have recently shown that there may be functional differences between these two subtypes [466, 469, 501, 502] and thus it was investigated whether ES-62 could exhibit its anti-inflammatory effects by modulating the cytokine profile of DN DCs.

Again, culture of this DC subtype with GM-CSF did not induce spontaneous production of TNF $\alpha$ , IL6 or IL12p40. However, as with CD4<sup>+</sup> DCs, analysis of the TNF $\alpha$  expression profile revealed significantly higher production of this cytokine by LPS-exposed cultures compared to those matured with ES-62 alone. Again, ES-62 pre-exposure was not able to reduce LPS-induced production of TNF $\alpha$  but instead, in this case, further increased it. By contrast, here pre-exposure to ES-62 was effective in suppressing LPS driven IL12p70 production (Figure 3.21). However, similar to the data obtained from CD8<sup>+</sup> DCs and CD4<sup>+</sup> DCs, DN DCs produced very low level of IFN $\gamma$  in response to all treatment and IFN $\alpha$  secretion could not be detected at all (Figure 3.21). Moreover, analyses of IL6 and TGF $\beta$  levels in the culture supernatants revealed only low production of these cytokines with no

significant differences amongst the different culture groups although exposure to ES-62 again tended to suppress TGF $\beta$  production (Figure 3.21).

Perhaps surprisingly, a significant increase in IL10 production was found in LPS, but not in ES-62, treated cultures. Furthermore pre-treatment of DN DCs with ES-62 was able to prevent such LPS-mediated production of this cytokine (Figure 3.21), suggesting that ES-62 does not direct the maturation of DN DCs towards a DCreg phenotype.

Thus, reviewing all the analyses of cytokine production by DC subtypes it has become clear that DC subtypes have quite different cytokine expression profiles and that these profiles can sometimes be altered by stimulation with ES-62. However, when analysing this data it has to be considered that any effect caused by interaction between DC subtypes or DCs and other immune cells cannot be seen in this culture system. Therefore, although the capability of the single subtype to produce a certain cytokine upon stimulation with different stimuli can be measured, there is still the possibility that in a physiological environment these DCs would behave differently. Furthermore, splenic DCs are mature DCs and some may have been activated *in vivo* by PAMPs. Thus, it is possible that due to the maturation and activation state of splenic DCs, ES-62 cannot achieve the same degree of immunomodulatory effect seen in bmDCs.

### **3.3.5 Exposure of individual splenic cDC subtypes to ES-62 results in differential priming of T cell responses**

The data presented so far have demonstrated that splenic DC subtypes react differently upon stimulation with ES-62 or LPS, either alone or in combination. As it has been shown in several studies that individual DC subtypes have the potential to differentially activate and polarise T cell responses, it was decided to confirm this data using an Ag-specific DC-T cell co-culture system and to determine whether pre-exposure of such splenic DC subtypes to ES-62 could not only alter the DC phenotype but also the T cell response elicited by each DC subtype.

### **3.3.5.1 The effect of ES-62/LPS exposure on the ability of CD8<sup>+</sup> cDCs to induce T effector cell responses**

CD8<sup>+</sup> DCs have been reported to be the best Th1 inducers [59, 61, 467] and consistent with this, this DC subtype secreted the highest amounts of pro-inflammatory cytokines of all subtypes tested (Figure 3.19). Hence it was quite surprising to find that LPS-treated CD8<sup>+</sup> DCs only induced a slight Ag-dependent increase of IL12p70 and TNF $\alpha$  levels. By contrast ES-62-CD8<sup>+</sup> DCs induced higher IL12p40 levels than LPS-CD8<sup>+</sup> DCs at all Ag concentrations and were more efficient at driving IL12p70 production at low Ag concentrations. Interestingly, ES-62 pre-exposure followed by LPS maturation resulted in low IL12p40 and p70 production although TNF $\alpha$  production was detected in an Ag-dependent manner (Figure 3.22).

Following analyses of pro-inflammatory cytokines, it was decided to determine whether ES-62 inhibited Th1 and/or induced Th2 responses by targeting CD8<sup>+</sup> DCs. To do this, the Th1 cytokines IL2 and IFN $\gamma$  and the Th2 cytokines IL4, IL5 and IL25 (IL17E) were measured. Analyses of the levels of IL2 and IFN $\gamma$  production by CD8<sup>+</sup> DC-T cell cultures revealed no significant difference in the production of these cytokines amongst treatment groups in response to Ag. Indeed, rather surprisingly, LPS-DCs induced an Ag-dependent decrease in IFN $\gamma$  levels (Figure 3.22). However, while as expected LPS-maturation of these DCs did not induce a rise in IL4 and IL5 levels, ES-62 DCs did indeed cause an Ag-dependent increase in their production which appeared to become desensitised at high Ag levels (10nM OVA peptide). Interestingly, ES-62-matured CD8<sup>+</sup> DCs induced IL25 (IL17E) production in the absence of Ag and increasing Ag concentrations suppressed this IL25 response. Collectively, these data suggest that CD8<sup>+</sup> DCs are very effective at mediating ES-62-induced classical Th2 cytokine production. Given these findings, it was interesting to note that subsequent exposure to LPS was able to prevent induction of such Th2-like responses. Thus, although LPS-matured CD8<sup>+</sup> DCs did not have a strong Th1 inducing effect, LPS was able to inhibit the ES-62-driven Th2-polarising capability of these DCs (Figure 3.22).

In addition to driving production of Th2-like cytokines, ES-62-matured CD8<sup>+</sup> DCs induced production of Treg-associated cytokines as evidenced by a significant rise in IL10 release and a low level production of TGFβ even in the absence of Ag. By contrast, LPS stimulation of these DCs did not induce IL10 production by such CD8<sup>+</sup> DC-T cell co-cultures although an increase in TGFβ could be seen at higher Ag concentrations (3-10 nM OVA peptide). A similar pattern was found in cultures containing ES-62 + LPS exposed DCs indicating that subsequent exposure to LPS inhibits ES-62 mediated induction of IL10 production (Figure 3.22).

IL17 is the main cytokine produced by Th17 cells which have been shown to require IL6 and TGFβ for their initial development and IL23 for expansion [287]. As IL23 contains the IL12p40 subunit which was up-regulated, in the absence of IL12p70 up-regulation, in T cell co-cultures primed by ES-62-matured CD8<sup>+</sup> DCs, the possibility of Th17 cell development was investigated. Indeed, whilst no production of IL17 could be found in cultures containing LPS- or ES-62 + LPS-matured DCs, an Ag-dependent increase in production of this cytokine (desensitised at high Ag levels (10 nM OVA peptide)) by T cells primed by ES-62-treated CD8<sup>+</sup> DCs could be detected, indicating that ES-62-, but not LPS-exposed CD8<sup>+</sup> DCs, might be able to induce Th17 production. Furthermore it appeared that exposure to LPS might have an inhibitory effect on the ability of such DCs to prime IL17 production as IL17 release was abolished in ES-62 + LPS-matured CD8<sup>+</sup> DC-T cell cultures (Figure 3.22).

As the development of Th17 cells has been reported to be IL6 and TGFβ dependent [283-285], it was decided to determine whether these two cytokines were up-regulated in co-cultures displaying enhanced IL17 production. Indeed, cultures containing ES-62-CD8<sup>+</sup> DCs showed low level constitutive release which, like IL17 production, was desensitised at 10 nM OVA and perhaps reflects consumption of these two cytokines by cells undergoing Th17 development. Interestingly, even though comparable levels of both IL6 and TGFβ were produced in LPS and ES-62 + LPS-exposed CD8<sup>+</sup> DC-T cell cultures, no IL17 production was observed. However, closer analysis of the Ag-dependency of such cytokine release

showed an inverse profile of induction by ES-62-CD8<sup>+</sup> DCs with the latter cultures showing a much higher TGFβ/IL6 ratio than that achieved by ES-62-matured CD8<sup>+</sup> DCs. Thus, one possible explanation for the lack of IL17 in these cultures could be that there was an imbalance of IL6 and TGFβ at different Ag concentrations which in turn inhibited production of Th17 cells (Figure 3.22).

To further investigate whether maturation of CD8<sup>+</sup> DCs by ES-62 and LPS, either alone or in combination resulted in induction of differential effector T cell responses it was decided to measure the expression of FoxP3, Tbet and GATA3, the master transcription factors for Treg, Th1 and Th2 cell development, respectively. Rather surprisingly, given that ES-62-CD8<sup>+</sup> DCs induced Th2-like cytokines, the lowest expression levels of GATA3 were found in the ES-62 treatment group and also the ratio of GATA3/Tbet did not show a relative increase in GATA3 expression. Indeed, the ES-62+LPS-CD8<sup>+</sup> DCs induced the highest expression of both Tbet and GATA3 despite the findings that neither Th1 nor Th2 cytokine levels were significantly increased in these cultures. Both transcription factors also showed elevated expression in the LPS treatment group (Figure 3.23).

Analysis of the correlation between FoxP3 mRNA expression and IL10 and TGFβ release demonstrated that T cells from cultures containing LPS-matured (LPS and LPS+ES-62) CD8<sup>+</sup> DCs showed an increase in FoxP3 mRNA and a corresponding increase in TGFβ levels. Interestingly also the ratio of FoxP3/Tbet revealed a relative increase in FoxP3. By contrast, T cells primed by ES-62-CD8<sup>+</sup> DCs displayed low levels of FoxP3 mRNA and no relative increase in FoxP3/GATA3 consistent with the finding that, at this Ag concentration, low levels of IL10 and no production of TGFβ could be detected (Figure 3.23). Taken together these data could indicate that the LPS-driven TGFβ production might drive FoxP3 up-regulation and consequent Treg effector function that could account for the low Th1, Th2, Th17 and pro-inflammatory cytokine production detected in such CD8<sup>+</sup>DC-T cell culture supernatants. However, although IL17 release by T cells primed by such LPS-matured CD8<sup>+</sup> DCs was not observed, T cells co-cultured with LPS-matured CD8<sup>+</sup> DCs displayed a strong up-regulation of IL17 mRNA perhaps suggesting that these cells have the capability to release IL17 on receipt of

appropriate signals. By contrast, the lowest level of IL17 mRNA was observed in T cells primed by ES-62-matured CD8<sup>+</sup> DCs despite these cells eliciting a strong IL17 release. Nevertheless, IL17 release appeared to be desensitised at 10 nM OVA, the concentration of Ag in the cultures in which IL17 mRNA levels were expected, indicating that not only was secretion of IL17 blocked at this Ag concentration but also that induction was inhibited at the transcriptional level.

#### **3.3.5.2 The effect of ES-62/LPS exposure on the ability of CD4<sup>+</sup> cDCs to induce T effector cell responses**

Whilst LPS-treated CD4<sup>+</sup> DCs induced a slight Ag-dependent increase in production of all three pro-inflammatory cytokines (IL12p70, IL12p40, TNF $\alpha$ ) in the DC-T cell cultures, ES-62-CD4<sup>+</sup> DCs had almost no effect. Interestingly however, subsequent exposure of ES-62-treated DCs to LPS converted them to an effector phenotype more closely resembling LPS-matured DCs at least at low Ag concentrations. However once Ag levels cross a certain threshold it seems that ES-62 is able to suppress LPS-CD4<sup>+</sup> DC-mediated T cell responses and hence inhibits secretion of pro-inflammatory cytokines (Figure 3.24).

As observed with CD8<sup>+</sup> DCs, LPS-maturation of CD4<sup>+</sup> DCs did not result in an Ag-dependent increase in IFN $\gamma$  production in such DC-T cell co-cultures although, as with the CD8<sup>+</sup> DC-T cell cultures significant amounts were produced spontaneously. Furthermore, and consistent with this, LPS-treated groups did not display production of IL2 which is normally secreted by activated T cells and increases production of Th1 cytokines such as IFN $\gamma$ . By contrast IL2 levels were very slightly increased in co-cultures primed by ES-62-CD4<sup>+</sup> DCs although no Ag-dependent IFN $\gamma$  production could be observed. Moreover, whilst subsequent maturation of ES-62-treated CD4<sup>+</sup> DCs with LPS reversed the ability of ES-62-DCs to induce IL2 production from T cells, it enhanced the Ag-dependent increase in IFN $\gamma$ , resulting in higher levels than those found in response to either ES-62 or LPS-matured CD4<sup>+</sup> DCs (Figure 3.24).

Following the analyses of IL2 and IFN $\gamma$  production, it was decided to assess the Th2-associated IL4, IL5 and IL25 (IL17E) response. In contrast to what was observed with CD8 $^{+}$  DCs, ES-62- and LPS-matured CD4 $^{+}$  DCs induced comparable levels of IL4, IL5 and IL25 (IL17E), and in a relatively Ag-independent manner. Moreover, the levels of IL4 and IL5 induced by such CD4 $^{+}$  DCs were much less than those observed in ES-62 CD8 $^{+}$  DC-T cell co-cultures (Figure 3.24).

Thus, as very similar levels of Th1 and Th2 cytokines were elicited by all the treatment groups it appears that this DC subtype is not capable, or is dependent on co-stimulation from another DC subtype or soluble factor to initiate Th1 or Th2 cell polarisation in response to either ES-62 or LPS. Similarly, whilst low levels of IL10 could be found in co-cultures containing ES-62-CD4 $^{+}$  DCs perhaps consistent with the induction of Tregs, these cytokines were not elicited in response to LPS or LPS + ES-62-CD4 $^{+}$  DCs.

As no strong evidence for Th1, Th2 and Treg polarisation was found in these cultures it was decided to determine whether CD4 $^{+}$  DCs could be involved in driving Th17 development. Analyses of IL17 levels in culture supernatants from all treatment groups revealed negligible levels of this cytokine, particularly in the ES-62-matured CD4 $^{+}$  DC group. Consistent with this, production of IL6 and TGF $\beta$ , the two cytokines needed to produce Th17 cells was also negligible suggesting that such CD4 $^{+}$  DCs are not effective in driving Th17 responses following maturation with either ES-62 or LPS (Figure 3.24). Indeed, apart from the strong Ag-independent secretion of Th2 cytokines, these T cells look unresponsive.

Perhaps consistent with the observed lack of polarisation of T cell cytokine production in response to any of the “matured” CD4 $^{+}$ DC groups was the finding that although the strongest expression of the transcription factors Tbet and GATA3 was seen under LPS exposure the ratio of Tbet/GATA3 did not significantly change from any of the co-cultures apart from the ES-62 + LPS group. Thus collectively it appears as if CD4 $^{+}$  DCs are not very good in polarising T cells towards a Th1 or Th2 phenotype on their own and indicates that these cells might be dependent on further stimulation from other cells. In addition to GATA3 and Tbet, FoxP3 levels



were determined to investigate whether ES-62 DC were able to induce Treg development. Indeed, ES-62 DCs induced an up-regulation of this transcription factor in co-cultured T cells. An even stronger increase in this transcription factor was seen in T cells cultured with ES-62 pre-treated LPS-matured DCs. By contrast, much less FoxP3 could be detected in T cells cultured with LPS DCs which showed an up-regulation of Tbet and GATA3. Consistent with this, analyses of the ratio of GATA3/FoxP3 and FoxP3/Tbet showed that FoxP3 expression was relatively increased in both ES-62 and ES-62 + LPS cultures whereas it was decreased in LPS exposed cultures. As regulatory cytokines were either not produced or produced at very low levels and also Th2 cytokines were not significantly elevated in cultures containing ES-62 or ES-62 + LPS-matured DCs the increase in FoxP3 might be a mechanism to suppress Th1 and pro-inflammatory cytokine production (Figure 3.25).

Similarly, IL17 mRNA expression was highest in T cells cultured with ES-62 + LPS DC and slightly increased in T cells cultured with ES-62 DCs. Again, this mRNA profile was not entirely consistent with the cytokine release profile which showed highest production of IL17 in LPS- and ES-62 + LPS- CD4<sup>+</sup> DC-T cell co-cultures but with no production in response to ES-62-CD4<sup>+</sup> DCs. However, as the levels of both IL17 mRNA and protein release were very low, it is rather difficult to analyse these data (Figure 3.25).

### **3.3.5.3 The effect of ES-62/LPS exposure on the ability of DN cDCs to induce T effector cell responses**

Whilst LPS-matured DN cDCs induced an Ag-dependent increase in the production of IL12p70 and TNF $\alpha$ , by contrast, ES-62-DCs induced lower levels of those cytokines and in an Ag-independent manner (Figure 3.26). Subsequent exposure of LPS restored the levels of pro-inflammatory cytokines in these cultures to similar levels to those found in response to LPS-treated co-cultures. Furthermore, production of the levels of IL12p40 observed in response to ES-62 bmDCs was also reduced.

Surprisingly, analyses of the co-culture supernatants showed no production of IL2, and a very similar Ag-independent IFN $\gamma$  production by all treatment groups indicating that T cells primed by DN DCs were producing IFN $\gamma$  regardless of the DC treatment (Figure 3.26). Similarly, very low levels of IL17 were induced by all groups of DN cDCs, also in an Ag-independent manner. By contrast, ES-62-treated DN cDCs caused an Ag-dependent increase in IL4 and IL5 whereas LPS maturation resulted in a loss of the Ag-dependent IL4 and IL5 release. Interestingly, pre-exposure to ES-62 was able to partially restore the Ag-dependent increase in IL4, but not IL5, production by LPS-matured DN-DCs. Thus, collectively these data indicate that ES-62 unlike LPS, acts to promote a Th2 phenotype in these co-cultures by up-regulating Th2 associated cytokines (IL4/IL5) and hence polarising the ratio of Th1/Th2 cytokines in favour of a Th2 response (Figure 3.26).

Consistent with previous reports [146] that ES-62 elicits a Th2/anti-inflammatory phenotype, these studies also showed that ES-62-matured DN cDCs were able to strongly increase the production of IL10 upon maturation with ES-62, but not LPS. To determine whether these cells were able to drive a Treg-like response, culture supernatants were analysed for TGF $\beta$ , another cytokine associated with the development and presence of Treg cells. Indeed, whilst co-cultures containing ES-62 DN cDCs showed enhanced production of both IL10 and TGF $\beta$ , albeit only at moderate Ag concentrations (3nM OVA peptide), LPS-matured DN cDCs were not able to induce IL10 production in T cells, although some TGF $\beta$  could be detected at high Ag concentrations (3-10 nM OVA peptide). Interestingly, ES-62 pre-treatment of such LPS-matured DN cDCs did not result in the production of IL10 indicating that pre-exposure to ES-62 could not inhibit the LPS-driven effect (Figure 3.26).

As observed with the other DC subtypes, analyses of FoxP3, GATA3 and Tbet mRNA expression indicated that such levels did not correlate with the functional responses of the co-cultures. For example, an up-regulation of the transcription factors GATA3 and Tbet was detected in T cells responding to LPS-DN cDCs or ES-62 + LPS-DN cDCs, despite the finding that ES-62-matured DN cDCs were the only DCs capable of driving IL4/IL5 responses and that IFN $\gamma$  levels were

comparable in all treatment groups (Figure 3.27). Rather surprisingly therefore, the ratio of GATA3/Tbet expression also only displayed minimal differences between LPS- and ES-62-DN DC-primed T cells.

Similarly, FoxP3 expression in the co-cultures containing DN DCs exposed to LPS was higher than in those responding to ES-62 DN cDCs despite the fact that IL10 was only produced by cultures primed with ES-62 DN-DCs. Furthermore, analyses of the ratio of FoxP3/Tbet and GATA3/FoxP3 displayed a relative decrease of FoxP3 in ES-62 and LPS exposed cultures. Interestingly however, these data partially corresponded to the cytokine expression profiles as TGF $\beta$  levels were highest in the LPS-DN cDC primed group, stimulated with 10 nM OVA peptide, indicating that FoxP3 up-regulation may correspond to TGF $\beta$  rather than to IL10 production (Figure 3.27).

Strangely, given that only very low levels of IL17 were produced, and to a comparable extent in all cultures the highest IL17 mRNA levels in T cells were detected in co-cultures containing DCs exposed to ES-62 (Figure 3.27).

### **3.3.6 Investigation of the influence of ES-62 on the cytokine production profile of pDCs (CD11c<sup>int</sup> CD11b<sup>-</sup> CD45R<sup>+</sup>)**

This subtype of DC has been shown have a very plastic character which allows it to drive different immune responses. Indeed, it has been shown in several studies that pDCs are involved not only in polarising towards Th1 and Th2 responses but also driving development of Treg cells [183, 184, 478-480, 503]. Furthermore, due to their unique TLR expression profile, comprising high expression of TLR7 and TLR9 and low expression of TLR2, pDCs have been strongly implicated in viral defence [95-97, 504]. TLR7 and TLR9 are located in endosomal compartments of the cell and have been shown to bind single stranded and double stranded RNA as well as synthetic CpG containing oligodeoxynucleotides (CpG-ODN) [89, 90, 92]. The only known surface receptor on pDCs from the TLR family is TLR2. TLR4 which is the main receptor for LPS and a putative ES-62 receptor [402] has not been found on pDCs. Therefore it was decided to determine whether ES-62 could

modulate pDC responses via alternative, as yet undefined mechanisms. Furthermore, as it has been suggested that LPS can exhibit some functions through TLR2, the effects of CpG and LPS on these DCs were compared as well as the ability of ES-62 to inhibit these responses were also investigated.

Firstly, to confirm that purified pDCs did not express any TLR4 on their surface, cells were stained for the receptor and flow cytometry was used to analyse its expression level. As expected, and consistent with the literature, no expression of the receptor could be detected. To exclude the possibility that the receptor was up-regulated at mRNA level to be expressed during maturation upon the right stimulation, quantitative RT-PCR was carried out and clearly showed that there was no evidence of TLR4 expression even at the transcriptional level. By contrast and as previously reported in literature [109], TLR9 was strongly up-regulated and TLR2 mRNA was present at low levels whereas no TLR3 mRNA could be detected. As this TLR expression pattern was therefore similar to that reported in literature, these data confirmed that the DC population truly represented pDCs and was not contaminated with cDCs (Figure 3.28).

Culture of pDCs with GM-CSF did not elicit significant production of any of the cytokines (IL12p40 and p70, TNF $\alpha$ , IFN $\alpha$ , TGF $\beta$ , IL6 and IL10) tested (Figure 3.29). Nevertheless, to determine the effects of CpG on the production of pro-inflammatory cytokine production in pDC cultures TNF $\alpha$ , IL12p40 and IL12p70 were measured. Whilst no expression of TNF $\alpha$  could be detected in any pDC culture supernatants (data not shown), IL12p40 was produced at very low levels. Furthermore a significant increase in IL12p70 could be detected in CpG-cultures compared to control cultures. Interestingly ES-62-exposed cultures produced similar levels of this cytokine to those found in CpG cultures and pre-treatment with ES-62 had no effect on CpG-mediated production. However, analysis of IL12p70 secretion surprisingly revealed significantly increased levels in the LPS treatment group compared to any other treatment group and moreover, pre-treatment with ES-62 successfully inhibited the LPS-driven increase in IL12p70 production (Figure 3.29).

pDCs have not yet been implicated in the polarisation of Th17 cells but as this Th cell type has only recently been discovered it was decided to investigate whether pDCs could be involved in initiating a Th17 response and if so whether ES-62 was capable of preventing it. Thus IL6 and TGF $\beta$  were measured in the culture supernatants and this revealed that pDCs were only capable of producing extremely low amounts of TGF $\beta$  (GM-CSF and GpG containing groups only) whilst no IL6 could be detected indicating that these DCs do not provide the cytokines necessary to initiate a Th17 response under any of the conditions tested (Figure 3.29).

Several studies have previously shown that pDCs can induce production of IL10 producing cells [184, 186]. Furthermore, it is known that IL10 can polarise T cells to become IL10-producing cells but under the culture conditions tested, no IL10 production by any of these pDC cultures could be detected (data not shown).

One cytokine that has always been associated with pDCs is IFN $\alpha$  as this cytokine is reported to be produced in vast amounts upon stimulation of pDCs with either viruses or CpG-ODNs [479, 505, 506]. To confirm this finding and to determine whether ES-62 could inhibit the production of this cytokine, IFN $\alpha$  levels were measured. To our surprise and contrary to all published data we did not find any IFN $\alpha$  production by pDC, in any of the treatment groups other than in the GM-CSF control group and the ES + CpG group (Figure 3.29).

### **3.3.7 The effect of ES-62/CpG/LPS exposure on the ability of pDCs to induce T effector cell responses**

Neither the CpG- nor the ES-62 + CpG-treatment groups induced Ag-dependent production of IL12p70, p40 and TNF $\alpha$  by pDC-T cell co-cultures although there were substantial levels of spontaneous cytokine secretion detected (Figure 3.30). Interestingly, LPS-pDCs did induce TNF $\alpha$  (Figure 3.31). Rather unexpectedly, ES-62-maturation of pDCs induced the production of IL12p70 and TNF $\alpha$  but this was suppressed at the higher Ag concentrations (3-10 nM OVA peptide). Interestingly, pre-exposure of pDCs to ES-62 before subsequent maturation with LPS almost

abolished production of IL12p70, and strongly reduced TNF $\alpha$  production, by these cultures whilst IL12p40 production was elevated (Figure 3.31) indicating as with the DN cDC cultures that IL12p40 and IL12p70 show inverse patterns of production.

Interestingly, as pDC cultures do not display any TNF $\alpha$  secretion (data not shown), the production of this cytokine in the co-cultures indicates that either T cells produce it or that pDCs require cell interaction with T cells to initiate its secretion. IL12p70 was produced in pDC cultures with highest levels detected in the LPS-treatment group, however ES-62-maturation only slightly induced its production suggesting that the high levels of IL12p70 observed in these co-cultures at low Ag concentrations are probably triggered by cell-cell interactions.

An interesting discovery was made when pDCs were analyzed for their ability to induce Th1 cytokine secretion in T cells. Whilst, CpG- and CpG + ES-62-matured pDCs induced low levels of IL2 production by T cells in an Ag-independent manner, no IFN $\gamma$  production was observed. Some IL2 production was also detected in co-cultures containing pDCs stimulated with LPS, ES-62 and LPS + ES-62, albeit at greatly reduced levels compared to the CpG containing groups. However, in contrast to the CpG-pDC- and CpG + ES-62-pDC-primed groups, T cells from all other co-cultures were able to induce IFN $\gamma$  production, albeit in an Ag-independent manner. This indicates that although the CpG-matured pDCs have been reported to be very good at activating T cells, in these studies they do not induce Th1 cell development. By contrast, LPS and also ES-62 are able to mature pDCs in such a way that they induce IFN $\gamma$  production in T cells and in an Ag-independent manner (Figure 3.30 and 3.31).

Another cytokine capable of inducing a Th1 response which is produced by pDCs is IFN $\alpha$ . In contrast to IFN $\gamma$  production, cultures containing pDCs matured with CpG, but not with LPS or ES-62, demonstrated a strong Ag-dependent up-regulation of IFN $\alpha$ . In addition, pre-treatment with ES-62 strongly inhibited the ability of pDCs subsequently matured with CpG to induce an increase in production of this cytokine. This indicates that pDCs are very plastic in their response to different stimuli as both LPS and CpG (not seen in these co-cultures) may be able

to induce Th1-like responses, albeit by inducing the production of different cytokines (Figure 3.30 and 3.31).

Analyses of the Th2-associated cytokines released in co-cultures containing such pDCs revealed very interesting data. Whereas high levels of IL4 and IL5 were induced by both CpG- and ES-62 + CpG-matured pDCs, even in the absence of Ag, IL4 but not IL5 could be detected in an Ag-dependent manner following priming with ES-62-matured DCs. Furthermore although LPS-exposed pDCs did not drive production of IL4 by T cells, ES-62 + LPS-pDCs led to Ag-dependent production of IL4. IL25 (IL17E), another Th2 cytokine, was affected differently, as although CpG- and ES-62 + CpG-pDC induced production of low levels of IL25 (IL17E) in an Ag-dependent manner, ES-62-, LPS-, and ES-62 + LPS-pDCs induced much higher levels of this cytokine by T cells. Interestingly, the highest levels of this cytokine were found to be induced in ES-62-pDC cultures (Figure 3.30 and 3.31).

Thus collectively these data indicate that ES-62, LPS and CpG all have an effect on pDC maturation and function, resulting in the differential induction of various Th1- and Th2-associated cytokines

As pDCs have been implicated in the development of IL10 producing cells, it was decided to analyse the ability of these cells to produce IL10 and TGF $\beta$  upon maturation with ES-62, LPS and/or CpG. Both IL10, and to a much lesser extent, TGF $\beta$  were produced in an Ag-dependent manner in cultures primed by pDCs that had been treated with ES-62. LPS-maturation of pDCs also induced an increase of both cytokines which was also apparently Ag-independent. Exposure to ES-62, prior to LPS maturation, modulated pDC priming of such T cell responses, in that whilst ES-62 + LPS-pDCs could induce cytokine secretion in the absence of Ag, at high Ag concentrations a sharp decrease in both cytokine levels was detected. In cultures primed by CpG-pDCs, whilst TGF $\beta$  levels showed an Ag-dependent rise, no IL10 could be detected. Furthermore pre-exposure of pDCs to ES-62 prior to maturation with CpG not only did not restore IL10 to levels observed with ES-62-pDCs but, in addition abolished production of TGF $\beta$  in these cultures (Figure 3.30 and 3.31). Considering that no IL10 was found, it is possible that the production of

TGF $\beta$  in CpG exposed cultures is not a sign for a Treg polarization of T cells but rather contributes to the formation of Th17 cells. Another possibility would be that IL10 produced upon CpG stimulation was taken up by cells in the process of developing into Tregs and hence no free IL10 could be found in the culture supernatant. However, as an increase in TGF $\beta$  and a mild increase in IL12p40 could be detected in co-cultures containing CpG-exposed pDCs, it was decided to determine whether Th17 cell development was promoted in these cultures. While some IL17 could indeed be found in CpG-pDC primed co-cultures, strongly increased levels of IL17 were only found at high Ag concentration in cultures primed by ES-62 + CpG-treated pDC which did not apparently produce TGF $\beta$ . However ES-62- or LPS-pDCs did not drive T cells towards a Th17 phenotype (Figure 3.30 and 3.31).

Analyses of the expression of FoxP3, GATA3 and Tbet mRNA in T cells from co-cultures containing pDCs showed a strong up-regulation of all of these in the CpG treatment group. This correlates very well with the cytokine data which also shows high spontaneous levels of IFN $\alpha$ , IL4 and IL5 in this group. Interestingly however, IFN $\gamma$  levels were not raised in this group though the GATA3/Tbet ratio and the FoxP3/Tbet ratio were nearly the same as in the control group. Similarly, expression of all three transcription factors was elevated in ES-62-pDC-primed cultures. Interestingly T cells from co-cultures containing pDCs pre-exposed to ES-62, and subsequently matured with CpG, showed similar levels of the transcription factors as found in T cells from ES-62 exposed cultures indicating that pre-exposure to ES-62 is capable of inhibiting the CpG-pDC driven expression of both transcription factors. In terms of cytokine expression, a decrease in the ES-62 + CpG group, compared to the CpG group, could also be detected however these levels were still higher than in the ES-62-treated culture. Consistent with the induction of IL4 and IL10 response, GATA3/Tbet and FoxP3/Tbet ratios were increased in the ES-62 group but not or only marginally in the ES-62 + CpG group. T cells from LPS and ES-62 + LPS-treated co-cultures did not display any up-regulation in FoxP3, GATA3 and Tbet. In the case of Tbet this was quite surprising as some IFN $\gamma$  production was found in these co-cultures (Figure 3.32). However



when analysing GATA3/Tbet and FoxP3/Tbet ratios hardly any difference to control groups could be detected.

### 3.4 Discussion

Hosts infected with filarial nematodes have been shown to exhibit defective pro-inflammatory immune responses to the parasite and to unrelated immune stimuli [384, 390, 507]. It is currently believed that this may be a result of the parasite releasing excretory-secretory proteins into the host which interact with the immune system to generate an environment conducive to parasite survival. However by modulating the host immune system to attenuate the development of pro-inflammatory responses detrimental to the parasite, immune reactions towards other stimuli are affected as well.

As sentry cells, DCs are localised in many different sites in the body and therefore require different receptors and surface components to properly interact with their environment. During recent years various subtypes have been discovered in different lymphatic organs, among them the spleen, LNs and Payer's patches. A DC is currently defined as a separate subtype if it cannot be transformed into any other phenotypically different DC, even if a common precursor might exist not too far back in development. Depending on their location DCs are exposed to different pathogens or foreign substances and it is therefore not surprising that these cells have varying potential to initiate different types of T cell responses. In part, this might be due to the fact that the combination of subtypes of DCs changes in different sites throughout the body. Another important factor that might contribute to the induced immune reaction is the interaction between DCs and other cells found in their vicinity. Furthermore the differentiation state at which DCs come in contact with foreign or inflammatory substances might be a relevant factor for developing their potential to initiate a T cell response.

#### **3.4.1 The effect of ES-62 on DC surface molecule expression is depending on the site of DC origin and possibly on the differentiation state of the cells.**

The mechanisms by which ES-62 modulates DC-maturation to promote a T effector response are unknown. One possibility would be to change the DC

phenotype or the ratio of DC subtypes at a particular site, as different DC subtypes have been proposed to mediate different immune functions and hence, even a minor change could have a dramatic effect on the overall T effector response. For instance, CD8<sup>+</sup> DCs have been proposed to drive Th1 responses: thus, an increase in their number could lead to enhanced Th1 responses whereas a reduction might tip the balance in favour of a Th2- or Treg- response. Therefore, as DCs are very plastic cells, their phenotype undergoing changes during differentiation, activation and even culture, it was hypothesised that ES-62 might be able to exert immunomodulatory effects by modulating the development of one or more DC precursors [69, 449, 452, 455] or alternatively, by altering the phenotype of a DC subtype to polarise T cell responses towards creating an environment that is beneficial to the parasite's survival.

Normally splenic DCs (1% of all spleen cells) comprise of around 20% pDCs, and 80% cDCs, which dependent on their subtype, express a combination of CD4, CD8, CD205 and CD11b. In this study following *in vitro* culture of spleen cell with GM-CSF, CD45R expression was observed on between 50% and 80% of CD11c<sup>+</sup> cells whereas CD205 and CD11b were only found on a maximum of 10% of CD11c<sup>+</sup> cells indicating that either more pDCs were produced or that the culture was beneficial to pDC survival or that the cell surface phenotype of the cultured cells was altered. In the steady state, spleen cDCs are replaced between 3 and 4 d [76, 78] whereas pDC turnover takes about 2 weeks [69]. Therefore it could be possible that, as a result of the *in vitro* culture, DCs have an even shorter life span which leads to a higher proportion of pDCs, although substantial cell death was not observed at the end of the culture period. Another possible explanation for this shift in DC cell surface molecule expression might reflect the finding that GM-CSF has been shown to induce enhanced production of cDCs from bone marrow [508] and peripheral blood cells [509] and creates an inflammatory environment which might mimic the initial environment DCs are exposed to after infection with parasites (Figure 3.4). However, in this case the % of CD45R<sup>+</sup> cells might have been expected to decrease.

In the spleen, the CD8<sup>+</sup> DC subpopulation expresses CD205 but this marker is not expressed by either the CD4<sup>+</sup> or the DN subpopulation. By contrast, these latter two subtypes express CD11b. Thus, as ES-62 induced up-regulation of CD205 and down-regulation of CD11b, these results could indicate that ES-62 might promote development or survival of CD8<sup>+</sup> DCs and at the same time reduce the number of CD4<sup>+</sup> and DN cDCs. As it has been reported that CD8<sup>+</sup> DCs produce high amounts of active IL12p70, this proposal was perhaps rather surprisingly supported by the increased amounts of this cytokine detected in these ES-62-matured CD11c<sup>+</sup> splenic DC cultures, as this subtype has been reported to be a strong Th1 inducer [59, 61, 467], and specialised in cross presentation of Ags to T cells by class I MHC complexes [462]. By contrast, CD45R expression did not vary between ES-62-treated and GM-CSF-DCs and this suggests that ES-62 has no influence on the selection of pDCs whereas LPS-treatment induced increased CD45R levels perhaps suggesting that, in an inflammatory environment, LPS enhances production or prolongs survival of pDCs (Figure 3.4).

By contrast, exposure to ES-62, LPS or ES-LPS does not increase expression of CD205 but instead appears to promote up-regulation of CD11b in bm cultures. Furthermore, an increase in CD11c<sup>+</sup>CD45R<sup>+</sup> expression is seen in bm cultures exposed to ES-62 compared to the GM-CSF control cultures. These responses were not reproduced when purified bm CD11c<sup>+</sup> DCs were exposed to ES-62 and/or LPS suggesting either that interaction with other cells modulates the effects of ES-62 and LPS on CD11c<sup>+</sup> DCs or that differentiation of the remaining DC progenitors can be influenced by these two substances.

Collectively, as ES-62 demonstrated differential effects in modulating the surface phenotype of whole bmDC, bm CD11c<sup>+</sup> DC and sp CD11c<sup>+</sup> DC cultures it is not clear how ES-62 influences DCs to change their surface marker expression. Furthermore, it is not known if, and to what extent, ES-62 can influence DC progenitors and whether these effects are location or cell interaction dependent.

### **3.4.2 Does the altered surface molecule expression profile of CD11c<sup>+</sup> DCs following culture with ES-62 reflect modulation of their cytokine profile?**

ES-62-matured DCs, derived either from the spleen or the bm, produced in general less IL12p40, IL6 and TNF $\alpha$  than DCs matured with LPS. As CD8<sup>+</sup> DCs have been reported to be Th1 inducers and the number of DCs expressing CD205, a surface marker found on CD8<sup>+</sup> DCs, was increased after ES-62-treatment possibly indicating an increase in their number, the low secretion of pro-inflammatory cytokines in these cells might be considered to be quite unexpected. However, as it has been shown that CD205 expression generally increases in culture [510] and thus an increase in this marker does not necessarily mean an increase in the number of the CD8<sup>+</sup> DC subtype. Presumably, and perhaps reflecting the plastic nature of DCs [76, 457, 510], this indicates that ES-62 suppresses pro-inflammatory responses regardless of the subtype of DCs, probably by interfering with DC signalling or by changing the microenvironment in culture, possibly via cell-cell interaction between DCs and T cells that cannot be seen in a homogenous DC culture system.

### **3.4.3 The effects of ES-62 on the cytokine profile of individual cDC subtypes**

Analyses of the cytokine profiles of mixed CD11<sup>+</sup> cDC cultures illustrates the overall pattern of how such DCs react to ES-62 or LPS, pathogen products which differentially induce anti-inflammatory versus pro-inflammatory responses, respectively. However, these data do not reveal whether the observed effect was caused by a strong alteration of the cytokine profile of one specific subtype or whether the responses of all subtypes were similarly modified. Furthermore, in mixed DC populations it is impossible to determine whether different subtypes are responsible for different immune reactions. Thus, for example, it could be that ES-62 actively induces particular DC subtypes to drive Th2 or Treg responses whereas it may only inhibit pro-inflammatory signals in others. However, other factors such as chemokine production and cell-cell interaction cannot be disregarded and as cultures containing individual DC subtypes are unlikely to

provide such an interactive environment, the cytokine profile of individual subtypes might not entirely represent what can be seen under physiological circumstances.

The parasite product, ES-62 only induces low level pro-inflammatory cytokine production from splenic DC cultures and such DCs exposed to ES-62 appear to be able to promote Th2-like and /or anti-inflammatory responses in DC-T cell co-cultures. Thus, as more information about DC subtypes and their specific functions has become available, it was thought possible that ES-62 may be inducing differential effects by altering the functions of specific subtypes depending on the type of immunomodulation necessary to achieve a parasite friendly environment.

#### **3.4.3.1 ES-62 does not inhibit production of proinflammatory/Th1-inducing cytokines by CD8<sup>+</sup> DC cultures**

Various studies have shown that, upon stimulation with pathogen-derived products, CD8<sup>+</sup> DCs are the best inducers of a Th1 response [59, 61, 467]. They are known to be superior in the production of IL12p70 compared to other DC subtypes and can crosspresent exogenous Ag via the MHC class I complex [462, 511]. Furthermore, in an *in vivo* study it was shown that Ag-pulsed splenic CD8<sup>+</sup> DCs which were cultured in the presence of GM-CSF, could polarize T cells to become Th1 cells when they were transferred into mice [59]. Therefore it was thought possible that that ES-62 might interact with this specific subtype to suppress release of pro-inflammatory/Th1 driving cytokines and/or promoting an increased production of Treg driving cytokines. Thus, it was surprising that similar levels of TNF $\alpha$ , IL12p70, IL12p40, IFN $\gamma$  and IL6 release were detected from CD8<sup>+</sup> DCs exposed to ES-62, LPS and ES-62 + LPS. However, the levels of IFN $\alpha$ , induced by ES-62, whilst clearly above those of the control group, did not reach the levels secreted by such DCs exposed to LPS or ES-62 + LPS (Figure 3.19).

In concurrence with the inability of ES-62-treated CD8<sup>+</sup> DCs to produce decreased levels of most pro-inflammatory cytokines, the low levels of TGF $\beta$  observed were similar in ES-62 and LPS-exposed cultures. Perhaps surprisingly therefore, given that TGF $\beta$  is a cytokine which has important functions in the development of

regulatory T cells, IL10 production was found to be most strongly up-regulated upon ES-62 exposure regardless of whether these DCs were subsequently matured with LPS or not (Table 3.1). As IL10 has been shown to suppress IL12p70 production and thus inhibit a Th1 response, a selective increase in IL10 upon stimulation with ES-62 could be a countermeasure of the parasite protein to reduce the pro-inflammatory response elicited by these DCs.

#### **3.4.3.2 ES-62-matured CD8<sup>+</sup> DCs do not promote Th1 responses**

Studies with CD8<sup>+</sup> DCs have shown that these cells produce high levels of IL12 and hence are very good inducers of Th1 responses [59, 61, 467]. Consistent with this, IL12p70 production was found to be produced in LPS and ES-62 exposed cultures, albeit not to the level described in literature. Furthermore IFN $\gamma$  and IFN $\alpha$  were also detected. Therefore, it was rather surprising to find that neither ES-62- nor LPS-matured CD8<sup>+</sup> DCs were able to drive Ag-specific Th1 responses as evidenced by the low Ag-independent levels of IL2 and IFN $\gamma$  (Figure 3.22). However the Th2 cytokines IL4, IL5 and IL17E were clearly increased by ES-62-matured CD8<sup>+</sup> DCs indicating that, at low Ag concentrations, ES-62 but not LPS promotes CD8<sup>+</sup> DCs to drive a Th2-like response. Furthermore, a similar profile of IL10, and albeit at a much lower level TGF $\beta$ , production was observed suggesting that Tregs might also be developed under these circumstances. Rather surprisingly, a slight rise in IL17A was also seen in these cultures which might indicate the development of Th17 cells (Figure 3.22). Interestingly, subsequent exposure of ES-62-treated CD8<sup>+</sup> DCs to LPS abolished the Th2/Th17-like phenotype (Table 3.1). However, further analyses are required, such as staining for intracellular cytokines to confirm whether more than one Th effector cell type is being developed. In addition further characterisation of the CD8<sup>+</sup> population might indicate that distinct subpopulations of those cells are responsible for these differential Th17 and Th2-like responses.

Given that CD8<sup>+</sup> DCs have been reported to be potent inducers of Th1 responses, it was quite interesting to see that ES-62 was able to modulate them so that they shifted this response not only towards a Th2/Treg phenotype but also rather

surprisingly, towards a Th17 phenotype in terms of cytokine production. However, analyses of FoxP3, GATA3, Tbet and IL17 mRNA expression in T cells from these cultures (at 10 nM OVA peptide) did not reflect these profiles, although at this concentration, these cytokine responses also appear desensitised or alternatively reflected cytokine consumption. Furthermore the mRNA data from LPS cultures appeared equally quite unexplainable as, completely contrary to the cytokine profile, FoxP3, GATA3 and IL17 were all up-regulated whilst Tbet remained at low expression levels. However, it has been suggested that GATA3 and Tbet expression does not always correlate with Th2 or Th1 development, respectively [512] (Figure 3.23).

#### **3.4.3.3 ES-62 and LPS elicit differential cytokine production by CD4<sup>+</sup> DCs**

CD4<sup>+</sup> DCs make up about 60% of all splenic cDCs and although some studies implicate a role for these DCs in Th2 development [61], a specific function for these DCs has not yet been defined. For example, it was shown that Ag-pulsed CD8<sup>-</sup> DCs (CD4<sup>+</sup> and the DN subpopulation), are able to induce higher levels of IL4 by T cells than CD8<sup>+</sup>DCs, despite both groups of DCs producing comparable levels of IFN $\gamma$  in these cultures [61]. Moreover, following culture for 24 h with GM-CSF, Ag-pulsed CD8<sup>-</sup> DCs are able to drive a Th2 response when transferred to mice [59]. As these DCs seem therefore to preferentially promote Th2 responses, it was postulated that ES-62 might promote their responses. Although ES-62 induced significantly less TNF $\alpha$ , IL12p40 and IL6 production by CD4<sup>+</sup> DCs compared to those exposed to LPS, pre-exposure to ES-62 did not suppress LPS-induced responses, a pattern that was also seen in mixed populations of CD11c<sup>+</sup> DCs (Figure 3.20). Moreover, CD4<sup>+</sup> DCs showed almost equal levels of IL12p70 production in control, ES-62 and LPS cultures. In addition, TGF $\beta$  and IL10 production were, if anything, decreased in ES-62 cultures suggesting that targeting of this subtype on its own does not secrete a cytokine profile likely to promote Th2 or Treg development upon exposure to the parasite product (Table 3.1).



#### **3.4.3.4 ES-62-matured CD4<sup>+</sup> DCs induce hyporesponsive Th responses relative to LPS-matured CD4<sup>+</sup> DCs**

In contrast to that reported for CD8<sup>+</sup> DCs, CD4<sup>+</sup>DCs have been associated with Th2 development in *in vivo* studies [59]. Nevertheless, LPS-matured CD4<sup>+</sup> DCs promoted Ag-dependent increases in TNF $\alpha$ , IL12p40 and IL12p70 and Ag-independent production of IFN $\gamma$ , IL4 and IL25 (IL17E). By contrast ES-62-matured CD4<sup>+</sup> DCs induced relatively little or no production of IL12p40, IL12p70, TNF $\alpha$  or IFN $\gamma$  compared to such LPS-matured DCs. Consistent with ES-62-matured CD4<sup>+</sup> DCs inducing hypo-responsive Th responses, exposure to ES-62 prior to LPS-induced maturation, resulted in suppression of LPS-CD4<sup>+</sup> DC priming of these Ag-specific pro-inflammatory responses (IL12, TNF $\alpha$ ). Although ES-62-matured CD4<sup>+</sup> DCs were able to induce IL4, IL5 and IL25 (IL17E) production this was generally in an Ag-independent manner and at a comparable level to that observed with LPS-matured CD4<sup>+</sup> DCs (Figure 3.24, Table 3.1). Consistent with this, analyses of mRNA levels of Tbet and GATA3 in ES-62-CD4<sup>+</sup> DC primed cultures revealed that these two transcription factors were not modulated, however FoxP3 mRNA was up-regulated (Figure 3.25). Thus, consistent with the low level IL10 production, it is possible that ES-62-matured CD4<sup>+</sup> DCs initiate a Treg phenotype which could be responsible for the observed lack of polarised Th2 or Th1 development. This DC phenotype appears to be very plastic with regard to the T cell polarisation and hence might *in vivo* depend on additional signals to prime T cells in a certain way.

#### **3.4.3.5 CD4<sup>-</sup>CD8<sup>-</sup> DN DCs show comparable production of pro-inflammatory and Th1 driving cytokines after ES-62 and LPS exposure**

Currently very little information is available about DN DCs as these DCs have generally been believed to be functionally similar to the CD4<sup>+</sup> DC subpopulation [455] and hence often analyzed together. However, analyzing the data from these studies suggested that this subtype may indeed have different functional abilities. The first thing that stood out was, that regardless of any treatment, levels of cytokine production by DN DCs were lower than those produced by the other DC subtypes. Further analyses showed that whereas LPS and ES-62 increased the

pro-inflammatory and Th1 driving cytokines, IL12p40 and IL12p70 to a comparable extent, ES-62 induced significantly lower TNF $\alpha$  levels. Interestingly IL10 levels were highest under LPS treatment, whereas TGF $\beta$  production did not show significant differences between the treatment groups. Furthermore, DN DCs pre-exposed to ES-62 and subsequently matured with LPS demonstrated significantly reduced levels of IL-12p70 relative to those observed in response to LPS alone indicating that ES-62 might act to prevent Th1 polarisation in this manner. However, as IL12p40 levels remain at the same level as in the LPS-treatment group and TNF $\alpha$  levels significantly increased, it appears that ES-62 cannot interfere with production of all pro-inflammatory cytokines once these cells have been exposed to a strong maturational signal such as LPS (Figure 3.21, Table 3.1).

#### **3.4.3.6 ES-62 promotes a mixed Th2/anti-inflammatory phenotype in Ag-specific DN DC-T cell cultures**

One of the most intriguing discoveries was that, as with the other purified DC subtypes, upon LPS maturation these DN DCs did not induce a strong effector Th1 polarisation as, although significant levels of IFN $\gamma$  were produced, these were comparable to those elicited by ES-62-matured DN DCs and in addition, they were released in an Ag-independent manner. Similarly, Ag-independent release of the Th2-cytokines IL4 and IL25 (IL17E), but not IL5, were detected. Moreover, low levels of IL10 and TGF $\beta$  were produced in an Ag-dependent manner and, perhaps consistent with this rather non-polarised profile, the mRNA data clearly showed an increase not only in Tbet, but also in GATA3 and FoxP3 expression (Figure 3.26 and 3.27; Table 3.1).

By contrast, although ES-62-matured DN-DCs induced comparable levels of IFN $\gamma$  to LPS-DN DCs, they also induced a Th2-like response characterized by increased IL4, IL5 and IL17E levels and an increase in GATA3 mRNA expression (Figure 3.26 and 3.27; Table 3.1). As these responses were different to those seen in CD4<sup>+</sup> DC-T cell cultures, they further confirmed that DN DCs constitute a functionally-different population of CD8<sup>-</sup> DCs.

Another interesting difference that further distinguishes DN DCs from the subtype is that subsequent maturation with LPS almost completely reverses the ES-62 effects on pro-inflammatory and Th2 cytokines.

#### **3.4.4 ES-62 has been shown to mediate its effects in a TLR4-dependent manner: can it modulate pDC responses?**

pDCs normally express high amounts of TLR7 and TLR9 [109] which are located in endosomes inside the cell and act as receptors for ligands such as single stranded RNA, single stranded CpG-DNA and imidazoquinolines. Furthermore, low expression of TLR2, which can bind LPS [513-516], has been reported on pDCs [109] and these present studies confirmed this finding at the mRNA level. However, no other TLR has been detected on such DCs and this has also been verified by the present studies. Therefore, as ES-62 has been shown to require the presence of TLR4, albeit not necessarily in a functional form [402], the question that was initially raised was whether the parasite product was able to interact with pDCs. Rather interestingly, ES-62 was capable of eliciting some cytokine response in pDC cultures indicating that the parasite product was potentially capable of signalling via a different receptor and/or inducing up-regulation of TLR4. Similarly, LPS was also able to stimulate pDCs perhaps due to signalling via TLR2 or possibly due to a later up-regulation of TLR4 after initial signalling through TLR2, as TLR expression has previously been shown to be altered by cytokine production [517].

Analyses of the cytokines produced by pDC cultures exposed to ES-62, LPS or CpG revealed completely different cytokine profiles indicating that these three substances probably signal via differential mechanisms in these cells. Signalling through TLR9 (CpG) is reported to produce high levels of IFN $\alpha$  and TNF $\alpha$  which are required by pDCs to fully differentiate into mature pDCs. It was therefore quite interesting to see that within the 24 h culture period neither TNF $\alpha$  nor IFN $\alpha$  were secreted into the culture supernatant in response to any of these stimuli. Indeed, IFN $\alpha$  levels were reduced below control levels although there was some very low production of IFN $\alpha$  in ES-62 + CpG exposed cultures which could indicate that pre-

exposure of ES-62 alters the pDCs and enhances CpG-induced production of IFN $\alpha$ .

Currently there is controversy regarding the production of IL12 by pDCs. It is usually suggested that these cells produce this cytokine only in very low amounts and that most of its production is due to cDC contaminations in the culture. Therefore, to rule out any contaminations in these studies, pDCs were purified by cell sorting according to their surface marker expression and the lack of contaminating cDCs was confirmed by flow cytometric analysis of TLR4 expression, which cannot be detected on freshly isolated pDCs. Furthermore TLR2, 4 and 9 mRNA expression was analysed and this confirmed that only TLR9 and minimal levels of TLR2 mRNA were present (Figure 3.28). Analyses of IL12p70 however showed very high levels of this cytokine in LPS-treated cultures whereas CpG- and ES-62-exposed cells only displayed a slight up-regulation of this cytokine. Furthermore whereas pre-exposure to ES-62 had negligible effects on CpG-mediated induction of IL12p70, it led to a drastic decrease in the production of this cytokine by LPS. This suggests that not only is LPS able to induce a Th1-driving cytokine profile but it also suggests that ES-62 can interfere or even block this signal. When IL12p40 was measured, this was completely suppressed below the low control basal levels in all but the ES-62 group.

Consistent with previous reports, TGF $\beta$  production was not up-regulated relative to control DCs upon CpG stimulation and indeed was completely abrogated in ES-62 or LPS cultures. Similarly, IFN $\gamma$ , IL10, IL6 were not produced in any of the culture groups. The lack of IL10 was rather surprising as pDCs have been shown to induce IL10 producing Th cells and thus IL10 production by pDCs would have been a possible mechanism for promoting such a phenotype. In conclusion, the obtained data suggests that pDCs can respond not only to CpG but also to ES-62 and LPS. A recent study comparing the functions of IKDCs [85] and pDCs also showed that pDCs can respond to LPS stimulation, albeit only minimally, but did not explain any possible reasons for this. However, because of the significant differences in the cytokine profiles it is likely that CpG uses completely different mechanisms to ES-

62 and LPS to activate pDCs. It is furthermore possible that all three substances utilize different signalling pathways and hence can drive unique T cell reactions.

#### **3.4.5 Could IKDCs be responsible for some of the observed pDC effects?**

Recently a new DC subtype has been detected, namely IKDC. This particular DC subtype shows strong similarity not only to NK cells but also to pDCs. For example, the surface markers CD45R and CD11c, which were used to separate pDCs from cDCs, are also found on IKDCs and hence IKDC contamination of such pDC cultures cannot be ruled out. In later experiments, however, an antibody against a new marker, PDCA1, which is only found on pDCs, was used to separate these cells from other DCs and such purified pDCs gave essentially identical responses (only data from these latter experiments are presented in the thesis). Studies have shown that IKDCs and pDCs have a slightly different cytokine production profile depending on the activation stimuli [85, 86, 470] although both cell types were more responsive to CpG than to the TLR4 agonist LPS. For example, it was demonstrated that IKDCs can produce substantial amounts of IFN $\gamma$  upon stimulation with CpG which far exceeded those produced by pDCs [85, 86, 470]. The opposite was true for IFN $\alpha$  and IL12 production which was found to be higher by pDCs [85]. In addition, and consistent with the results discussed above, a slight increase in IL12 upon LPS-stimulation was found in pDC but not IKDC cultures [85]. Thus, in conclusion although IKDC contamination cannot be ruled out entirely, it is believed to be unlikely.

#### **3.4.6 pDCs drive different T cell responses depending on the maturational stimulus**

The high functional plasticity of pDCs and hence their ability to prime naïve T cells in different ways has been demonstrated in various studies [17, 184, 186, 478, 480]. For example, it has been shown that pDC stimulation with CD40L and IL3 leads to the polarization of T cells towards a Th2 phenotype characterized by the production of IL4 and IL5 [479]. In contrast virus-induced maturation of pDCs results in the production of Th1 cells [478]. However, in both cases, such pDCs

also induced T cell mediated production of IL-10. Moreover, freshly isolated pDCs have been shown to only induce IL10 production by T cells and hence it has been suggested that pDCs normally function to promote Treg development [184, 186].

Several studies have shown that CpG-stimulated pDCs can induce production of cytokines associated with different T effector populations. Consistent with this, IFN $\alpha$  and TGF $\beta$  were found to be elicited in the present study in an Ag-dependent manner whereas IL4 and IL5, although produced at high levels, appeared to be induced in an Ag-independent way. Generally, pre-exposure of such pDCs to ES-62 was able to suppress production of all CpG-promoted cytokines except IL-17A which by contrast was increased (Figure 3.30 and Table 3.1). This suggested rather surprisingly and distinct from the results found with cDC co-cultures, that prior exposure to ES-62 seems to act to reduce Th2/anti-inflammatory response elicited by pDC co-cultures. Consistent with this, even though FoxP3 mRNA from T cells from ES-62 + CpG exposed cultures remained up-regulated, a strong decrease in GATA3 could be seen (Figure 3.32). By contrast, although pDCs only exposed to ES-62 rather surprisingly elicited a strong Ag-independent production of IFN $\gamma$ , they also induced strong Ag-dependent induction of IL4, IL10 and Ag-independent IL17E responses.

Analyses of the T cell responses from cultures containing LPS-matured pDCs or ES-62 + LPS-matured pDCs showed some quite interesting responses. For example, whereas CpG was not able to induce IFN $\gamma$  production, LPS-pDC induced similar levels to those found in ES-62 cultures. By contrast IFN $\alpha$  which was up-regulated in CpG-pDC containing co-cultures was induced at much reduced levels (x 10 fold) under LPS treatment. This indicates that not only both CpG and LPS are able to induce Th1-like responses but also that these two substances achieve this in a different way. Furthermore, LPS was not able to induce significant levels of IL4 and IL5. However ES-62-pre-exposure followed by LPS-maturation induced an increase in the production of IL4 and, to a lesser degree, IL5 suggesting that ES-62 can promote Th2 development by pDCs even if additional stimuli such as LPS occur (Figure 3.31 and Table 3.1).

### 3.4.7 Does ES-62 drive TH17 development?

Th17 cells are characterized by their ability to produce high levels of the pro-inflammatory cytokine IL17A. IL17A has been shown to induce other cytokines such as IL6 and GM-CSF as well as chemokines that are important in neutrophil recruitment and activation [518-522]. Furthermore, in recent years several studies have demonstrated that IL17A has many different functions in inflammatory processes and in the development and progression of autoimmune disease [297, 523-528]. For example a direct role for this cytokine has been found in experimental arthritis models [290, 297, 529, 530]. In such models, a Th1-biased immune reaction is found which is characterized by an increase of pro-inflammatory, especially TNF $\alpha$ , and Th1 cytokines. IL17A has been found to induce the production of TNF $\alpha$  and IL1 in macrophages [531] and consistent with this, the development of collagen-induced arthritis was not only suppressed in IL17A deficient mice [297] but also blocking of this cytokine resulted in a reduction of joint inflammation and cartilage destruction [529, 530]. Surprisingly therefore, several recent investigations have also provided evidence for the involvement of IL17A in the pathological processes of allergic asthma [532-534] which, in contrast to arthritis, is a Th2 biased immune response [535] characterized by eosinophilia and increased levels of the cytokines IL4, IL5 and IL13. Indeed a recent study in IL17A deficient mice demonstrated reduced levels of IL4, IL5 and IgE indicating that this cytokine can also have an effect on the development of a Th2 response [533]. However, in the co-cultures containing CD4<sup>+</sup> DCs or DN DCs no significant rise in IL17A could be detected in any treatment group and CD8<sup>+</sup> DC-T cell cultures only showed a trend of increased IL17 production after ES-62 exposure (Table 3.1). A new hypothesis regarding Th17 cells was published recently indicating the existence of regulatory Th17 cells which have no inflammatory function [536]. These cells were reported to produce high levels of IL10 and as this cytokine was produced in significant amounts in ES-62-treated CD8<sup>+</sup> DC-T cell co-cultures, it is possible that the these IL17 producing cells were indeed regulatory IL17 cells. pDC containing co-cultures were also analysed for IL17 production and although low, production could be found in almost all culture groups but no IL17 was found in ES-62-treated co-cultures. Thus collectively this data indicated that ES-62 was not

promoting development of Th17 cells. However, as hardly any IL17 was produced in any treatment group, possibly because these DCs were derived from healthy mice, it cannot be ruled out that ES-62 is able to suppress IL17 production under conditions such as arthritis where it has been found to be produced. Thus, investigating the ability of T cells co-cultured with ES-62-treated DCs derived from CIA or asthma model mice might provide a better insight whether ES-62 is able to modify this type of T cell response

#### **3.4.8 Does ES-62 promote a T regulatory phenotype?**

DCs have been shown to play a role in inducing T regulatory cells which have the capacity to down-regulate proliferation and cytokine production of Th1 and Th2 cells [537]. Therefore it is not surprising that many pathogens use T regulatory cells to reduce an effector response [325, 348, 538, 539]. Paradoxically during infection these Tregs are also of benefit to the host as they prevent excessive damage during an immune response. Currently there are two known subsets, the natural occurring CD4<sup>+</sup>CD25<sup>+</sup> FoxP3-dependent Tregs and the induced/adaptive Tregs. Studies investigating the induction of Tregs suggested that IL10 was a possible costimulatory factor [322, 540] and it has been shown that IL10 is also secreted by certain Tregs [541].

Results of this study showed that ES-62 might be promoting Treg polarisation. Firstly FoxP3 levels were higher in the ES-62, than in the LPS-treated CD4<sup>+</sup> DC-T cell co-cultures and secondly an increase in IL10 levels was found in all ES-62-treated cDC-T cell co-cultures (Figures 3.22-3.27). Interestingly, it was also discovered that ES-62 + LPS-exposed co-cultures exhibited the highest levels of FoxP3.

However, to date there is no definite proof that ES-62 can induce Treg development as the levels of FoxP3 detected were usually low and whilst the IL10 produced is indicative of Tregs, it could also be produced by other T effector cells.



### **3.4.9 ES-62 exposed DCs from the bone marrow and the spleen differentially activate T cells.**

DCs have the capacity to activate and polarise T cell effector function. Many studies have shown that most DC populations are able to induce both a Th1 and a Th2 response depending on the amount of Ag they are exposed to [542-544]. For example, stimulation of OVA transgenic CD4<sup>+</sup> T cells with LPS-matured DCs bearing low Ag concentrations has been shown to induce IL4 responses whereas high Ag doses resulted in the production of IFN $\gamma$  [94, 542-544]. Furthermore the Th1 or Th2 decision has been reported to be DC stimulant dependent. For example, CD8<sup>+</sup> and CD8<sup>-</sup> splenic DCs exposed to purified protein derivative (PPD) from *Mycobacterium tuberculosis* are able to induce IFN $\gamma$ -producing T cells whereas stimulation with zymosan enhanced priming of IL4-producing effector cells [545]. Similarly, bm-derived DCs were shown to respond to LPS or CpG by promoting a Th1 response [94], whilst recent studies in this lab determined that the parasite protein ES-62 induced development of Th2 cells [146]. Interestingly, a study with *Candida albicans* illustrated that different developmental stages of a pathogen can stimulate DCs to promote different T cell responses [267]. All these studies clearly show the high plasticity of DCs upon exposure to different Ags in different environments.

The ability of ES-62-exposed DCs to promote an anti-inflammatory T cell response was confirmed by the low level production of IL12p40, IL12p70 and TNF $\alpha$ . The only exception was the high TNF $\alpha$  secretion in co-cultures containing cells (DCs + DC/myeloid progenitors) from whole bmDC cultures where maturation with ES-62 induced the highest levels of the cytokine. Interestingly, recent studies suggest that maturation by TNF $\alpha$ , despite inducing high levels of surface co-stimulatory molecules, only promotes low level secretion of pro-inflammatory cytokines [546]. It is therefore possible that interactions of ES-62 with other cells in these whole bmDC-T cell cultures results in increased TNF $\alpha$  production to an extent that desensitises DC cytokine production. As the increase in production of TNF $\alpha$  is an Ag-dependent effect it is possible that this mechanism reflects interaction between

T cells and cells such as monocyte progenitors and is used by ES-62 to counteract the pro-inflammatory stimulus.

Whereas both whole bmDCs and CD11c<sup>+</sup> bmDCs induced high levels of IL6 in co-cultures (Figure 3.8 and 3.9), CD11c purified spDCs (Figure 3.14) were unable to do so indicating that the location at which and the conditions under which these DCs develop affect their ability to produce cytokines. In addition, a similar pattern could be observed with IL5. However, analyses of IL10 generally showed similarities in the response of all the DC populations tested, particularly that they often simply induced IL10 in response to ES-62 maturation.

The capacity of ES-62 to act to prevent Th1 responses could be seen in both the lack of priming of IFN $\gamma$  production or alternatively in its ability to induce an Ag-dependent decrease in IFN $\gamma$  levels (Figure 3.8, 3.9, 3.14). IFN $\gamma$  production is promoted by IL12 and IFN $\gamma$  in turn induces DCs to produce more IL12. Therefore it was reassuring to see that IFN $\gamma$  secretion generally correlated with the expression pattern of IL12p70. Analyses of the transcription factors from co-cultures containing ES-62-treated spDCs showed an Ag-dependent decrease in T-bet and an increase in GATA3 expression (Figure 3.15) while cultures containing ES-62-treated bmDCs perhaps surprisingly displayed an increase in both (Figures 3.10 and 3.12). However, the ratio of GATA3/Tbet in these latter cultures shows a relative increase of GATA3 (Figures 3.11 and 3.13). Thus, it is not surprising that, despite this increase in T-bet, IFN $\gamma$  levels in ES-62 exposed bmDC co-cultures decrease with Ag dose, as it has been shown that GATA3, which is highly expressed in these T cells as well, is a strong inhibitor of IFN $\gamma$  production [277, 547, 548]. Furthermore it has been reported that an inhibition of GATA3 is a far stronger inducer of IFN $\gamma$  than an up-regulation of Tbet [549].

While a rise in GATA3 generally resulted in a concomitant increase in IL4 in ES-62-treated cultures, IL4 production, despite high GATA3 levels, was abolished if ES-62 exposed spDCs were subsequently matured with LPS (Figure 3.15). However, discrepancies between GATA3 expression and IL4 production have recently been reported that suggest that such findings are due to the fact that GATA3 only

induces a structural change in the IL4 locus which is necessary for its production [512]. Thus, although ES-62 might be able to inhibit Th1 induction and unmask the IL4 locus, subsequent DC maturation with LPS might result in the lack of induction/secretion of Th2 cytokines. Interestingly, an increase in GATA3 expression was only seen in cultures primed by purified CD11c<sup>+</sup> DCs that had been exposed to ES-62 + LPS whereas T cells primed by exposure to whole bm DC cultures, matured with this combination of pathogen products, exhibited equivalent GATA3 levels to those seen in LPS-treated cultures (Figure 3.10).

#### **3.4.10 Does an interaction of DCs and/or T cells with other cells in culture have an effect on the ES-62 mediated T cell response?**

Functional differences between cultures containing purified immature CD11c<sup>+</sup> DCs and those containing GM-CSF selected bm cells were observed. Furthermore, differences between spleen- and bone marrow-derived CD11c<sup>+</sup> DCs have been detected, suggesting that, *in vivo*, interactions between cell populations may take place which potentially could substantially alter the cytokine profiles secreted in *in vitro* cultures. The importance of such specific cell-cell contacts and the specific immunologic microenvironment has indeed been shown recently in the spleen. For example, CD8<sup>+</sup> DCs reside in the T cell area whereas CD8<sup>-</sup> DCs are located in the marginal zone where they come into close contact with macrophages. Therefore, these two DC populations are exposed to different cytokines and interact with a different cell repertoire. However, during an immune response, the CD8<sup>-</sup> cells have been shown to migrate to the T cell area of the spleen [73] presumably to interact with either T cells or other DCs to initiate the appropriate immune response. Likewise a bidirectional interaction between DCs and NK cells has also been reported. Thus, it has been shown that not only do DCs have the ability to activate NK cells but also that NK cells, once activated, can induce the maturation of immature DCs. Both cell-cell contact and cytokine production are necessary for these processes to take place [550, 551].

As the exact mechanism by which ES-62 exerts its immunomodulatory functions has not been found, it is not possible to determine to which extent the

microenvironment and the influence of other cells contribute to its DC-mediated subversion of the immune response. Considering the differences seen in our cultures, it is however very likely that an interaction between different cells takes place and that this interaction may be influenced by ES-62 to a certain extent. In this context it is also possible that ES-62 has an effect on cells in the vicinity of DCs which might influence the development and maturation status of these DCs. In addition ES-62 might have the ability to influence development of DCs but it is still unclear if it can alter the subtype composition or indeed if it is able to induce formation of a new subtype.

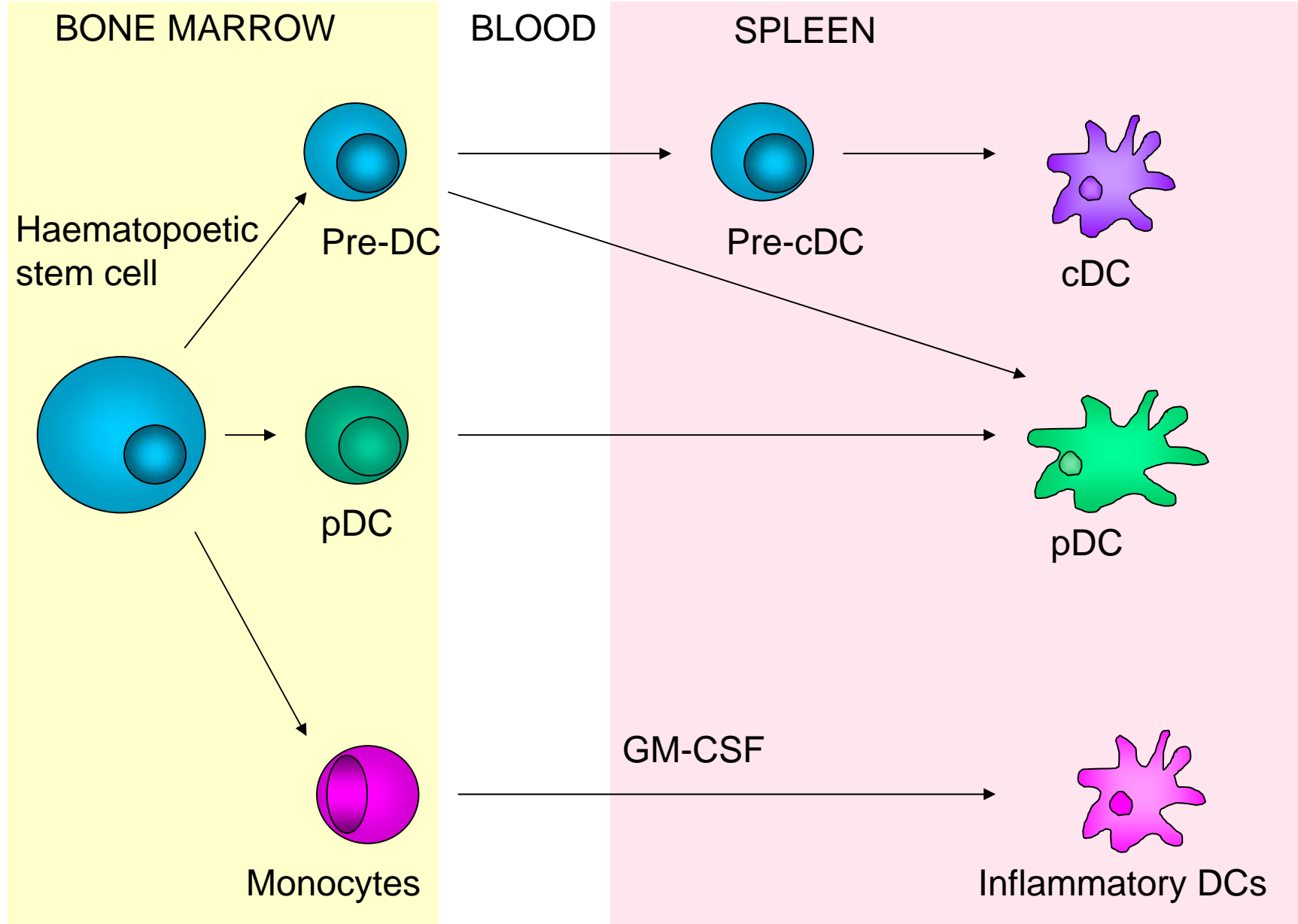
#### **3.4.11 Potential additional mechanisms for ES-62 to modulate T cell responses *in vivo***

Using the above described *in vitro* culture systems has the benefit of being able to investigate the reaction of single DC subtypes to ES-62 and to further define their innate functions and abilities to prime T cells. However as these *in vitro* cultures do not necessarily represent a physiological environment it is hard to predict whether these DCs would behave similarly *in vivo*. One of the biggest problems in such an artificial culture system is the lack of cell tissue interaction which plays an important role *in vivo*. For instance different DC subtypes are located in different areas of the lymphoid organs and hence are surrounded by different cells in a different microenvironment. This not only means that they are activated at different time-points but also that other cells and thus different cytokines and chemokines contribute to this process. The lack of relative cell-cell contact is the major problem in these subtype cultures as for example it has been shown that CD8<sup>-</sup>DC subtypes migrate to the CD8<sup>+</sup> DC compartments upon inflammatory stimuli presumably to interact with each other. Similarly, pDCs that become activated also migrate to the lymphoid tissues. Thus whilst purified subtype cultures allow analyses of specific DC responses, any crosspresentation or receptor interaction between different DC populations cannot take place and an effect caused by cytokines produced by one specific DC subtype on other subtypes cannot be seen.

Therefore, although these *in vitro* studies have characterised some of the effects of ES-62 on DC subtypes, further *in vivo* studies are necessary to confirm these data under more physiologic conditions *in situ*.

### **Figure 3.1 Development of splenic dendritic cells**

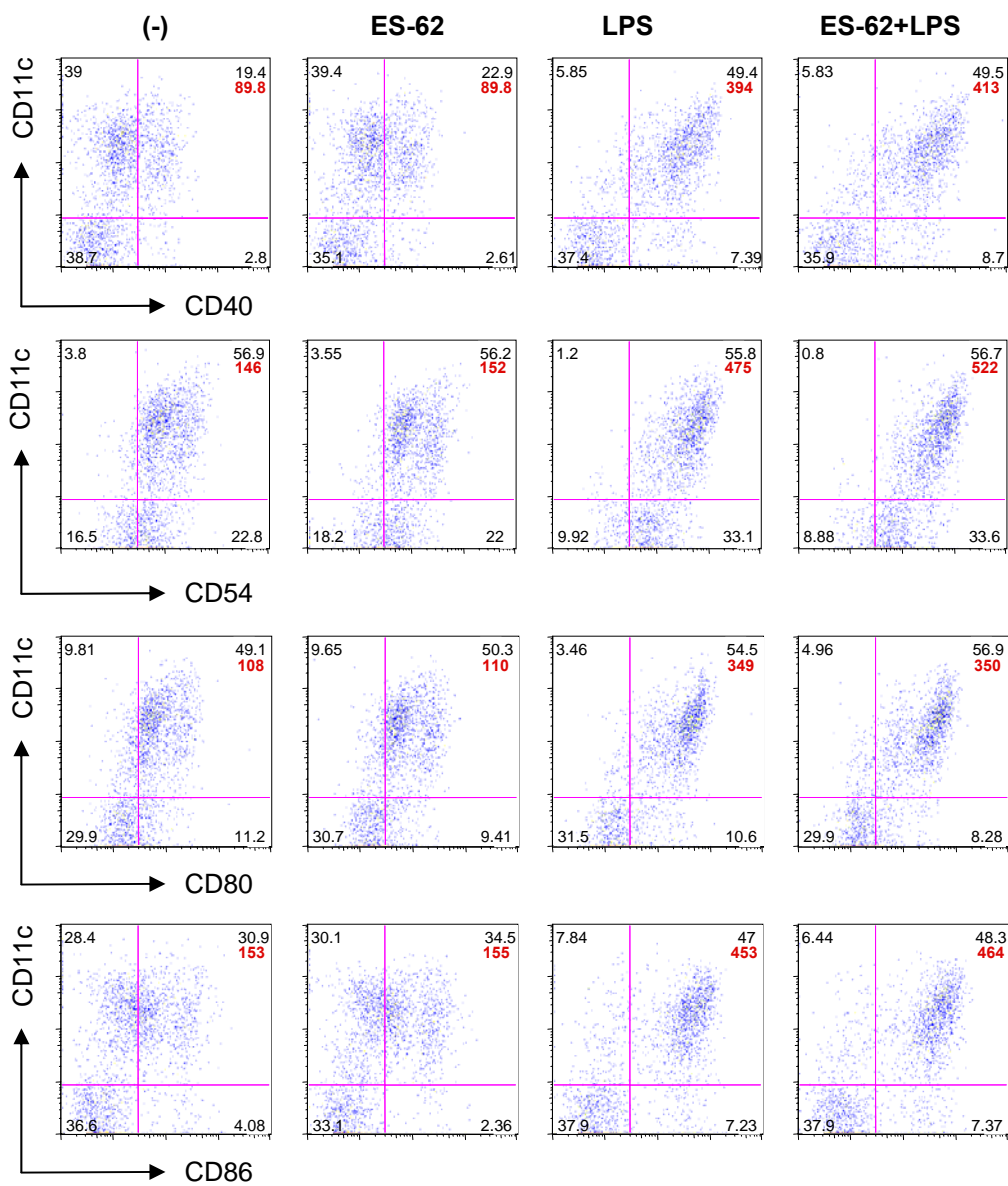
All blood cells including dendritic cells have their ultimate origin in the haemopoietic stem cell in the bone marrow. To generate spleen DCs the haematopoietic stem cell has to develop into an early DC precursor (pre DC) cell which is capable of giving rise to all conventional DCs (cDCs) and to plasmacytoid (pDCs). These pre-DCs then undergo further maturation and become pre-cDCs which are thought to leave the bone marrow and migrate towards the spleen. Pre-cDCs can still give rise to all cDCs but not to pDCs. Within the spleen these cells are thought to differentiate into immature cDCs. Under inflammatory conditions monocytes in the bone marrow can differentiate into “inflammatory” DCs under the influence of GM-CSF. Furthermore, within the bone marrow fully mature pDCs can be found which are thought to be able to migrate to various locations in the body.



**Figure 3.2 Effects of ES-62 and/or LPS on costimulatory molecule expression by murine bm-derived dendritic cells generated from BALB/c mice**

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either matured or not with ES-62 (2  $\mu$ g/ml) for 24 h on d 6 and subsequently in the absence or presence of LPS (1  $\mu$ g/ml) for an additional 24 h on d 7. On d 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 annotated. Additionally, the mean fluorescent intensity (MFI) (in red) of the double positive population is shown in the upper right quadrant. The data presented are from a single experiment representative of at least 5 other independent experiments.

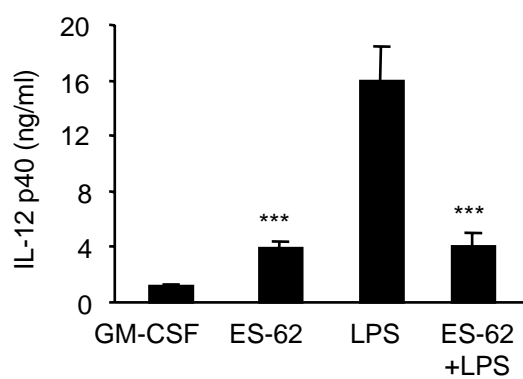




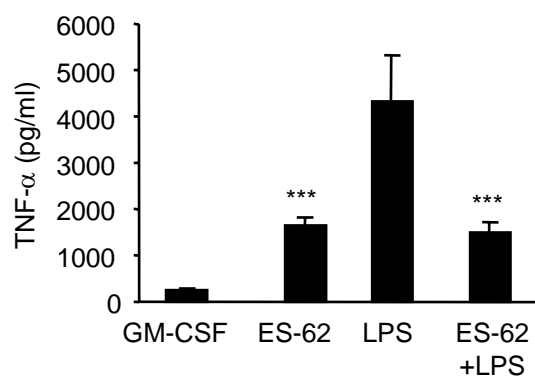
**Figure 3.3 Effects of ES-62 pre-exposure on LPS-induced bmDC pro-inflammatory cytokine production**

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either matured with GM-CSF alone or with ES-62 (2 µg/ml) for 24 h on d 6 and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 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 GM-CSF, ES-62 + LPS compared to LPS.

**A**



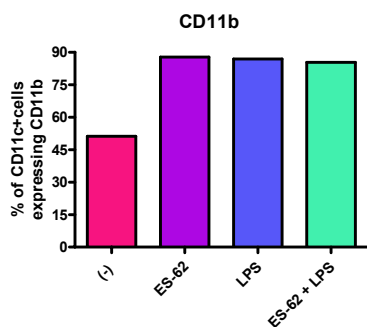
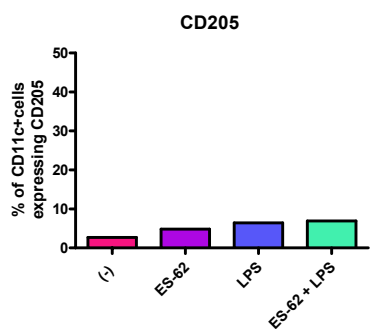
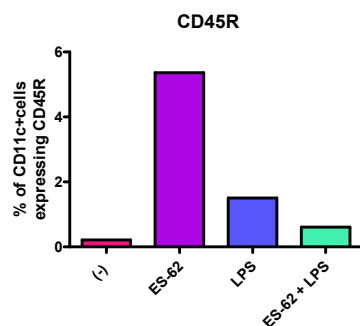
**B**



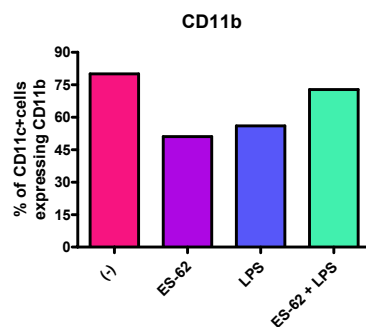
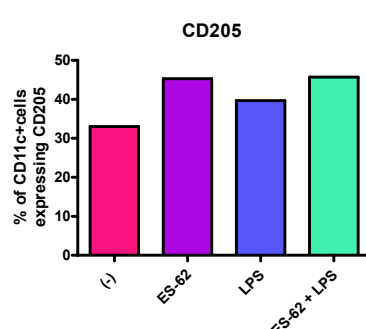
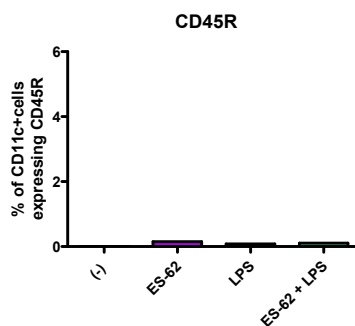
**Figure 3.4 Effects of ES-62 and/or LPS on surface molecule expression by murine bone marrow- and spleen-derived dendritic cells generated from BALB/c mice**

Bone marrow-derived DCs were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d and then either purified on the basis of their CD11c expression or used unpurified. Spleen-derived dendritic cells were purified on the basis of their CD11c expression without previous culture. These whole bmDC, CD11c<sup>+</sup> bmDCs and CD11c<sup>+</sup> spDCs were then either matured or not with ES-62 (2 µg/ml) for 24 h on d 6 and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, cells were harvested and cell surface expression of DC surface marker was analysed by flow cytometry as described in Materials and Methods. Data are presented % of CD11c<sup>+</sup> cells expressing the surface marker. The data presented are from a single experiment representative of at least 2 independent experiments.

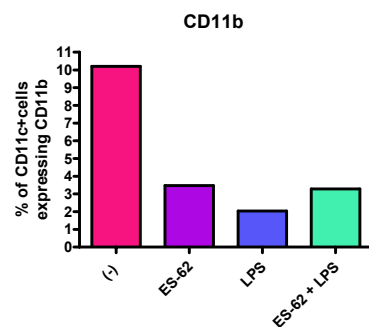
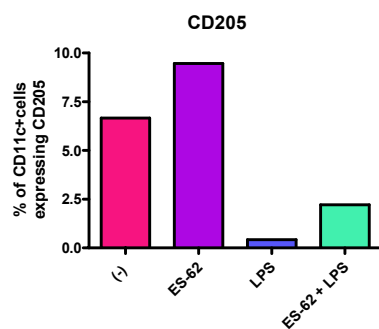
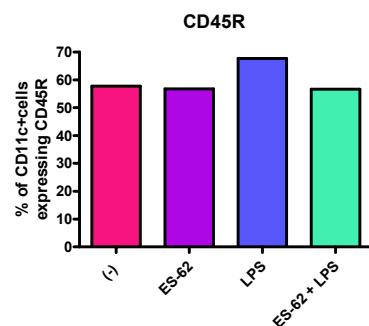
## whole bm DCs



## CD11c<sup>+</sup> bm DCs



## CD11c<sup>+</sup> sp DCs



### **Figure 3.5 Effects of ES-62 and/or LPS on CD11c purified bmDC cytokine production**

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then purified on the basis of their CD11c expression and either matured or not with ES-62 (2 µg/ml) for 24 h on d 6 and then subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, cytokines in culture supernatants were measured by ELISA. Data are presented as means ± SD and are representative of at least 4 independent experiments.

Significance: \*  $p < 0.05$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.005$

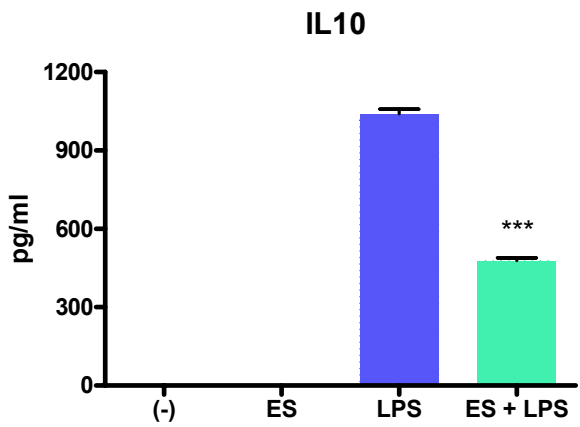
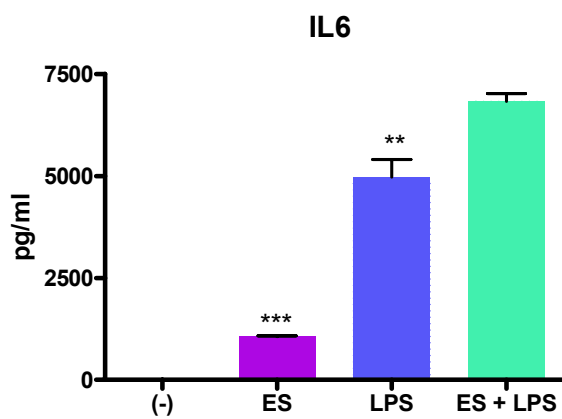
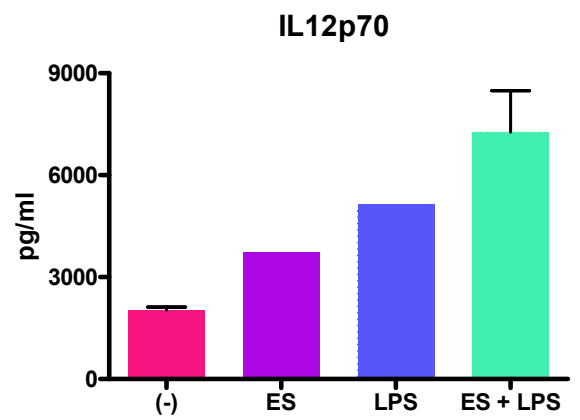
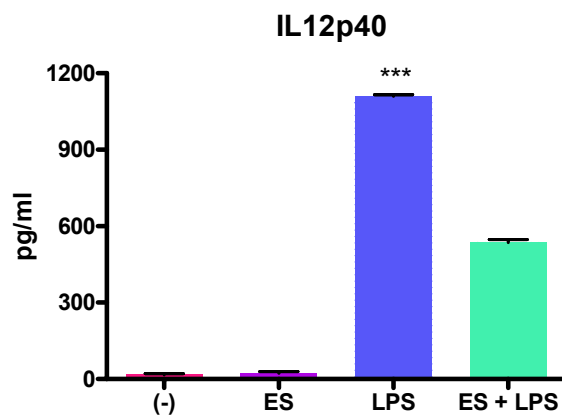
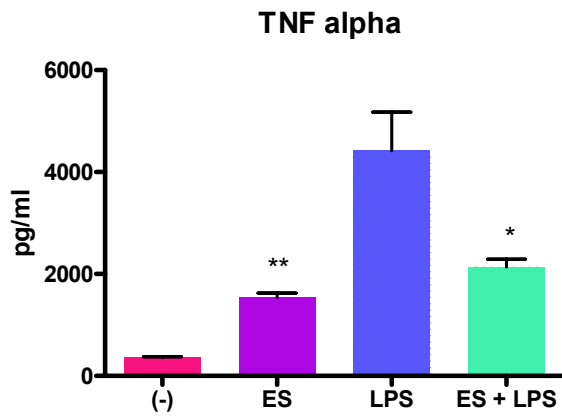
TNFα: \*\* LPS vs ES-62, \* LPS vs ES-62 + LPS

IL12p40: \*\*\* LPS vs ES-62 and ES-62 + LPS

IL6: \*\*\* ES-62 vs LPS and ES-62 + LPS, \*\* LPS vs ES-62 + LPS

IL10: \*\*\* LPS vs ES-62 + LPS

# CD11c<sup>+</sup>bmDC



### Figure 3.6 Effects of ES-62 and/or LPS on whole bmDC cytokine production

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either matured or not with ES-62 (2 µg/ml) for 24 h on d 6 and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8 cytokines in culture supernatants were measured by ELISA. Data are presented as means ± SD and are representative of at least 4 independent experiments.

Significance: \*  $p < 0.05$ , \*\* $p < 0.001$ , \*\*\*  $p < 0.005$

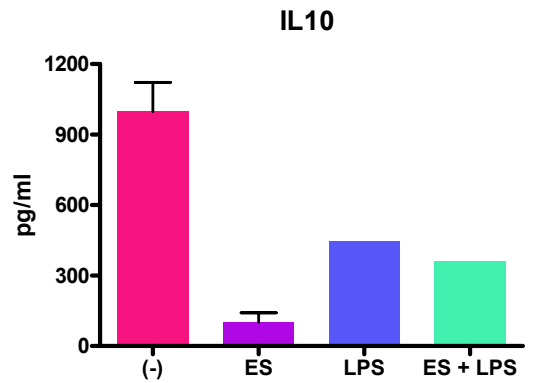
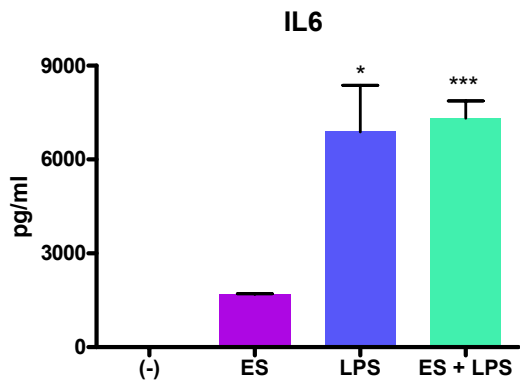
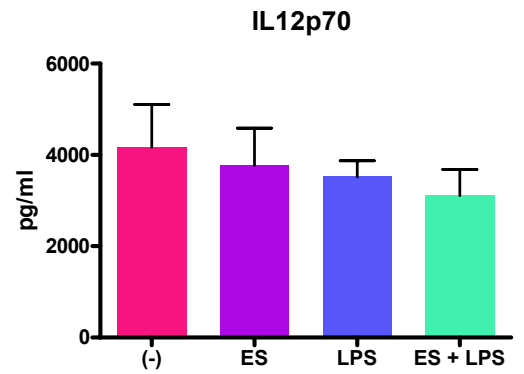
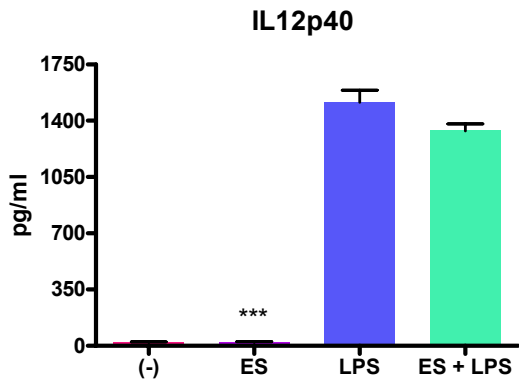
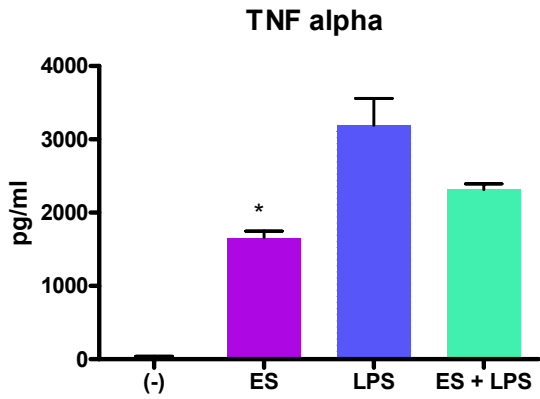
TNFα: \* ES-62 vs LPS

IL12p40: \*\*\* ES-62 vs LPS and ES-62 + LPS

IL6: \* ES-62 vs LPS, \*\*\* ES-62 vs ES-62 + LPS



## whole bmDC



**Figure 3.7 Effects of ES-62 and/or LPS on CD11c purified spleen DC cytokine production**

CD11c<sup>+</sup> splenic dendritic cells were purified and either matured or not with ES-62 (2 µg/ml) for 24 h and then subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3 cytokines in culture supernatants were measured by ELISA. Data are presented as means ± SD and are representative of 2 independent experiments.

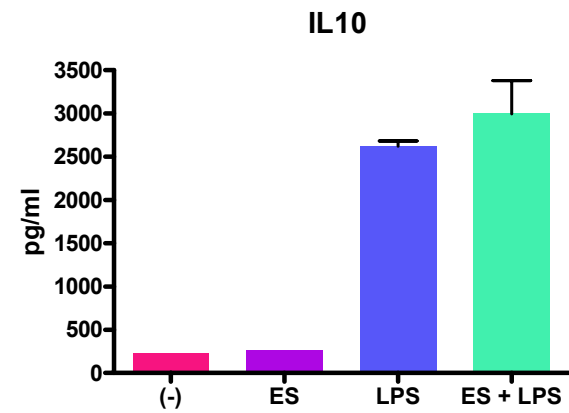
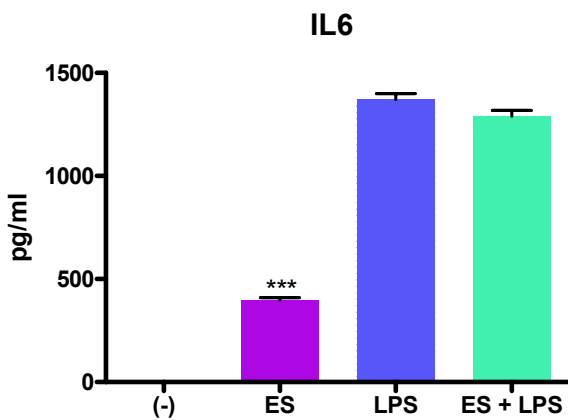
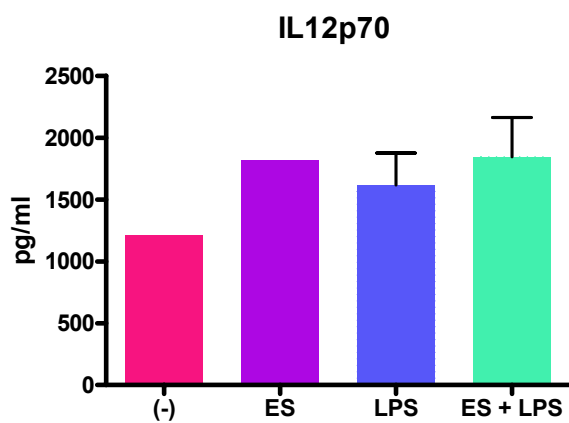
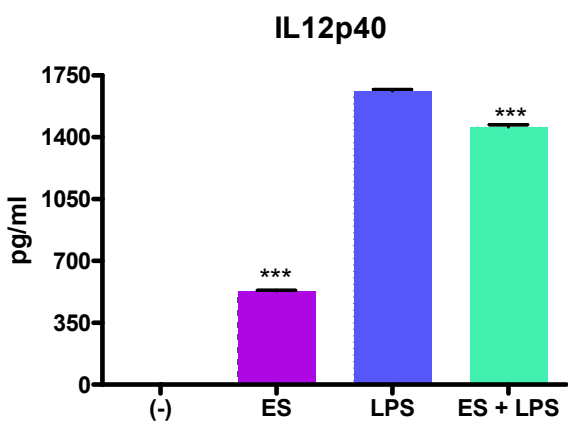
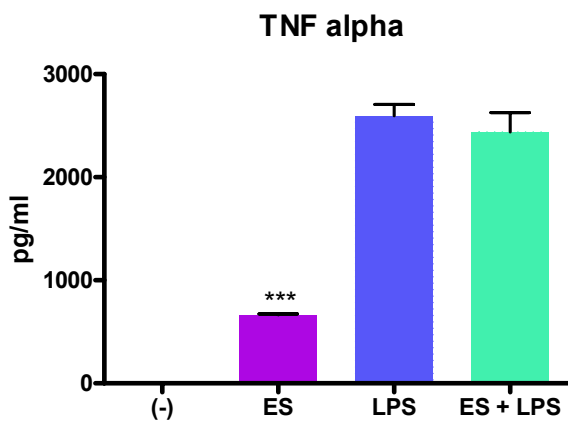
Significance: \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

TNFα: \*\*\* ES-62 vs LPS and ES-62 + LPS

IL12p40: \*\*\* ES-62 vs LPS and ES-62 + LPS, \*\*\* LPS vs ES-62 + LPS

IL6: \*\*\* ES-62 vs LPS and ES-62 + LPS

# CD11c<sup>+</sup>spDC



**Figure 3.8 Analyses of Ag-specific T cell responses elicited by CD11c<sup>+</sup> bmDCs matured in the presence of ES-62**

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then purified on the basis of their CD11c expression and either matured or not with ES-62 (2 µg/ml) on d 6 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x 10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means ± SD and are representative of two independent experiments. The various combinations of DC maturational stimuli are indicated in the key.

Significance: \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

IL12p40: \*, \*\*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS, \*\*LPS vs ES-62+LPS

IL12p70: \*\*\* ES-62 vs LPS

IL6: \* ES-62 vs LPS, \*, \*\*, \*\*\* ES-62 vs ES-62+LPS, \*\*\*LPS vs ES-62+LPS

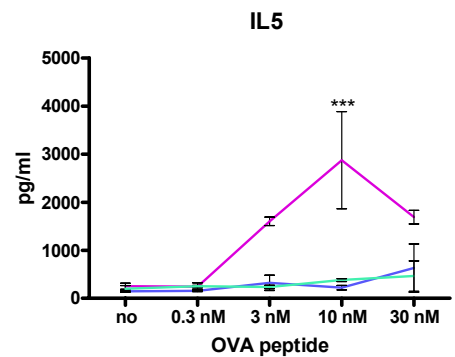
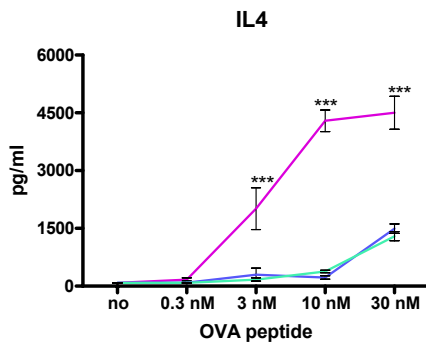
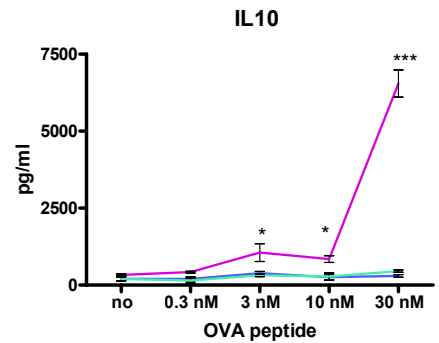
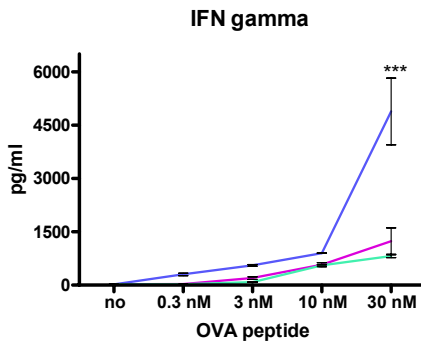
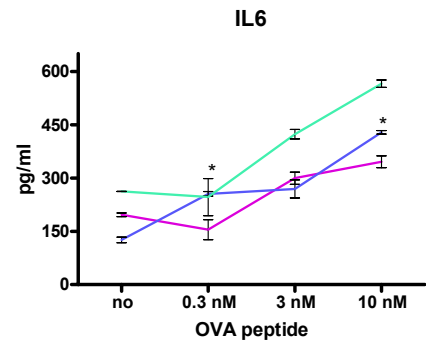
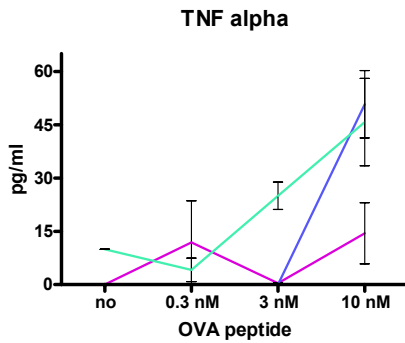
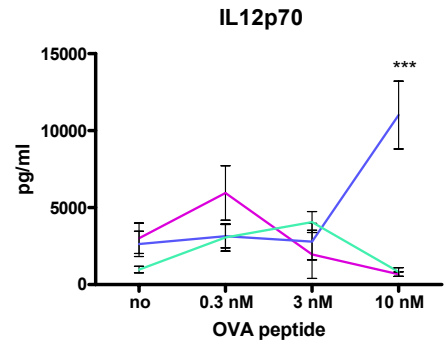
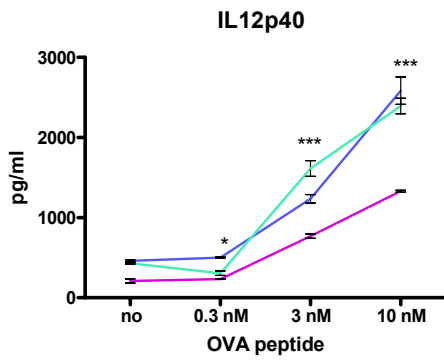
IFNγ: \*\*\* ES-62 vs LPS, \*\*\*LPS vs ES-62+LPS

IL10: \*, \*\*\* ES-62 vs LPS, \*, \*\*\* ES-62 vs ES-62+LPS

IL4: \*\*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS

IL5: \*\*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS

# CD11c purified bmDCs + T cells



■ ES-62 ▲ LPS ▼ ES-62 + LPS

**Figure 3.9 Analyses of Ag-specific T cell responses elicited by whole bmDCs matured in the presence of ES-62**

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either matured or not with ES-62 (2 µg/ml) on d 6 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, bmDC ( $2 \times 10^4$  cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means ± SD and are representative of two independent experiments. The various combinations of DC maturational stimuli are indicated in the key.

Significance: \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

IL12p40: \*, \*\*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS, \*\*, \*\*\* LPS vs ES-62+LPS

IL12p70: \*ES-62 vs ES-62+LPS

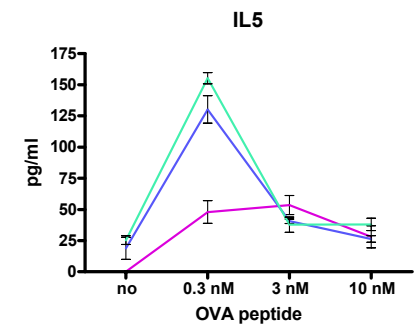
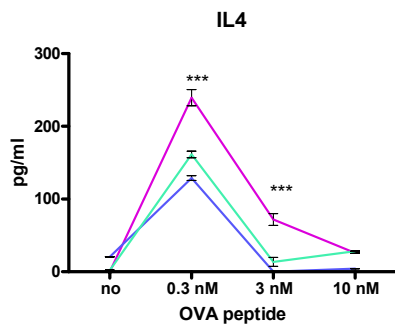
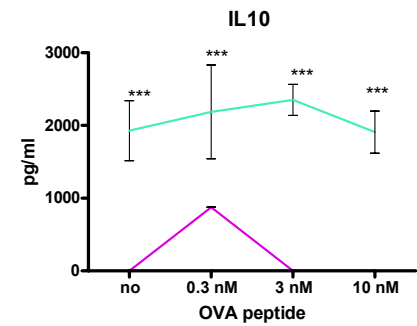
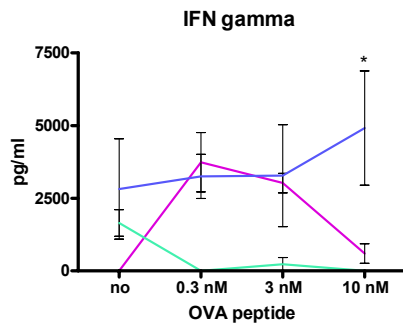
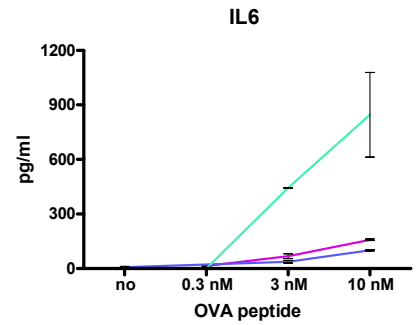
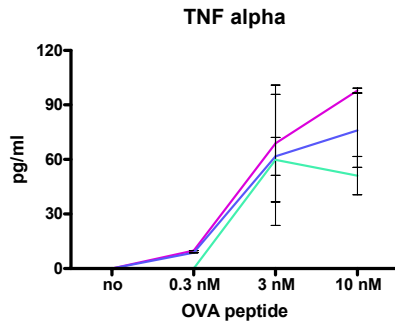
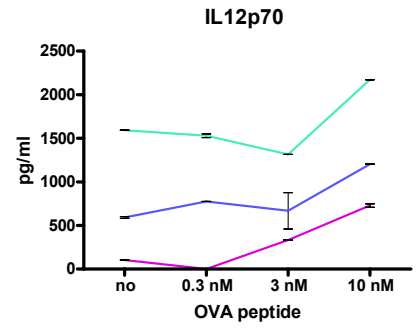
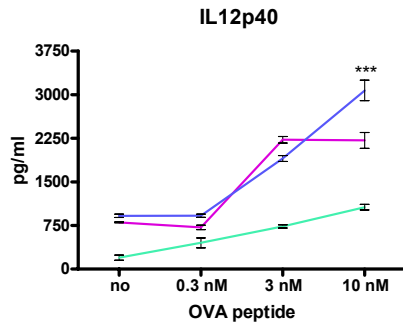
IFNγ: \* ES-62 vs LPS, \* ES-62 vs ES-62+LPS, \*\* LPS vs ES-62+LPS

IL10: \*\*\* ES-62 +LPS vs LPS, \*\*\* ES-62 vs ES-62+LPS

IL4: \*\*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS, \*\* LPS vs ES-62+LPS

IL5: \*\*\* ES-62 vs LPS, \*, \*\*\* ES-62 vs ES-62+LPS, \* LPS vs ES-62+LPS

# whole bmDCs + T cells



■ ES-62 ▲ LPS ▼ ES-62 + LPS

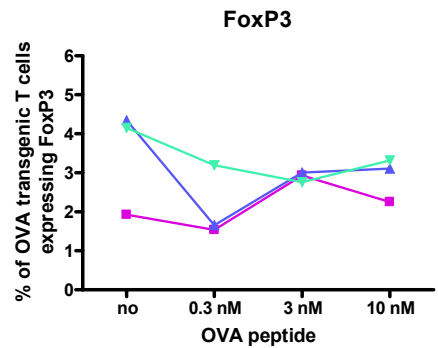
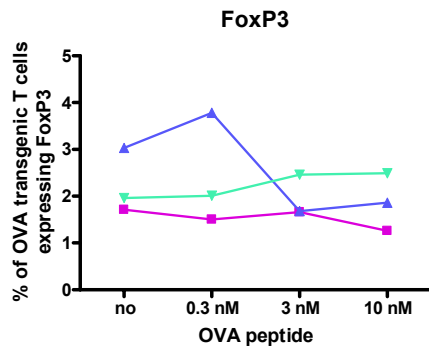
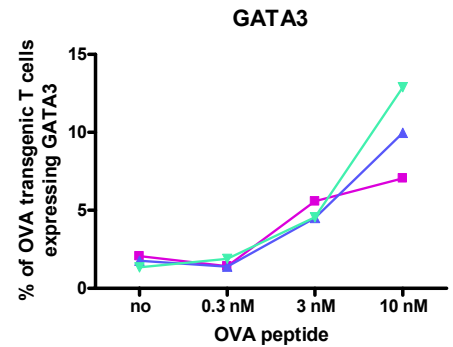
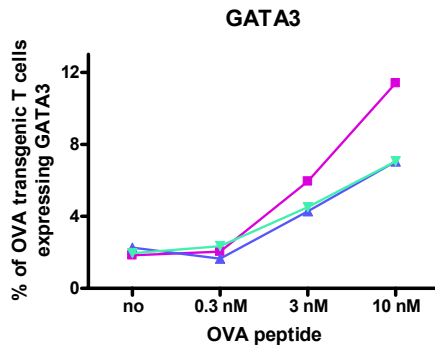
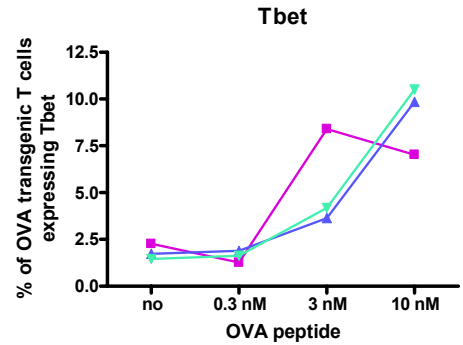
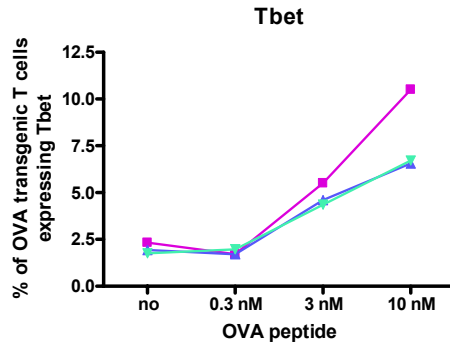
**Figure 3.10 Analyses of the expression of GATA3, Tbet and FoxP3 in T cells primed by CD11c<sup>+</sup> bmDCs or whole bmDCs matured in the presence of ES-62**

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either purified on the basis of their CD11c expression or used without further purification and matured with or without ES-62 (2 µg/ml) on d 6 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before T cells were stained for the OVA-specific TCR and Tbet, FoxP3 and GATA3 expression and analysed by flow cytometry. Data are representative of two independent experiments.



**whole bm DC –T cell  
culture**

**CD11c purified bm DC –T cell  
culture**



—■— ES-62    —▲— LPS    —▼— ES-62 + LPS

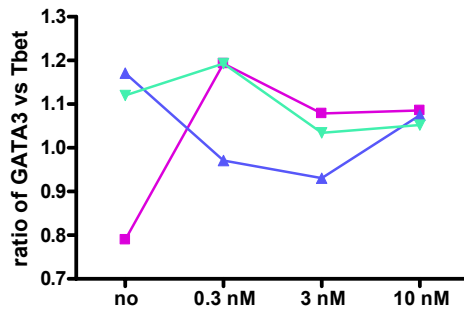
**Figure 3.11 Analyses of the ratios of expression of GATA3, Tbet and FoxP3 in T cells primed by CD11c<sup>+</sup> bmDCs or whole bmDCs matured in the presence of ES-62**

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either purified on the basis of their CD11c expression or used without further purification and matured with or without ES-62 (2 µg/ml) on d 6 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before T cells were stained for the OVA-specific TCR and Tbet, FoxP3 and GATA3 expression and analysed by flow cytometry. Data is presented as the ratio of GATA3/Tbet, GATA3/FoxP3 and FoxP3/Tbet and is representative of two independent experiments.

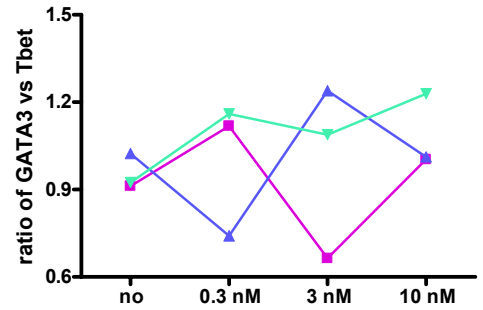
## whole bm DC –T cell culture

## CD11c purified bm DC –T cell culture

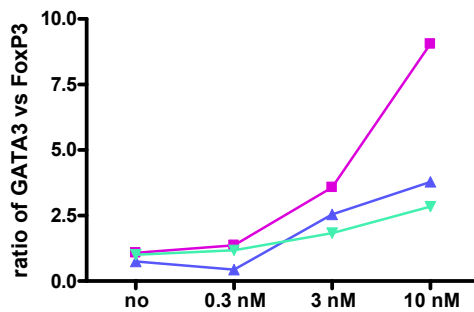
### GATA3/Tbet



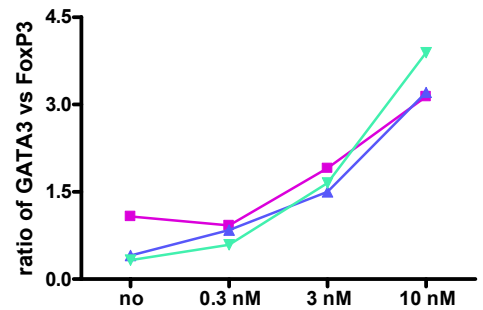
### GATA3/Tbet



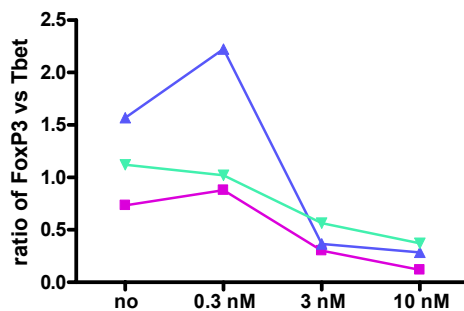
### GATA3/FoxP3



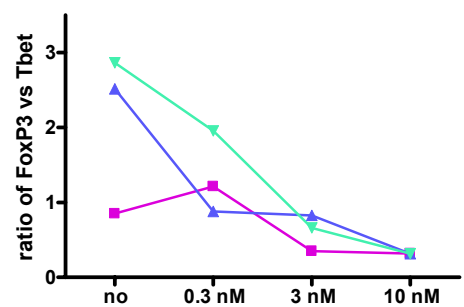
### GATA3/FoxP3



### FoxP3/Tbet



### FoxP3/Tbet



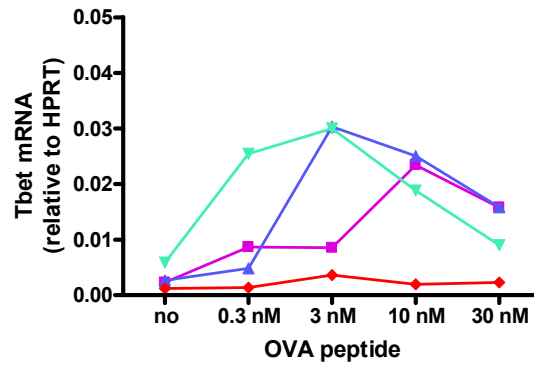
■ ES-62   
 ▲ LPS   
 ▼ ES-62 + LPS

**Figure 3.12 Analyses of the expression of GATA3, Tbet and FoxP3 in T cells primed by CD11c<sup>+</sup> bmDCs matured in the presence of ES-62**

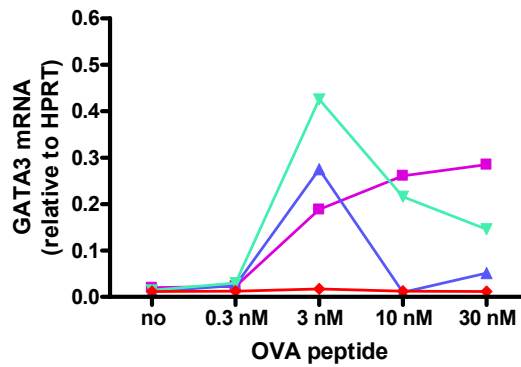
Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either purified on the basis of their CD11c expression or used without further purification and matured with or without ES-62 (2 µg/ml) on d 6 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before cellular RNA was extracted and quantitative PCR for Tbet, FoxP3 and GATA3 was carried out. Data are representative of two independent experiments.

# CD11c purified bm DC –T cell culture

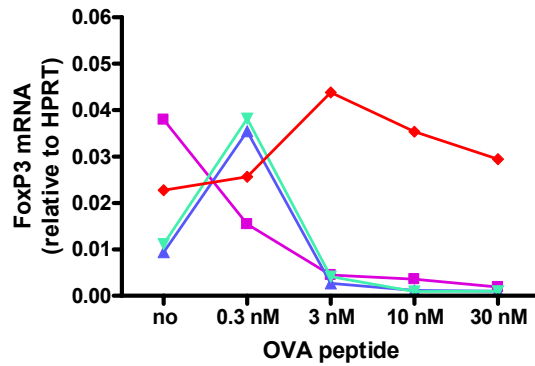
## Tbet



## GATA3



## FoxP3



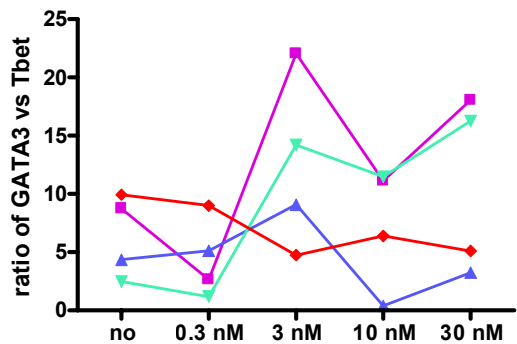
—■— ES-62    —▲— LPS    —▼— ES-62 + LPS    —◆— T cells only

**Figure 3.13 Analyses of the ratios of expression of GATA3, Tbet and FoxP3 in T cells primed by CD11c<sup>+</sup> bmDCs matured in the presence of ES-62**

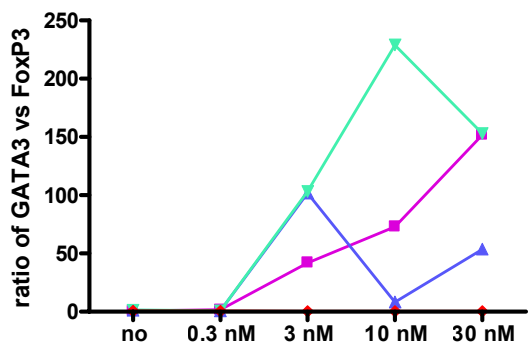
Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either purified on the basis of their CD11c expression or used without further purification and matured with or without ES-62 (2 µg/ml) on d 6 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before cellular RNA was extracted and quantitative PCR for Tbet, FoxP3 and GATA3 was carried out. Data are presented as the ratio of GATA3/Tbet, GATA3/FoxP3 and FoxP3/Tbet and is representative of two independent experiments.

CD11c purified bm DC –T cell culture

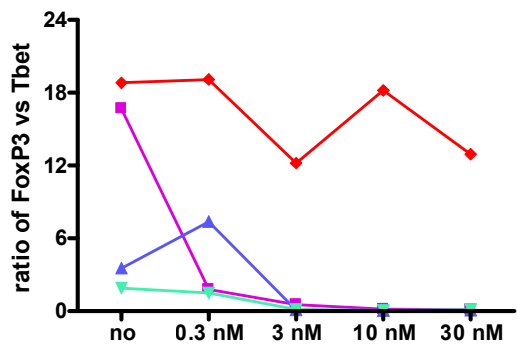
GATA3/Tbet



GATA3/FoxP3



FoxP3/Tbet



ES-62 LPS ES-62 + LPS T cells only

**Figure 3.14 Analyses of Ag-specific T cell responses elicited by CD11c<sup>+</sup> spDCs matured in the presence of ES-62**

CD11c<sup>+</sup> splenic dendritic cells were purified and either matured or not with ES-62 (2 µg/ml) for 24 h and then subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means ± SD and are representative of two independent experiments. The various combinations of DC maturational stimuli are indicated in the key.

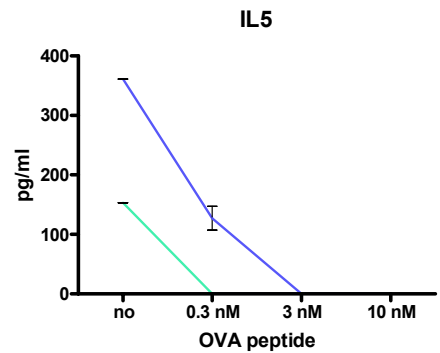
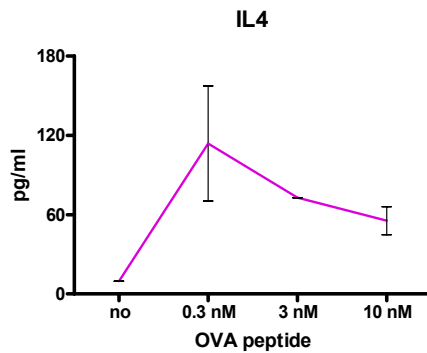
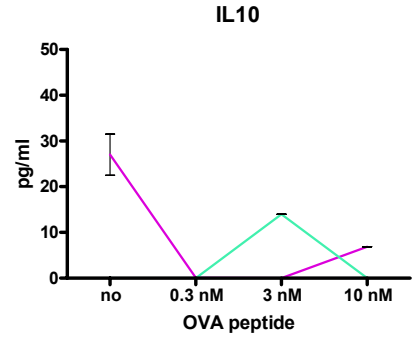
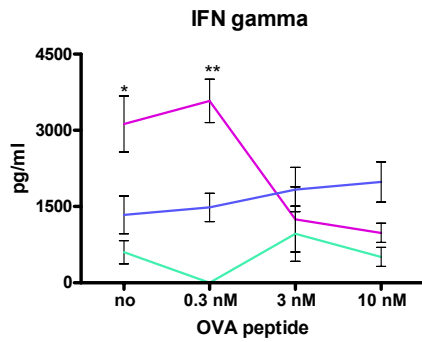
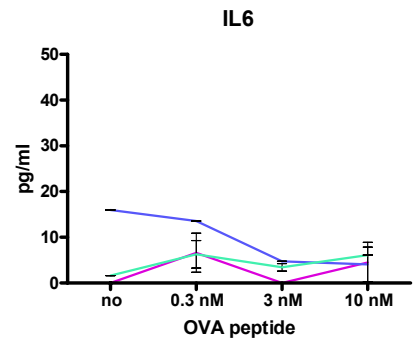
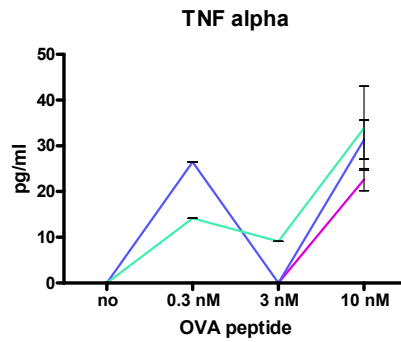
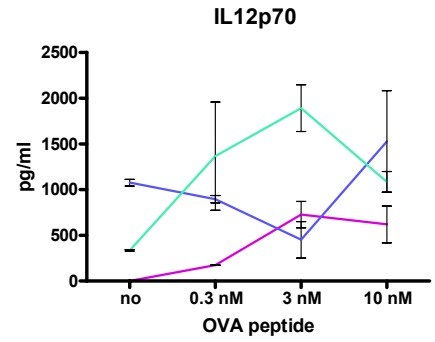
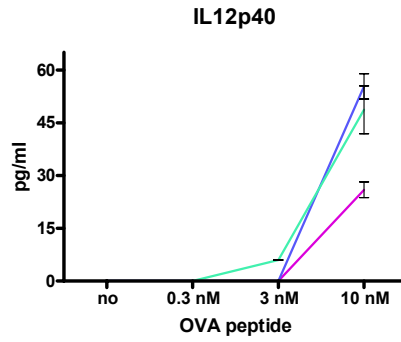
Significance: \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

IL12p70: \*ES-62 vs ES-62+LPS, \*\* LPS vs ES-62+LPS

IFNγ:\*, \*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS



# CD11c purified spDCs + T cells



ES-62

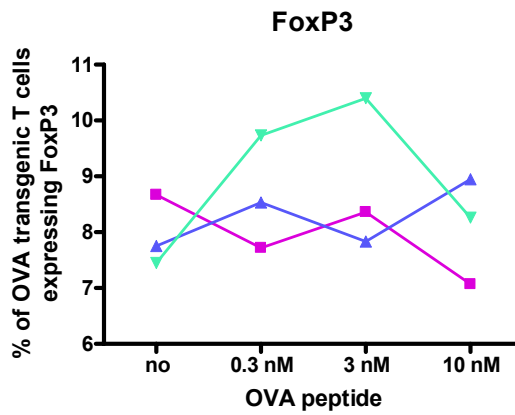
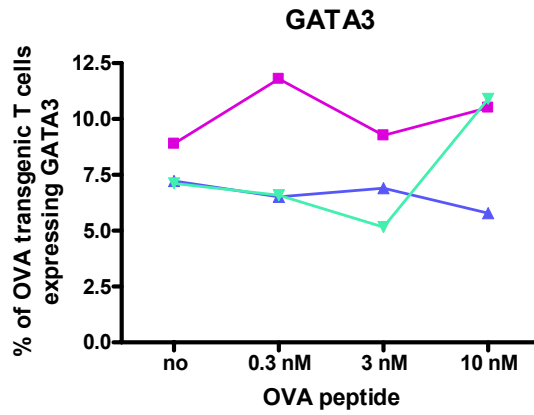
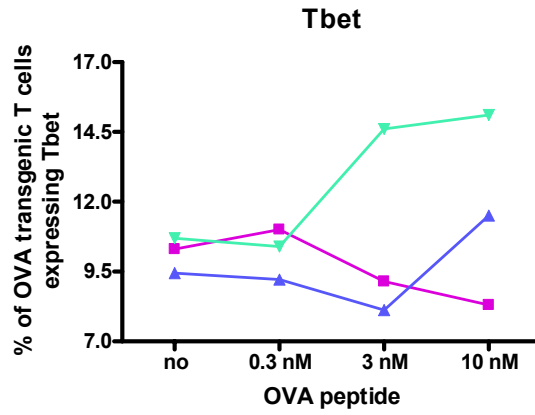
LPS

ES-62 + LPS

**Figure 3.15 Analyses of the expression of GATA3, Tbet and FoxP3 in T cells primed by CD11c<sup>+</sup> spDCs matured in the presence of ES-62**

CD11c<sup>+</sup> splenic dendritic cells were purified and either matured or not with ES-62 (2 µg/ml) for 24 h and then subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before T cells were stained for Tbet, FoxP3 and GATA3 expression and analysed by flow cytometry. Data are representative of two independent experiments.

## CD11c purified sp DC –T cell culture



ES-62

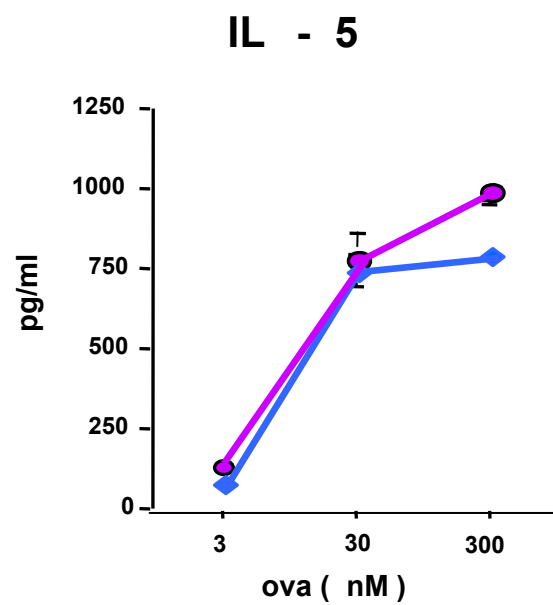
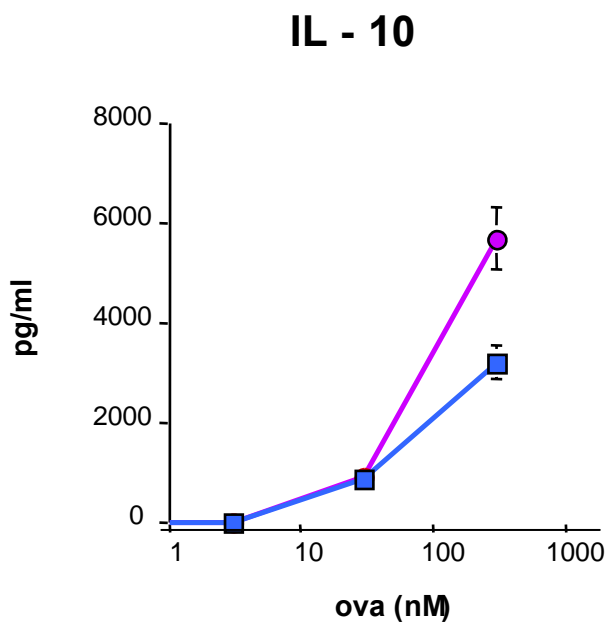
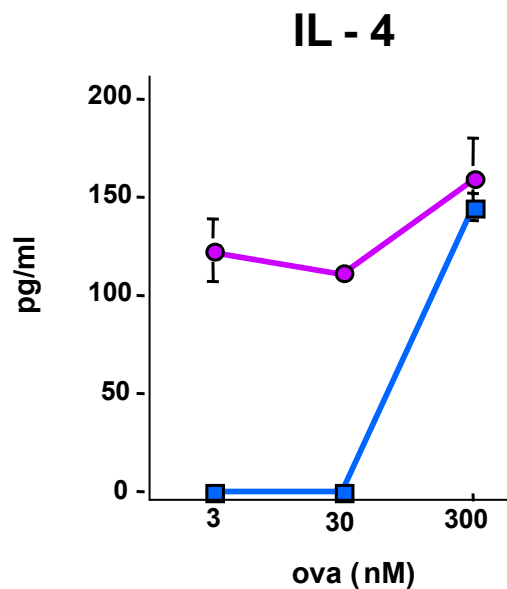
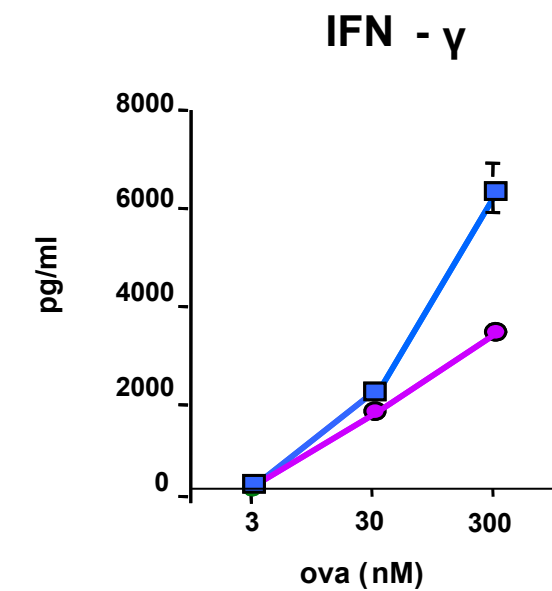
LPS

ES-62 + LPS

**Figure 3.16 Analyses of Ag-specific T cell responses elicited by CD11c<sup>+</sup> spDCs matured in the presence of ES-62 or LPS**

CD11c<sup>+</sup> splenic dendritic cells were purified and either matured with ES-62 (2 µg/ml) or LPS (1 µg/ml) for 24 h. bmDC (2 x 10<sup>4</sup> cells/well) pulsed with Ag were then co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means ± SD and are representative of two independent experiments. The various combinations of DC maturational stimuli are indicated in the key.

## CD11c purified spDCs + T cells



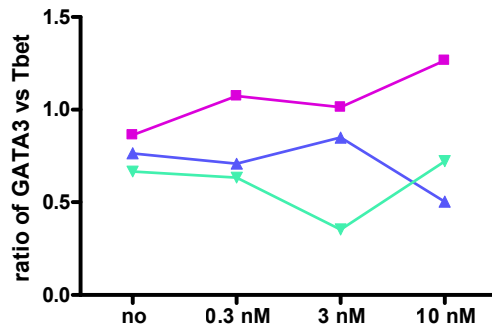
■ LPS  
● ES- 62

**Figure 3.17 Analyses of the ratios of expression of GATA3, Tbet and FoxP3 in T cells primed by CD11c<sup>+</sup> spDCs matured in the presence of ES-62**

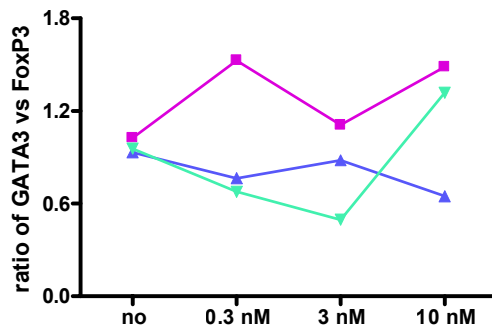
CD11c<sup>+</sup> splenic dendritic cells were purified and either matured or not with ES-62 (2 µg/ml) for 24 h and then subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before T cells were stained for Tbet, FoxP3 and GATA3 expression and analysed by flow cytometry. Data is presented as the ratio of GATA3/Tbet, GATA3/FoxP3 and FoxP3/Tbet and is representative of two independent experiments.

# CD11c purified sp DC –T cell culture

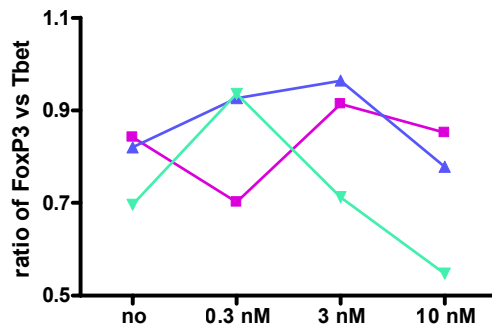
## GATA3/Tbet



## GATA3/FoxP3



## FoxP3/Tbet



ES-62

LPS

ES-62 + LPS

### Figure 3.18 The effects of ES-62 on cytokine production in reactivated DC-T cell co-cultures

Bone marrow-derived dendritic cells were grown from BALB/c mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either purified on the basis of CD11c expression or used without further purification. Splenic DCs were purified by CD11c expression without previous culture. All DC groups were then matured with or without ES-62 (2 µg/ml) for 24 h and then subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h. On d 8, bmDC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x 10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h. At that time PMA (500 µg/ml) and ionomycin (50 µg/ml) were added to the culture for another 24 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key. Significance: \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

whole bmDC-T cell co-culture:

IL12p40:\*,\*\*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS, \*\*,\*\*\* LPS vs ES-62+LPS

TNFα: \*\*\* ES-62 vs LPS and ES-62+LPS, LPS vsES-62+LPS

IFNγ: \* ES-62 vs LPS, \*\* ES-62 vs ES-62+LPS

IL4: \*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS

IL10: \* ES-62 vs ES-62+LPS

CD11c<sup>+</sup> bmDC-T cell co-culture:

IL12p40:\*\*\* ES-62 vs LPS, \*\*\* ES-62 vs ES-62+LPS

TNFα: \*\*\* ES-62 vs LPS, \*,\*\*\* ES-62 vs ES-62+LPS

IFNγ: \*\*\* ES-62 vsLPS, \*\* ES-62 and LPS vs ES-62+LPS

IL4: \*,\*\* ES-62 vs ES-62+LPS, \*\* LPS vs ES-62+LPS

CD11c<sup>+</sup> spDC-T cell co-culture:

TNFα: \*\* ES-62 vs LPS, \* ES-62 vs ES-62+LPS, \*,\*\*,\*\*\* LPS vs ES-62+LPS

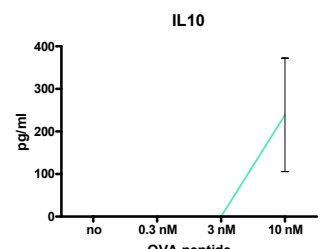
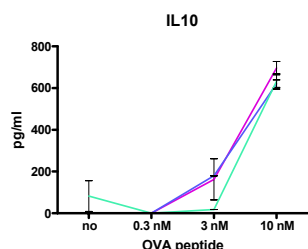
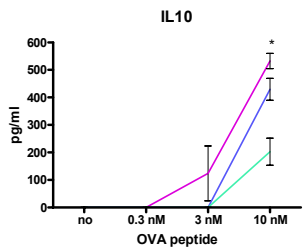
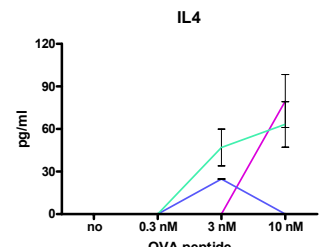
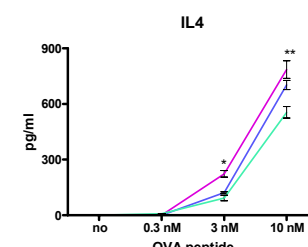
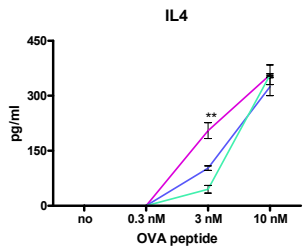
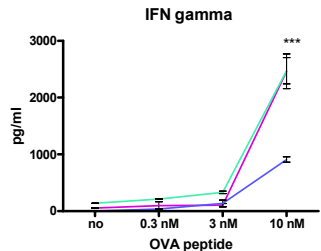
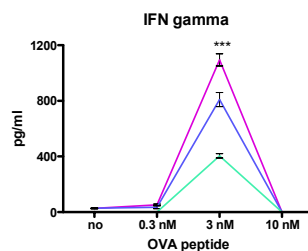
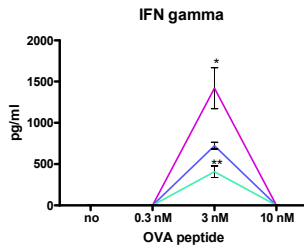
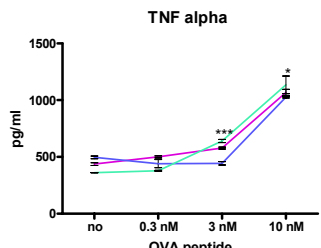
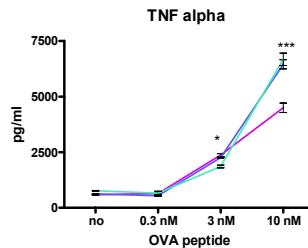
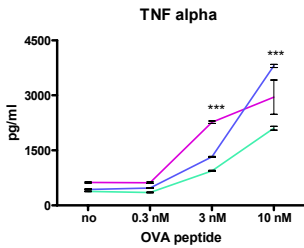
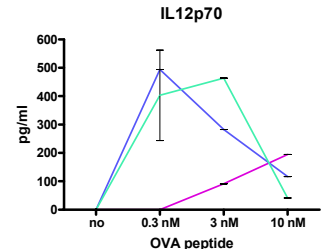
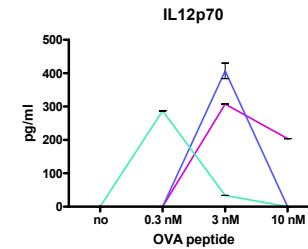
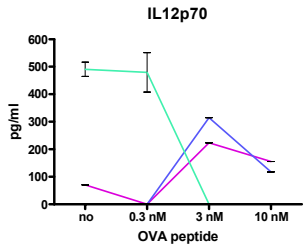
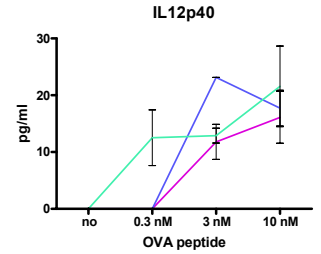
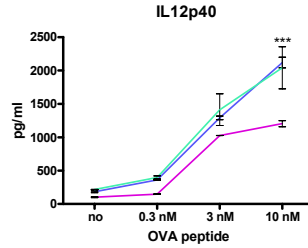
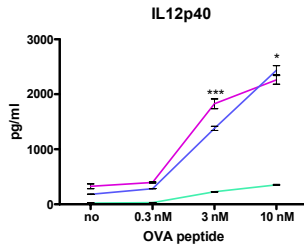
IFNγ: \*\*\* ES-62 vsLPS, \*\* ES-62 and LPS vs ES-62+LPS



**whole bm DC  
+ T cells**

**CD11cpurified bm DC  
+ T cells**

**CD11cpurified sp DC  
+ T cells**



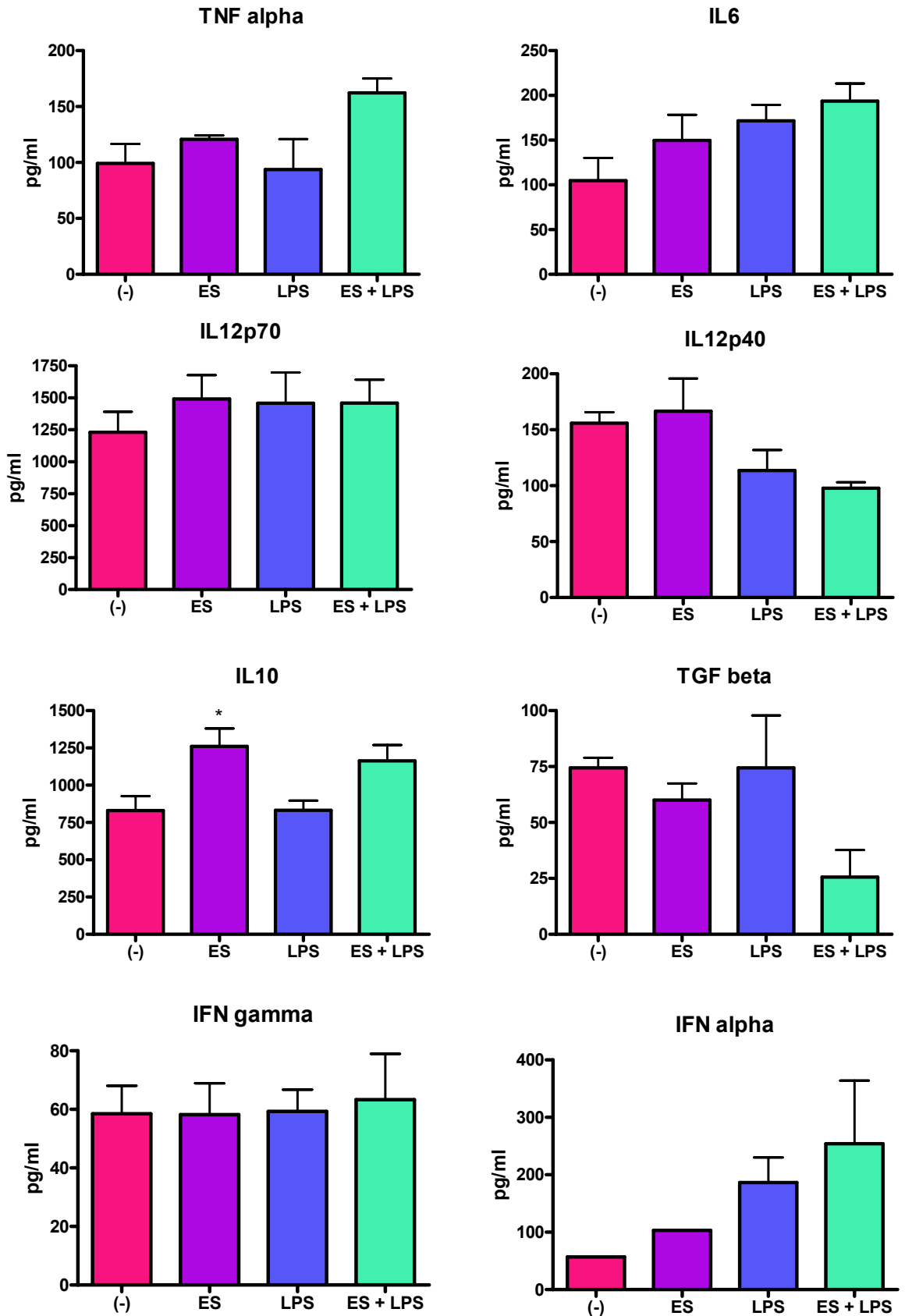
ES-62 LPS ES-62 + LPS

**Figure 3.19 Effects of ES-62 and/or LPS on spleen CD8<sup>+</sup> DC cytokine production**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the CD8<sup>+</sup> DC population was purified by high speed FACS sorting. CD8<sup>+</sup> DC were cultured and either matured with GM-CSF alone or with ES-62 (2 µg/ml) for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3 resulting cytokine production in culture supernatants were measured by ELISA. Data are presented as means ± SD and are representative of 2 independent experiments.

Significance: IL10: ES-62 vs LPS \* p<0.05

# CD8<sup>+</sup> DCs



### **Figure 3.20 Effects of ES-62 and/or LPS on spleen CD4<sup>+</sup> DC cytokine production**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the CD4<sup>+</sup> DC population was purified by high speed FACS sorting. CD4<sup>+</sup> DC were cultured and either matured with GM-CSF alone or with ES-62 (2 µg/ml) for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3 resulting cytokine production in culture supernatants were measured by ELISA. Data are presented as means ± SD and are representative of 2 independent experiments.

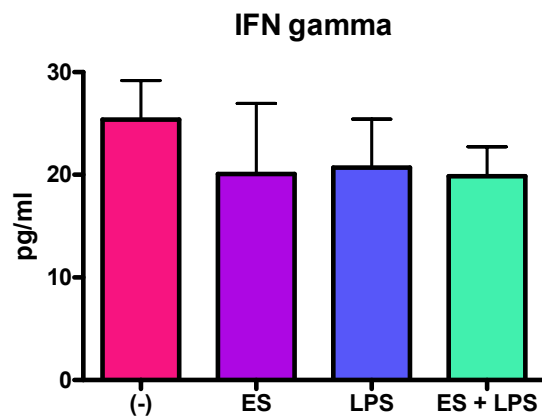
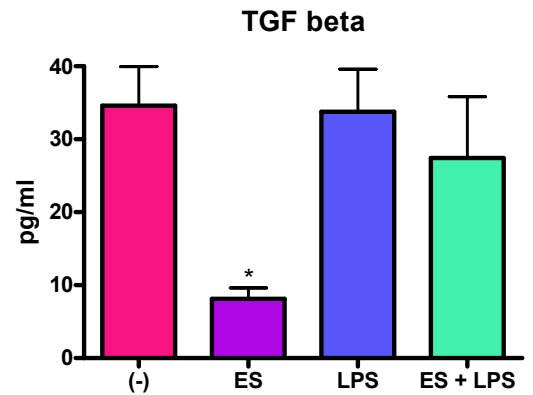
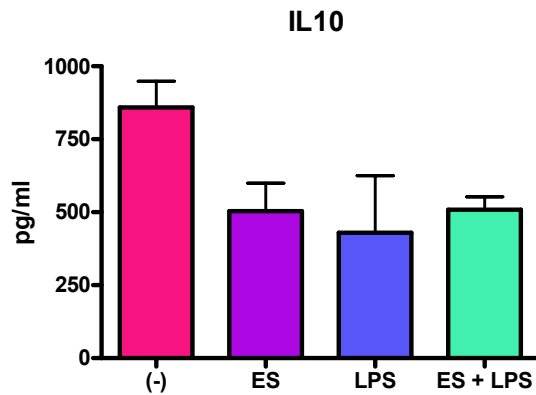
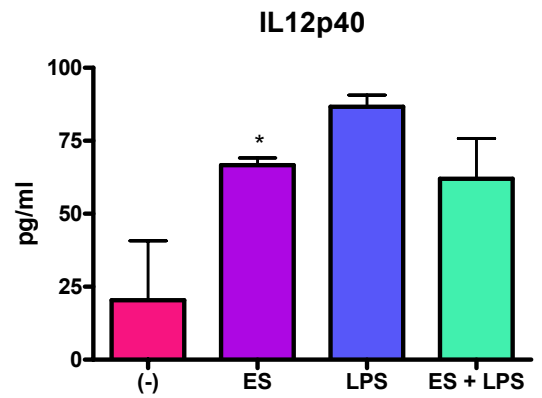
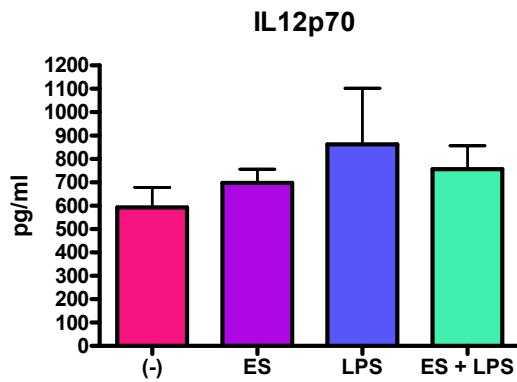
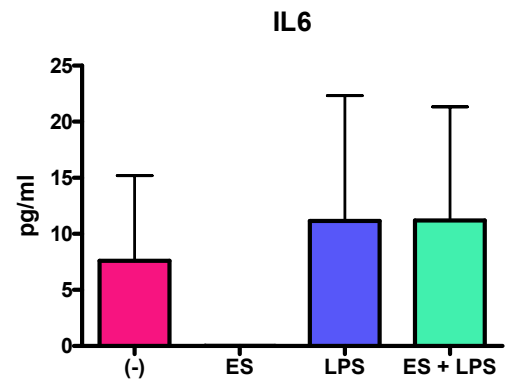
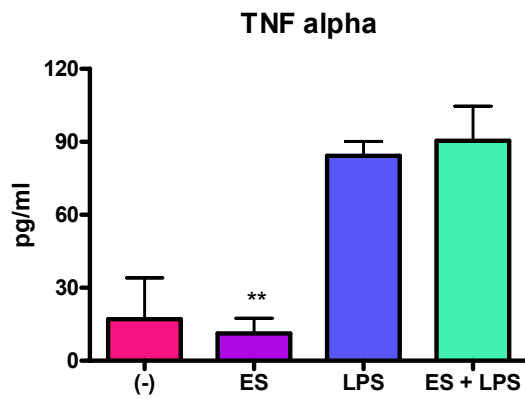
Significance: : \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

TNFα: \*\* ES-62 vs LPS and ES-62 + LPS

IL12p40: \* ES-62 vs LPS

TGFβ: \* ES-62 vs LPS

# CD4<sup>+</sup> DCs



### **Figure 3.21 Effects of ES-62 and/or LPS on spleen DN DC cytokine production**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the DN DC population was purified by high speed FACS sorting. DN DC were cultured and either matured with GM-CSF alone or with ES-62 (2 µg/ml) for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3 resulting cytokine production in culture supernatants were measured by ELISA. Data are presented as means ± SD and are representative of 2 independent experiments.

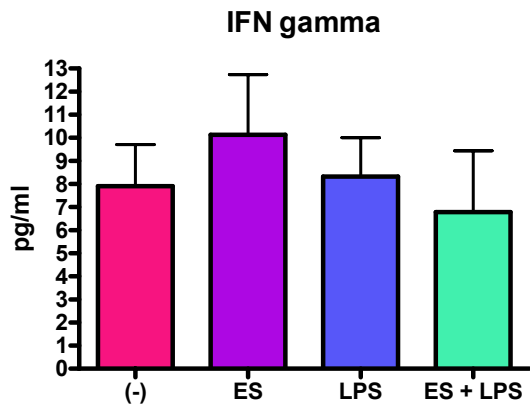
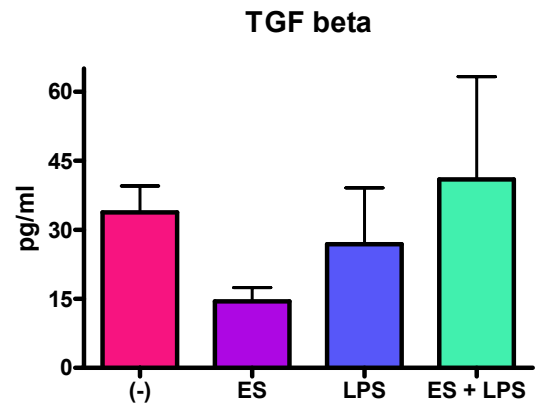
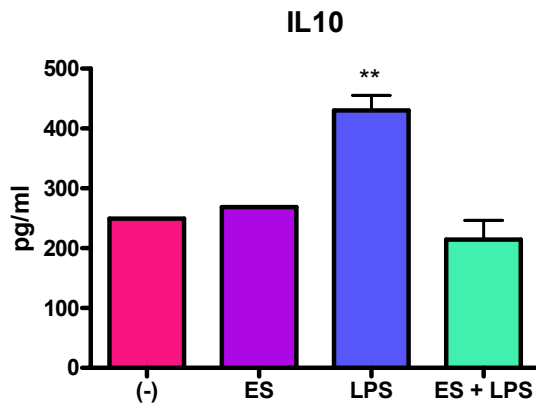
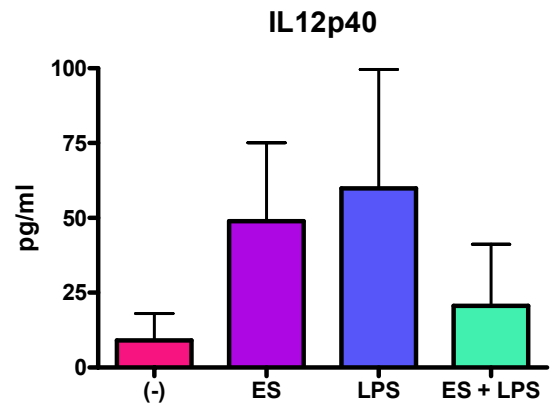
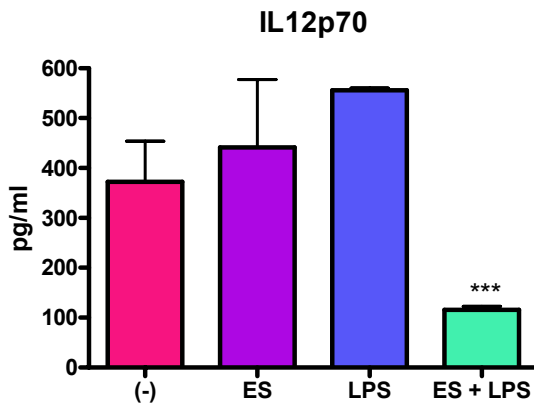
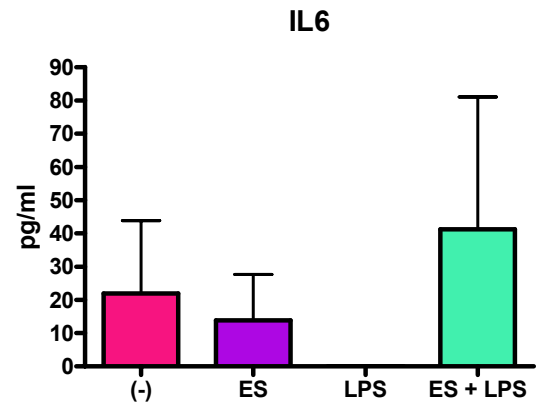
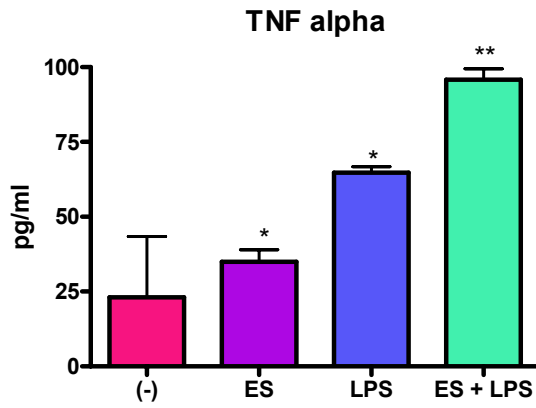
Significance: \*  $p < 0.05$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.005$

TNF $\alpha$ : \* ES-62 vs LPS, \*\* ES-62 vs ES-62 + LPS, \* LPS vs ES-62 + LPS

IL12p70: \*\*\* ES-62 + LPS vs LPS

IL10: \*\* ES-62 + LPS vs LPS

# DN DCs



**Figure 3.22 Analyses of Ag-specific T cell responses elicited by CD8<sup>+</sup> spDC matured in the presence of ES-62 or LPS**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the CD8<sup>+</sup> DC population was purified by high speed FACS sorting. CD8<sup>+</sup> DC were then matured with or without ES-62 (2 µg/ml) on d 1 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, DC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x 10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key.

Significance: \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

IL12p40: \*\* ES-62 vs ES-62 + LPS, \* ES-62 vs LPS

IL12p70: \* ES-62 vs LPS

IL17A: \*\*, \*\*\* ES-62 vs LPS

IL4: \*\*\*, \* ES-62 vs LPS, ES-62 vs ES-62+LPS

IL5: \*\*\*, \*\* ES-62 vs LPS and ES-62 + LPS

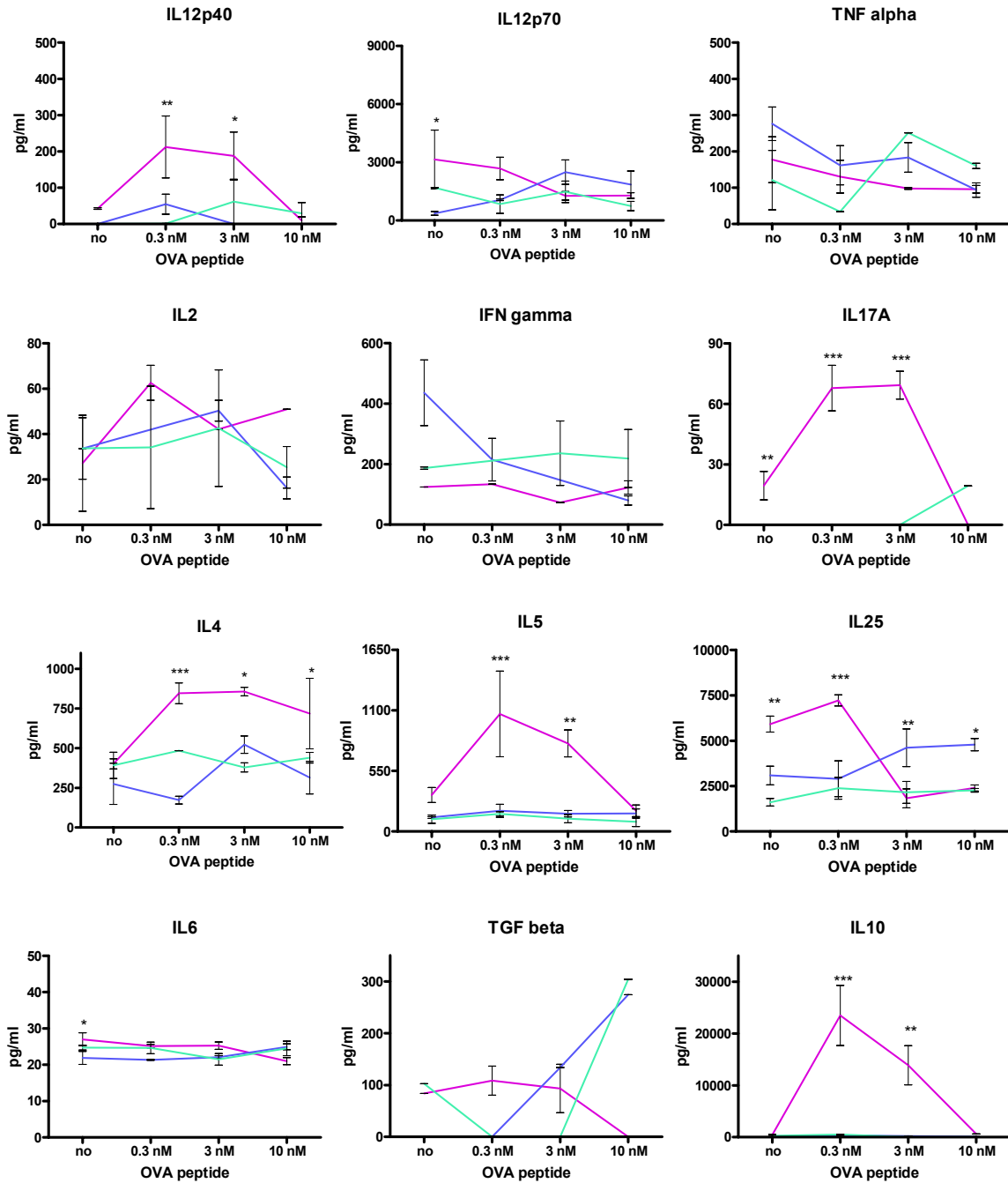
IL25: \*\*, \*\*\* ES-62 vs LPS, \*\*, \* LPS vs ES-62, \*\*\* ES-62 vs ES-62+LPS

IL6: \* ES-62 vs LPS

IL10: \*\*\*, \*\* ES-62 vs LPS and ES-62 + LPS



# CD8<sup>+</sup>spDC + KJ1.26 T cells



ES-62

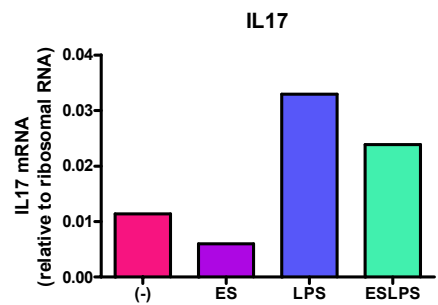
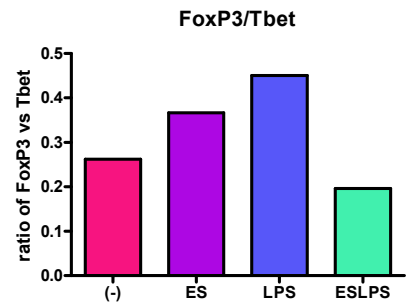
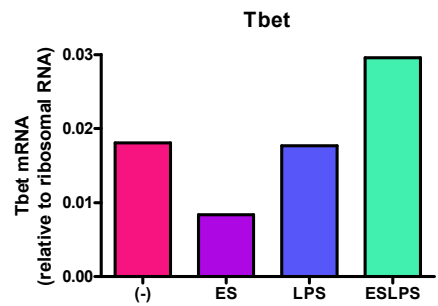
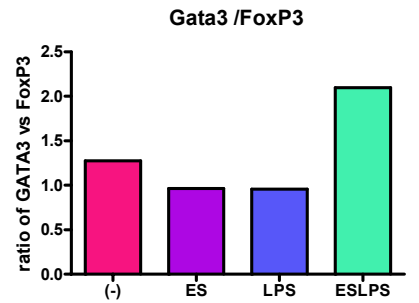
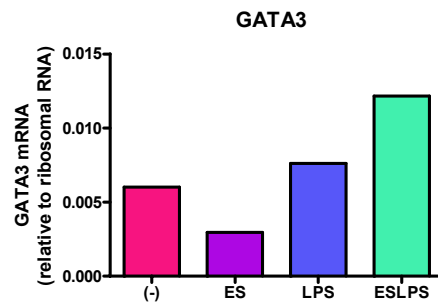
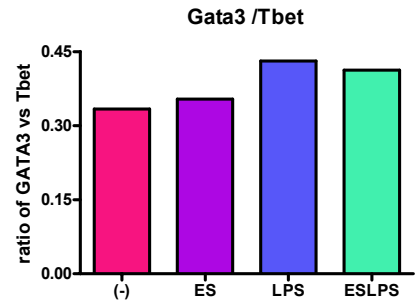
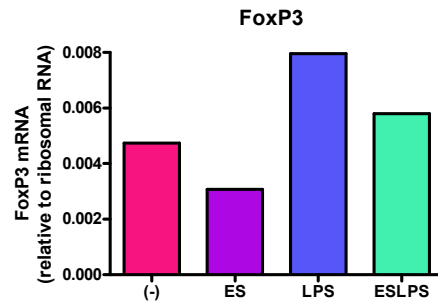
LPS

ES-62 + LPS

**Figure 3.23 Analyses of the expression of GATA3, Tbet and FoxP3 in T cells responding to CD8<sup>+</sup> spDC matured in the presence of ES-62 or LPS**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the CD8<sup>+</sup> DC population was purified by high speed FACS sorting. CD8<sup>+</sup> DC were then matured with or without ES-62 (2 µg/ml) on d 1 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, DC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before RNA was extracted and quantitative PCR for the transcription factors GATA3, Tbet, FoxP3 and IL17A was carried out. Data is presented as the expression levels of the transcription factors and as the ratio of GATA3/Tbet, GATA3/FoxP3 and FoxP3/Tbet and is representative of two independent experiments.

**CD8<sup>+</sup>DC +  
KJ1.26 T cells**



**(-) ES-62 LPS ES-62 + LPS**

**Figure 3.24 Analyses of Ag-specific T cell responses elicited by CD4<sup>+</sup> spDC matured in the presence of ES-62 or LPS**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the CD4<sup>+</sup> DC population was purified by high speed FACS sorting. CD4<sup>+</sup> DC were then matured with or without ES-62 (2 µg/ml) on d 1 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, DC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x 10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key.

Significance: \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

IL12p40: \* LPS vs ES-62 and ES-62+LPS

IL12p70: \* LPS vs ES-62 , \* ES-62 vs ES-62+LPS

TNFα: \* LPS vs ES-62 + LPS, \*,\*\* ES-62 vs ES-62+LPS

IL2: \*\* ES-62 vs LPS and ES-62 + LPS

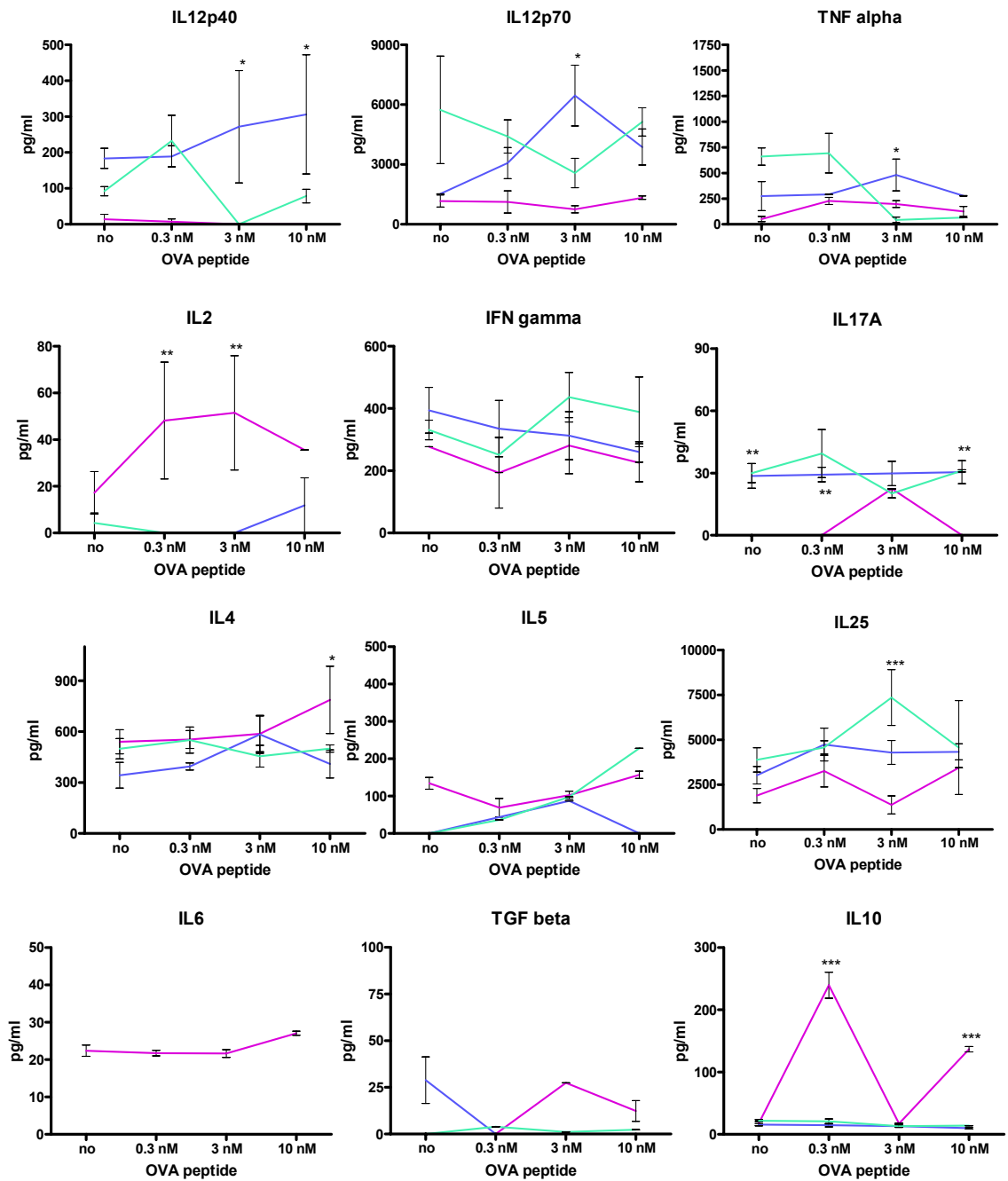
IL17A: \*\* LPS vs ES-62, \*\*,\*\*\* ES-62 vs ES-62+LPS

IL4: \* ES-62 vs LPS

IL25: \*\*\* ES-62 vs ES-62+LPS

IL10: \*\*\* ES-62 vs LPS and ES-62 + LPS

# CD4<sup>+</sup>spDC + KJ1.26 T cells



ES-62

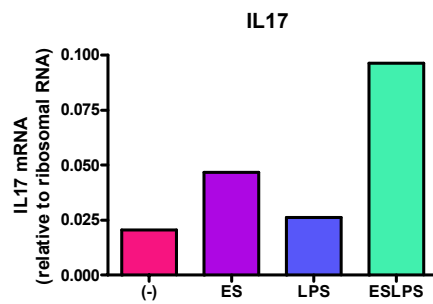
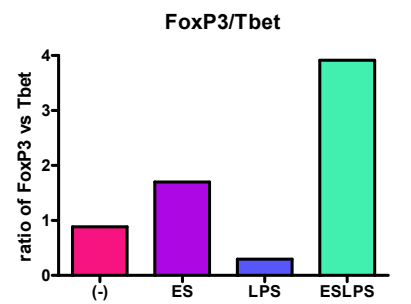
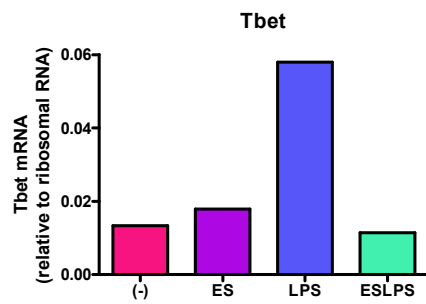
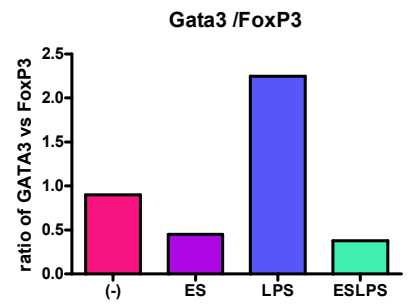
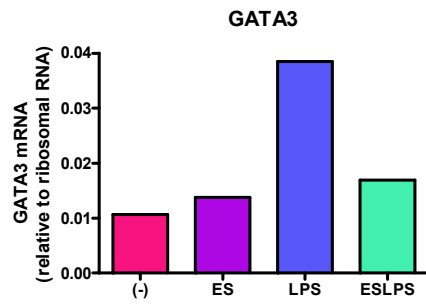
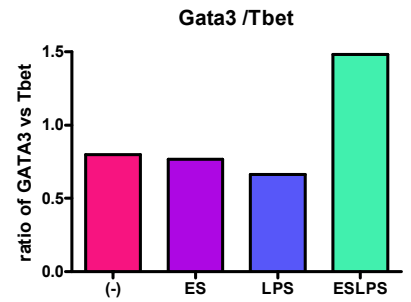
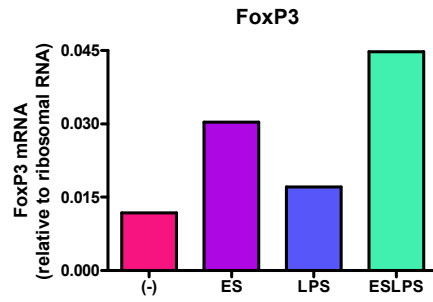
LPS

ES-62 + LPS

**Figure 3.25 Analyses of the expression of GATA3, Tbet and FoxP3 in T cells primed by CD4<sup>+</sup> spDC matured in the presence of ES-62 or LPS**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the CD4<sup>+</sup> DC population was purified by high speed FACS sorting. CD4<sup>+</sup> DC were then matured with or without ES-62 (2 µg/ml) on d 1 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, DC (2 x 10<sup>4</sup> cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) at the indicated concentrations of Ova peptide for 72 h before RNA was extracted and quantitative PCR for the transcription factors GATA3, Tbet, FoxP3 and IL17A was carried out. Data is presented as the expression levels of the transcription factors and as the ratio of GATA3/Tbet, GATA3/FoxP3 and FoxP3/Tbet and is representative of two independent experiments.

**CD4<sup>+</sup>DC +  
KJ1.26 T cells**



(-)
  ES-62
  LPS
  ES-62 + LPS

**Figure 3.26 Analyses of Ag-specific T cell responses elicited by DN spDC matured in the presence of ES-62 or LPS**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the DN DC population was purified by high speed FACS sorting. DN DCs were then matured with or without ES-62 (2 µg/ml) on d 1 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, DC ( $2 \times 10^4$  cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means  $\pm$  SD. The various combinations of DC maturational stimuli are indicated in the key.

Significance: \*  $p < 0.05$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.005$

TNF $\alpha$ : \* ES-62 vs LPS vs LPS

IL17A: \* LPS vs ES-62

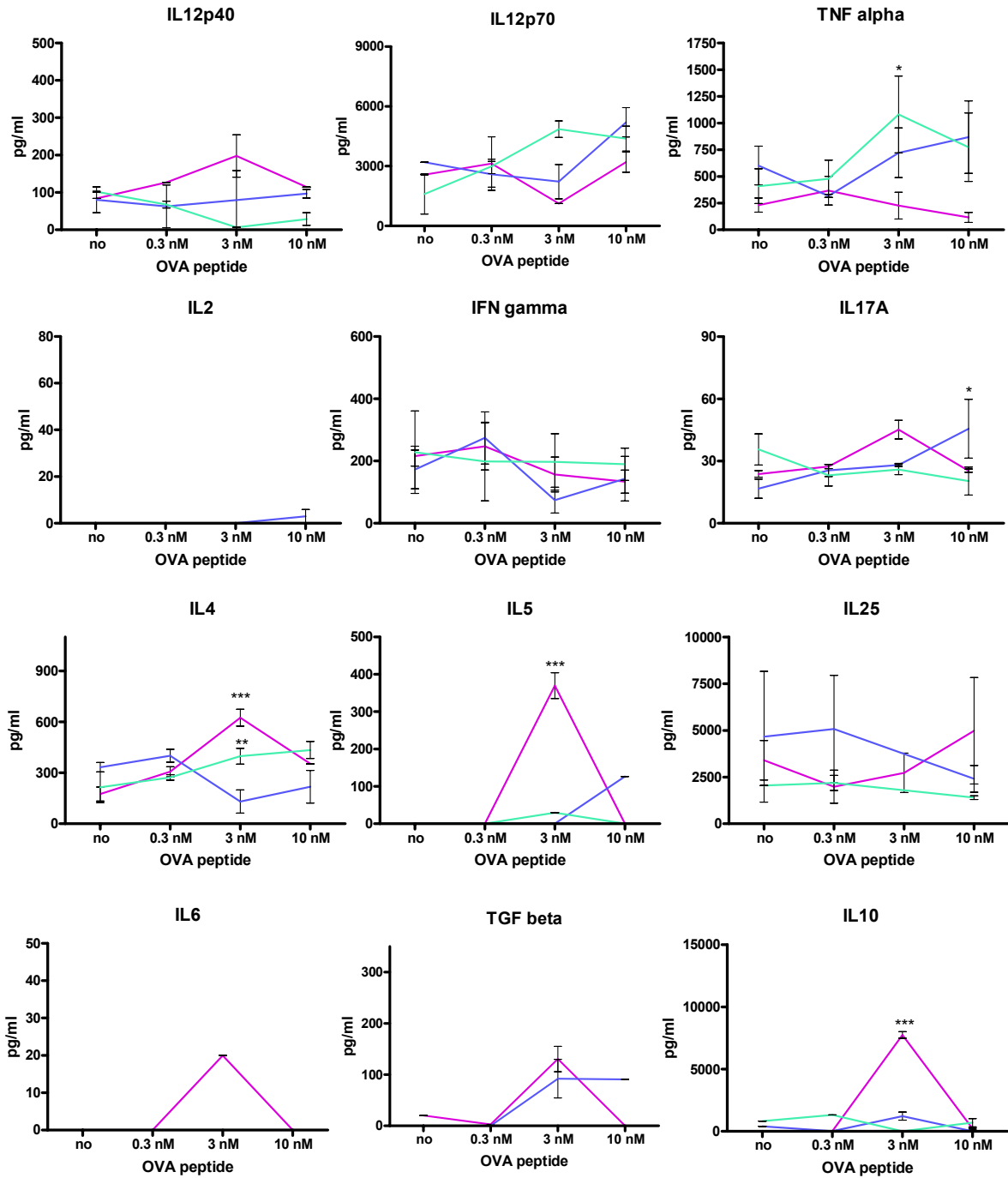
IL4: \*\*\* ES-62 vs LPS, \*\* ES +LPS vs LPS

IL5: \*\*\* ES-62 vs LPS

IL10: \*\*\* ES-62 vs LPS and ES-62 + LPS



## DN spDC + KJ1.26 T cells



ES-62

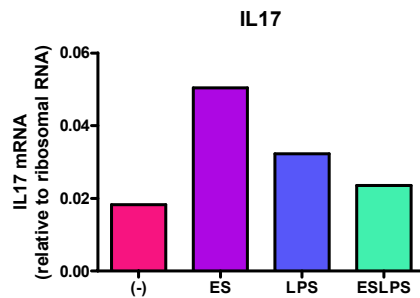
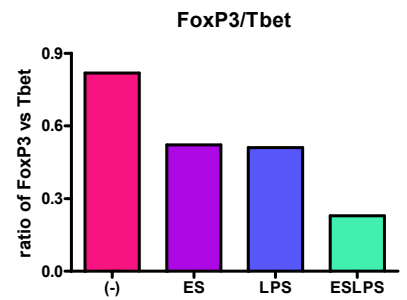
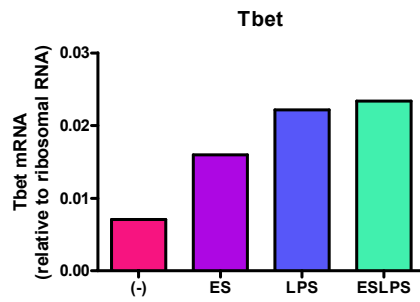
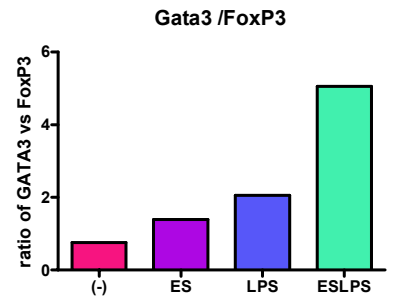
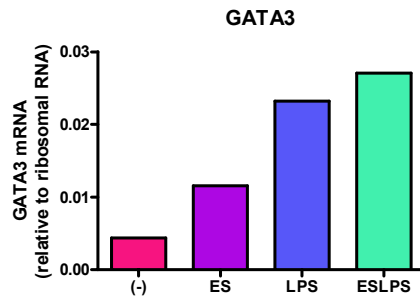
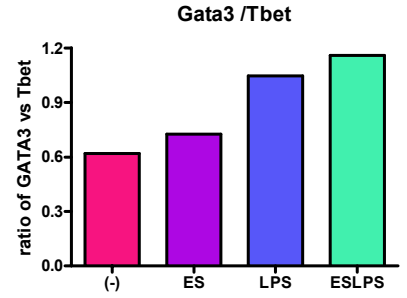
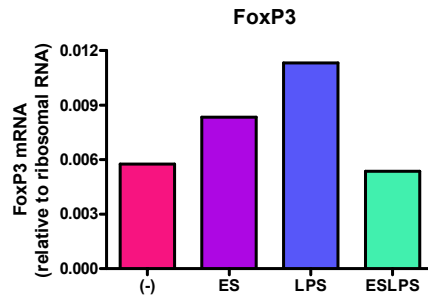
LPS

ES-62 + LPS

**Figure 3.27 Analyses of the expression of GATA3, Tbet and FoxP3 in T cells responding to DN spDC matured in the presence of ES-62 or LPS**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the DN DC population was purified by high speed FACS sorting. DN DCs were then matured with or without ES-62 (2 µg/ml) on d 1 for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, DC ( $2 \times 10^4$  cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) at the indicated concentrations of Ova peptide for 72 h before RNA was extracted and quantitative PCR for the transcription factors GATA3, Tbet, FoxP3 and IL17A was carried out. Data is presented as the expression levels of the transcription factors and as the ratio of GATA3/Tbet, GATA3/FoxP3 and FoxP3/Tbet and is representative of two independent experiments.

DN DC +  
KJ1.26 T cells

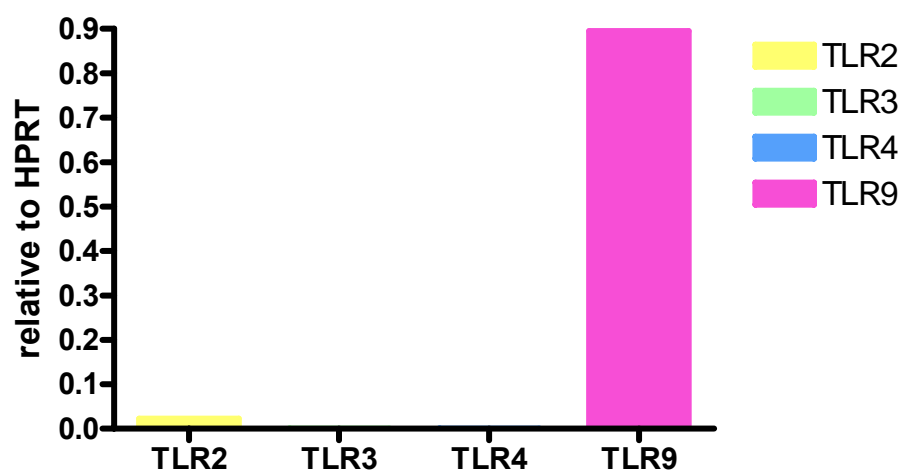


Legend: (-) ES-62 LPS ES-62 + LPS

### **Figure 3.28 TLR expression pattern in pDCs**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the pDC population was purified by high speed FACS sorting. RNA was extracted from these cells and transcribed into cDNA before quantitative PCR for TLR2, TLR3, TLR4 and TLR9 was carried out. The data represents one single experiment.

### TLR expression of pDCs

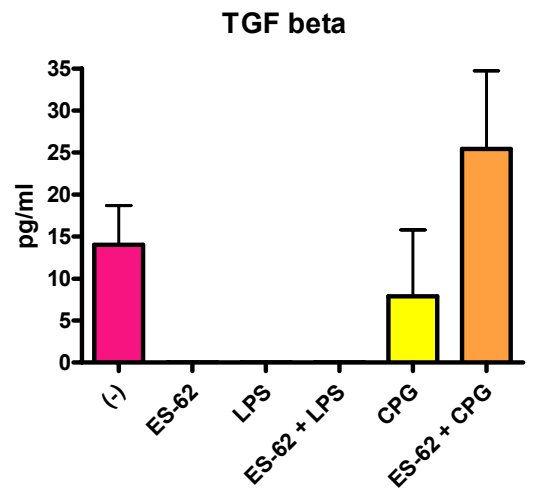
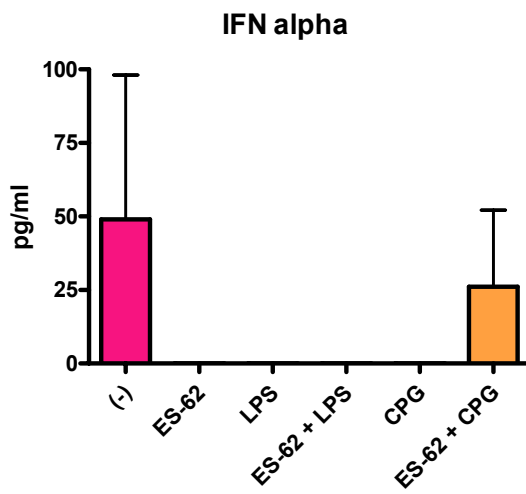
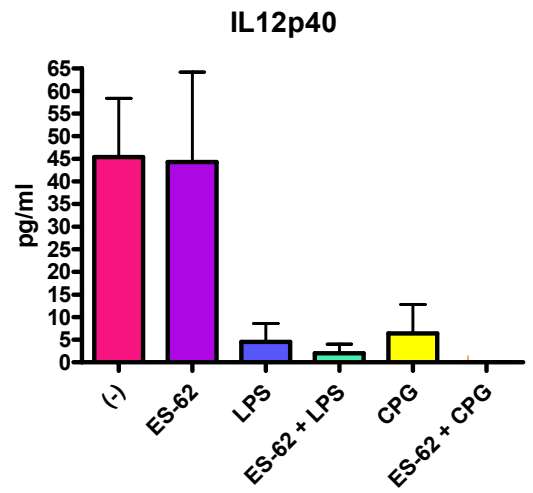
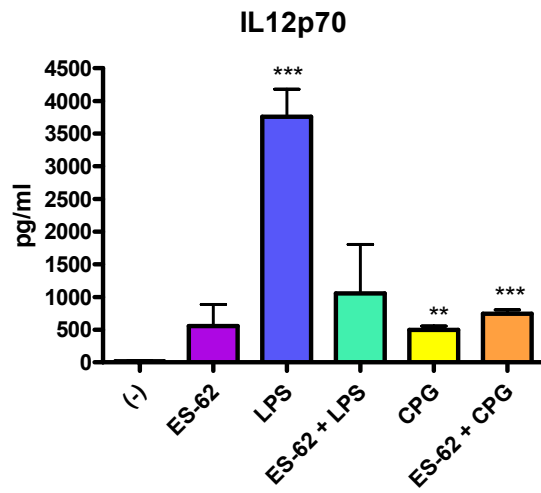


### **Figure 3.29 Effects of ES-62 and/or LPS on spleen pDC cytokine production**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the pDC population was purified by high speed FACS sorting. pDC were cultured and either matured with GM-CSF alone or with ES-62 (2  $\mu$ g/ml) for 24 h and subsequently in the absence or presence of CpG-ODN (40  $\mu$ g/ml) or LPS (1  $\mu$ g/ml) for an additional 24 h on d 2. On d 3 resulting cytokine production in culture supernatants were measured by ELISA. Data are presented as means  $\pm$  SD and are representative of 2 independent experiments.

Significance: IL12p70: \*\*\*  $p < 0.005$  vs all other groups, \*\*\*  $p < 0.005$  CpG vs (-), \*\*  $p < 0.01$  ES-62 + CpG vs (-)

# pDCs



**Figure 3.30 Analyses of Ag-specific T cell responses elicited by spleen pDC matured in the presence of ES-62 or CpG**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the pDC population was purified by high speed FACS sorting. pDC were cultured and either matured with GM-CSF alone or with ES-62 (2 µg/ml) for 24 h and subsequently in the absence or presence of CpG-ODN (40 µg/ml) for an additional 24 h on d 2. On d 3, pDC ( $2 \times 10^4$  cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key.

Significance: \* p<0.05, \*\*p <0.001, \*\*\* p<0.005

IL12p70: \*\*\* ES-62 vs CpG and ES-62 +CpG

TNFα: \* , \*\*\* ES-62 vs CpG and ES-62 +CpG

IFNγ: \*\*\* ES-62 vs CpG and ES-62 +CpG

IFNα: \*\*\* CpG vs ES-62 and ES-62 +CpG, \* , \*\* ES-62 +CpG vs ES-62

IL4: \* , \*\* , \*\*\* CpG and ES-62 +CpG vs ES-62

IL25: \*\*\* ES-62 vs CpG and ES-62 +CpG

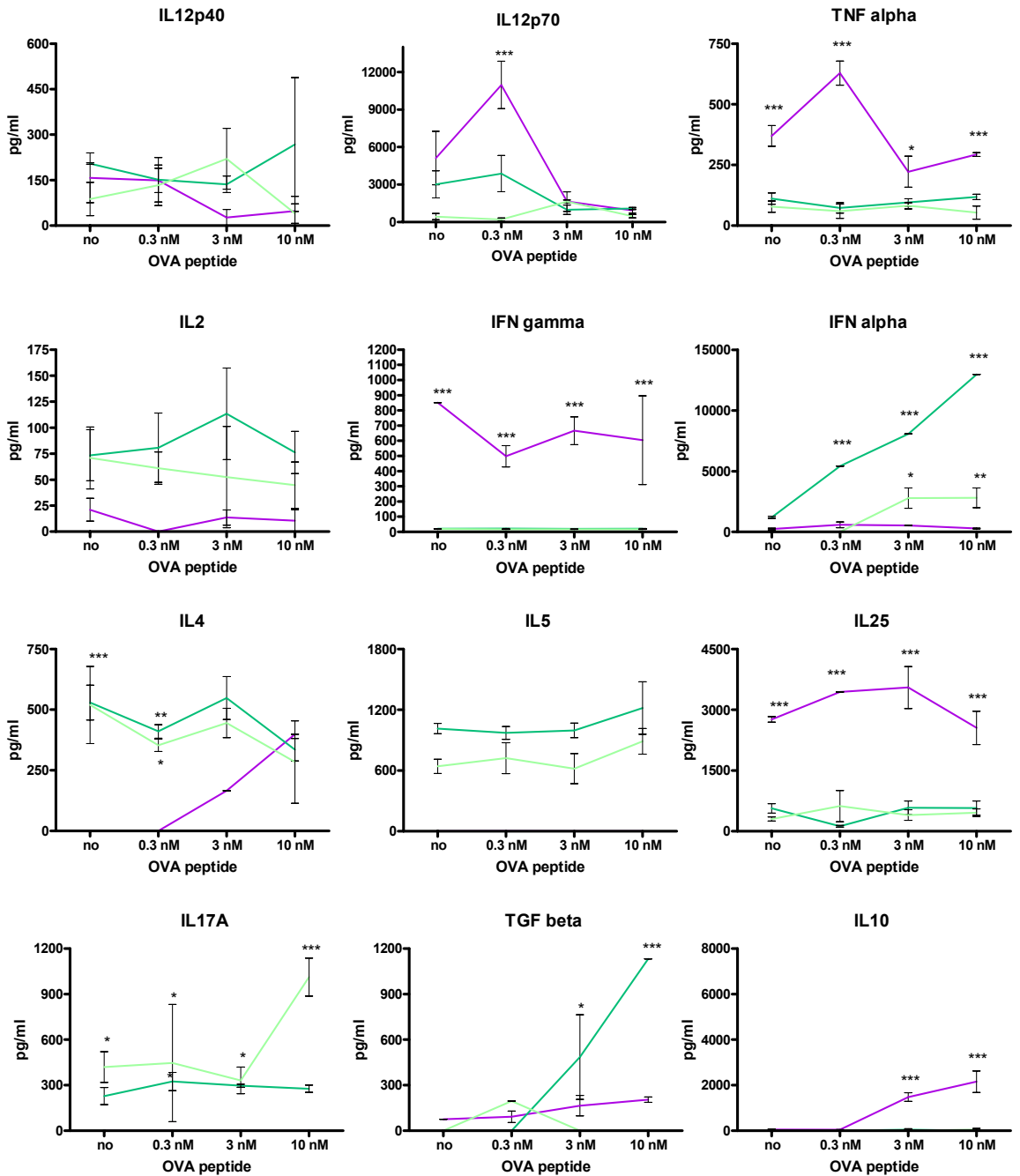
IL17A: \* , \*\*\* CpG vs ES-62, \*\*\* CpG vs ES-62 +CpG

TGFβ: \* , \*\*\* CpG vs ES-62 and ES-62 +CpG

IL10: \*\*\* ES-62 vs CpG and ES-62 +CpG



## pDC + KJ1.26 T cells



— ES-62 — CPG — ES-62 + CPG

**Figure 3.31 Analyses of Ag-specific T cell responses elicited by spleen pDC matured in the presence of ES-62 or LPS**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the pDC population was purified by high speed FACS sorting. pDC were cultured and either matured with GM-CSF alone or with ES-62 (2 µg/ml) for 24 h and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 2. On d 3, pDC ( $2 \times 10^4$  cells/well) were co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) at the indicated concentrations of Ova peptide for 72 h before cytokine production was measured from culture supernatants by ELISA. Data are presented as means  $\pm$  SD. The various combinations of DC maturational stimuli are indicated in the key.

Significance: \*  $p < 0.05$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.005$

IL12p70: \*\*\* ES-62 vs LPS and ES-62 + LPS

TNF $\alpha$ : \* LPS vs ES-62 and ES-62 + LPS. \*\* ES-62 vs LPS

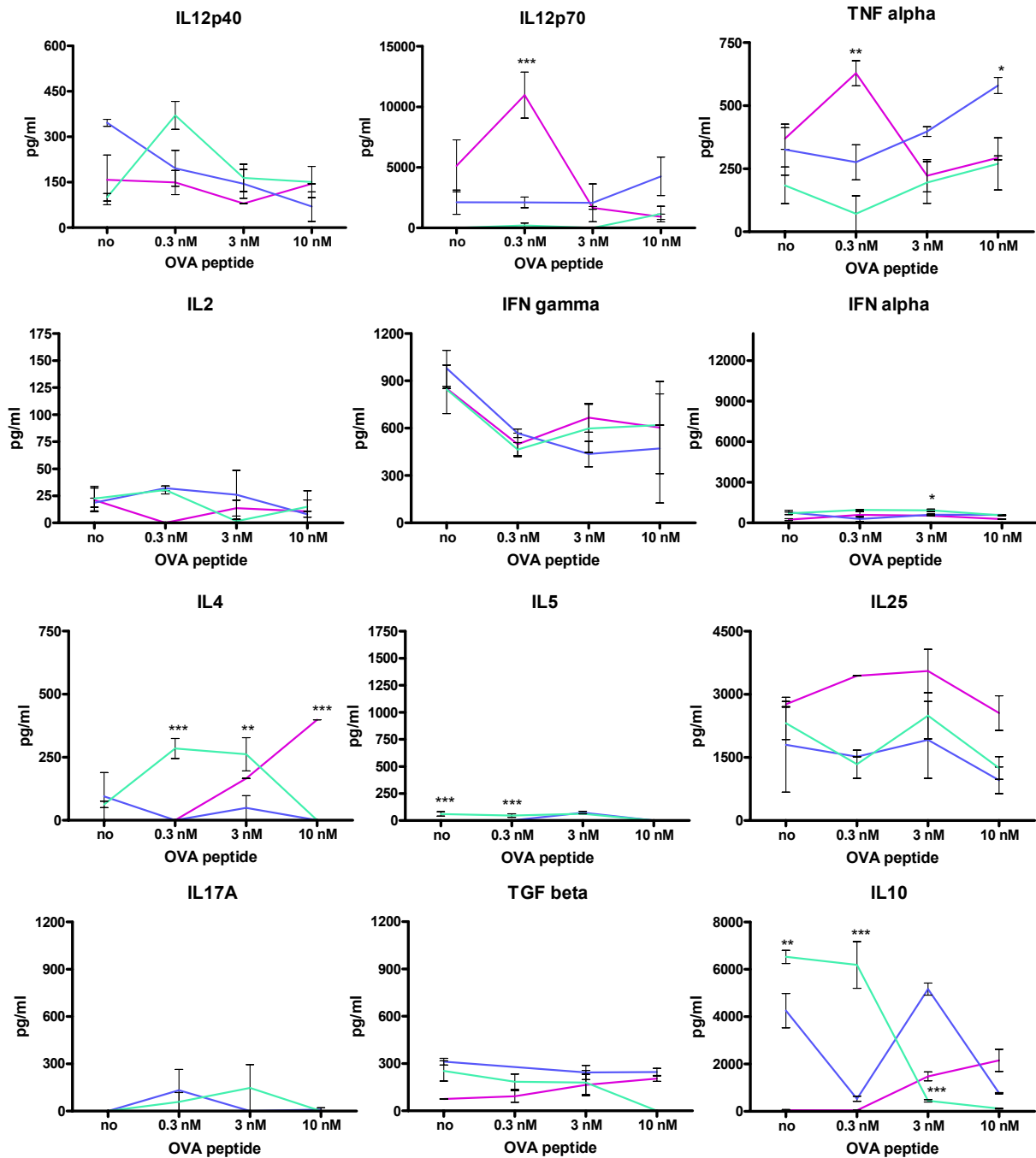
IFN $\alpha$ :\* ES-62 + LPS vs LPS

IL4: \*\*\*, \*\* ES-62 + LPS vs LPS , \*\*\* ES-62 vs LPS

IL5: \*\*\* ES-62 + LPS vs LPS

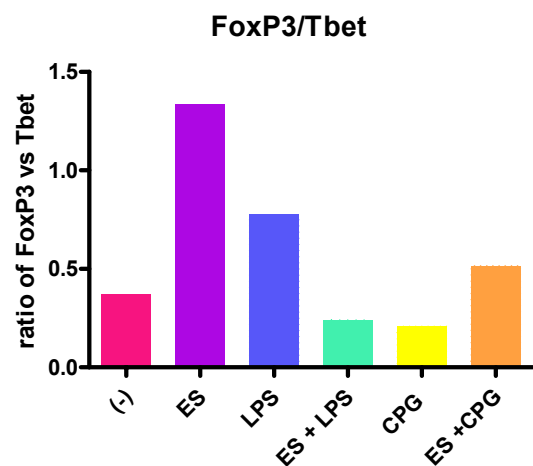
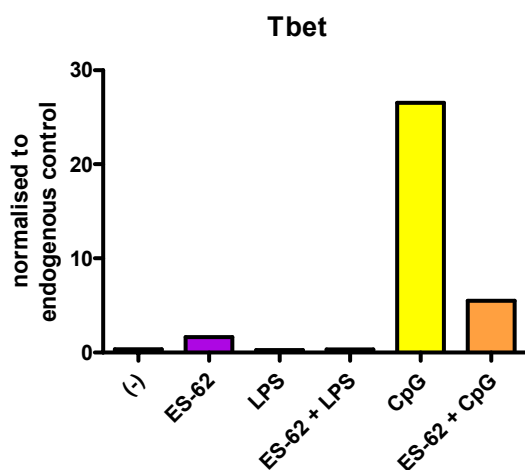
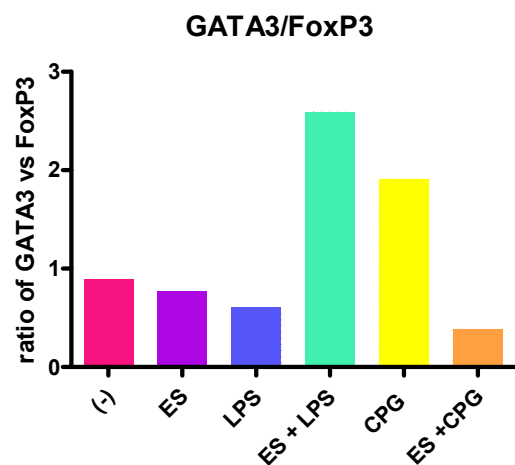
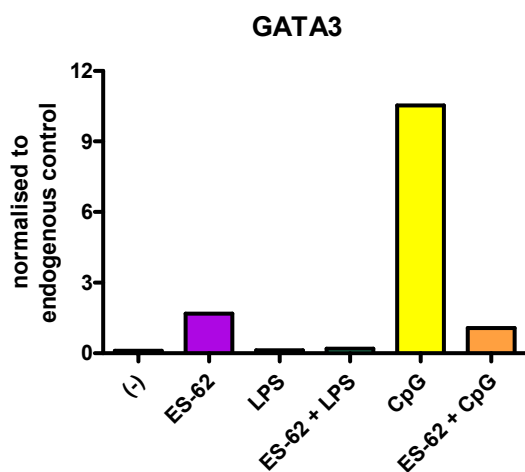
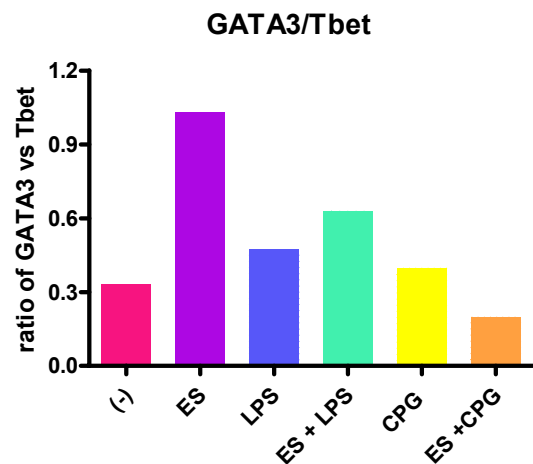
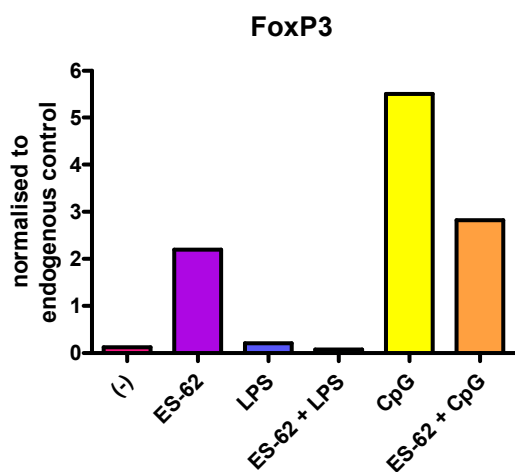
IL10: \*\*, \*\*\* ES-62 + LPS vs LPS, \*\*\* ES-62 vs ES-62+LPS, \*\*\* ES-62 vs LPS

## pDC + KJ1.26 T cells



**Figure 3.32 Analyses of the expression of GATA3, Tbet and FoxP3 in T cells responding to spleen pDC matured in the presence of ES-62, CpG or LPS**

Spleen-derived dendritic cells were stained for individual subtype phenotypic markers and then the pDC population was purified by high speed FACS sorting. pDC were then matured with or without ES-62 (2  $\mu\text{g/ml}$ ) on d 1 for 24 h and subsequently in the absence or presence of CpG-ODN (40  $\mu\text{g/ml}$ ) or LPS (1  $\mu\text{g/ml}$ ) for an additional 24 h on d 2. On d 3, DC ( $2 \times 10^4$  cells/well) were co-cultured with DO.11.10 Tg  $\text{CD4}^+\text{CD62L}^+$  T cells ( $2 \times 10^5$  cells/well) at the indicated concentrations of Ova peptide for 72 h before RNA was extracted and quantitative PCR for the transcription factors GATA3, Tbet and FoxP3 was carried out. Data is presented as the expression levels of the transcription factors and as the ratio of GATA3/Tbet, GATA3/FoxP3 and FoxP3/Tbet and is representative of two independent experiments.



**Table 3.1 Overview of the ability of ES-62, LPS or CpG-treated individual DC subtypes to prime T cells and to promote cytokine production in an Ag-dependent manner**

	CD8 <sup>+</sup> DCs	CD4 <sup>+</sup> DCs	DN DCs	pDCs
<b>ES-62</b>	IL17A ↑ IL4/IL5/IL25 ↑↑ IL10 ↑↑↑ IL12p40 ↑ IFNγ ↔	IL10 ↑↑ TGFβ ↑ IL4/IL5 ↑ IL2 ↑	IL4/IL5 ↑↑ IL10 ↑↑↑ TGFβ ↑ IFNγ ↔	IL4 ↑↑ IL25 ↑↑ IL12p70/TNFα ↑↑ IL10 ↑
<b>LPS</b>	IL12p70 ↑	IL12p40/p70/TNFα ↑	IL12p70/TNFα ↑ IL17A ↑ TGFβ ↑	TNFα ↑
<b>ES-62+LPS</b>	N/D	IL25 ↑↑ IFNγ ↔ IL5 ↑	IL12p70/TNFα ↑ IL4 ↑	IL4 ↑↑
<b>CpG</b>	N/A	N/A	N/A	IFNα ↑↑↑ TGFβ ↑↑↑
<b>ES-62+CpG</b>	N/A	N/A	N/A	IFNα ↑ IL17A ↑↑↑

## **Chapter 4**

**Characterisation of the mechanisms by which ES-62  
mediates its immunomodulatory effects on DCs**



## **4.1 Introduction**

### **4.1.1 Toll-like receptors**

TLRs belong to the family of pattern recognition receptors (PRRs) with homology to *Drosophila* Toll. These receptors are intrinsically suited to recognising pathogen-associated molecular patterns (PAMPs) and signs of endogenous tissue damage. To date, 11 TLRs have been detected on murine DCs [552] and their discovery on APCs and their characterisation have advanced our understanding of the relative specificity of pathogen recognition by the innate immune system. Indeed it has been shown that responses to a diverse range of pathogen products, as well as several host proteins that are associated with stress and cellular damage such as heat shock proteins and components of the extracellular matrix, are mediated through these receptors. During the last few years TLR signalling pathways have been studied extensively and four adapter proteins have been discovered. Among those adapter proteins myeloid differentiation primary-response protein 88 (MyD88) has been studied extensively as it has been found to play a key role in almost all TLR signalling pathways. However, lately, MyD88-independent signalling pathways have also been described.

#### **4.1.1.1 Toll-like receptor-mediated signalling**

TLRs are type I transmembrane proteins that share considerable homology with the IL1 receptor. Both families have a conserved cytoplasmic region of about 200 amino acids referred to as the Toll-interleukin 1 receptor (TIR) domain which contains three highly homologous regions crucial for signalling. Despite the similarity of the cytoplasmic domains the extracellular regions of the TLRs differ markedly. For example, IL1 receptor members have three immunoglobulin-like domains whereas TLRs contain leucine rich repeats. Upon pathogen binding, the TIR dimerises and undergoes conformational changes that allow recruitment of downstream signalling molecules such as the adaptor molecule MyD88, TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing Interferon  $\beta$  (IFN $\beta$ ) (TRIF), and TRIF related adaptor protein (TRAM) (Figure 4.1). All these adaptor molecules contain the TIR domain [552, 553].

#### 4.1.1.1.1 The MyD88-dependent TLR signalling pathway

MyD88 is an important adaptor protein used by all TLRs with the exception of TLR3. It encodes for a carboxy-terminal TIR domain and for an amino-terminal death domain (DD) through which it forms a dimer upon recruitment to the receptor complex [554, 555]. Following signalling via the TIR domain, MyD88 facilitates association with IL1 receptor associated kinase 4 (IRAK4) through a DD interaction which leads to phosphorylation of IRAK4. Subsequently IRAK4 recruits IRAK1 (another kinase of the same family) which autophosphorylates residues in its N-terminus and enables tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) to bind to this complex. The phosphorylated IRAK1 and TRAF6 then dissociate from the receptor to associate with a preformed complex consisting of transforming-growth-factor- $\beta$ -activated-kinase (TAK1), TAK1-binding protein (TAB1) and TAB2 or TAB3 at the plasma membrane. IRAK1 is subsequently degraded and the new complex translocates to the cytosol where TAK1 is activated with the help of ubiquitin-conjugating enzyme 13 (UBC13). This leads to the activation of the inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase (IKK) which phosphorylates I $\kappa$ B leading to its degradation and consequent release of NF- $\kappa$ B. NF- $\kappa$ B activation by this MyD88-dependent pathway results in the expression of inflammatory cytokine genes including TNF $\alpha$ , IL6 and IL1 (reviewed [553]) (Figure 4.1 and 1.1). Consistent with this, studies have shown that MyD88-deficient mice are not capable of producing TNF $\alpha$  or IL6 when exposed to IL1 or microbial products that bind to TLR2, TLR4, TLR5, or TLR9 [556]. It has further been demonstrated that IRAK1-deficient mice display a diminished, albeit not abolished, response to IL1 and LPS and that IRAK4 deficient mice show virtually no response to any bacterial component [557-560]. These results indicate that IRAK4 is required for MyD88 signalling and that it might function upstream of IRAK1. Further research into the signalling pathways of TLR led to the discovery of a second TIR domain containing adaptor protein, TIRAP which, unlike MyD88, does not have a DD [561, 562]. However it was shown that TIRAP is essential for MyD88 signalling through TLR2 and TLR4 as TIRAP-knockout mice show defective inflammatory cytokine production upon stimulation with either TLR2 or TLR4 ligands [562, 563]. Interestingly, TIRAP-deficient mice show a normal response to TLR3, TLR7 and

TLR9 ligands suggesting that TIRAP is only involved in TLR2- and TLR4-mediated MyD88-dependent signalling [563, 564] (Figure 4.2).

The presence of a MyD88-independent TLR signalling pathway was first described for TLR3 [19]. Since then, studies in MyD88-deficient mice have revealed further MyD88-independent pathways used by other TLRs and have led to the identification of two more adaptor proteins involved in those pathways.

#### **4.1.1.1.2 The MyD88-independent TLR signalling pathway**

It has been shown in several studies that TLR mediated signalling in response to LPS can either be mediated by MyD88 and/or transduced independent of this pathway. For example, in macrophages from MyD88-deficient mice TLR2 ligands cannot activate NF- $\kappa$ B whereas upon stimulation with LPS, a TLR4 ligand, activation occurs albeit with delayed kinetics. Analysis of gene expression in such macrophages revealed that LPS stimulation results in activation of the transcription factor IFN regulatory factor 3 (IRF3) in a MyD88-independent manner. Subsequently this leads to the induction of INF $\beta$  and IFN-inducible genes [565, 566]. However, stimulation with TLR2 ligands does not up-regulate these IFN-inducible genes and hence, it is currently believed that TLR2 does not use a MyD88-independent pathway [567] (Figure 4.1 and 4.2). Consistent with this, maturation of DCs as measured by the up-regulation of co-stimulatory molecules such as CD40, CD80 and CD86 and the ability to induce T cell proliferation, has been shown to be possible in a MyD88-dependent and independent manner for TLR4 ligands but only in a MyD88-dependent manner for TLR2 ligands [568]. It has further been shown that with the exception of TLR4, the TLR-mediated induction of inflammatory cytokines appears to be solely controlled in a MyD88-dependent manner [566, 569] and that the TLR4 MyD88-independent pathway induces the production of inflammatory cytokines via the production of INF $\beta$  [570] (Figure 4.1 and 4.2).

Two TIR containing adaptor molecules have been identified that play a role in the TLR4-associated MyD88-independent pathway, namely TRIF and TRAM. TRIF

was initially identified as a TLR3-binding molecule but targeted deletions of TRIF in mice revealed that it also played a role in TLR4 signalling [566, 571] as TRIF-deficient mice showed impaired activation of IRF3 and decreased activation of IFN-inducible genes in response to both TLR3 and TLR4 ligands [566]. Furthermore, it was shown that inflammatory cytokine production following TLR4 stimulation, but not after TLR2, TLR5, TLR7 and TLR9 ligation, was impaired in TRIF-deficient mice indicating that both the MyD88-dependent and the MyD88-independent pathways are required for the full expression of inflammatory cytokines through TLR4 [566, 569] (Figure 4.2).

TRAM, the fourth adaptor protein, has been shown to associate with TRIF and TLR4. Studies in TRAM-deficient mice established that the protein plays an essential role in MyD88-independent signalling through TLR4 as it has been shown that production of inflammatory cytokines in response to TLR4 ligands is impaired in these animals [572-574]. However, even though TLR3 also uses the TRIF-based MyD88-independent signalling pathway, TRAM does not seem to play a role in transducing such TLR3 signals [574].

Stimulation of TLRs consequently leads to the production of inflammatory cytokines. If these cytokines are produced in excess they have been associated with serious systemic disorders and high mortality rates. Hence upon stimulation of TLRs and production of inflammatory cytokines several molecules are activated that negatively regulate TLR signalling and the TLR mediated response. Among these molecules are IRAK-M, suppressor of cytokine signalling 1 (SOCS1), MyD88s, single immunoglobulin IL1R related molecule (SIGIRR) and ST2 [575-580] (Figure 4.3). Although, the molecular mechanisms by which TLRs activate the immune system have been elucidated over the past few years and a rapid progress in our understanding of how TLRs function in the recognition of pathogens has been made, many mechanisms in TLR signalling are yet not fully understood and several questions remain to be answered.

#### 4.1.2 The mannose receptor

The mannose receptor family consists of four receptors, namely the mannose receptor (MR), Dec-205, Endo-180 and the phospholipase A<sub>2</sub> receptor. All of these receptors belong to the group of type I transmembrane receptors with an N-terminal cysteine rich domain, a fibronectin type II domain and, with the exception of Dec-205, a unique series of eight tandemly expressed C-type lectin-like domains (CTLD) [581]. Among these receptors the MR has been studied most extensively and has been implicated in a variety of functions. It has been shown that the MR recognises oligosaccharides terminating in mannose, fucose or N-acetyl glucosamine through its CTLD [582] and efficiently internalises both endogenous and microbe-derived molecules and thus plays an important role in the maintenance of tissue homeostasis [583].

The MR is a pathogen-recognition receptor and has been detected on macrophages [584], inflammatory dermal DCs [585] and *in vitro* generated bone marrow-derived mouse and monocyte-derived human DCs [586], but not on murine lymphoid organ DC populations *in vivo*. It has been reported to bind a variety of pathogens including *Leishmania donovani* [587], *Streptococcus pneumoniae* [588] and *Trypanosoma cruzi* [589]. Ag uptake via the MR in DCs has been shown to mediate delivery of mannosylated Ags to MHCII compartments and results in enhanced presentation to T cells [590, 591]. Furthermore the MR is necessary for cross-presentation of soluble Ags such as OVA [592].

The MR also recognises endogenous molecules including lysosomal hydrolases [593], tissue plasminogen activator [594] and neutrophil-derived myeloperoxidase [595] which are secreted during inflammatory reactions and is thought to play an important role in clearing such molecules. However the MR also binds molecules targeted in autoimmune disease such as thyroglobulin [596], collagen type II [597] and collagen type IV [598] and thus it has been suggested that the MR can play a role in autoimmunity as the uptake of these molecules by DCs through this receptor could lead to inappropriate presentation to the adaptive immune system [599]. Therefore interaction with this receptor or modulation of its downstream

signalling pathways could be one mechanism of ES-62 to mediate its immunomodulatory effects.

#### **4.1.3 Platelet activation factor and its receptor**

Platelet-activating factor (PAF) was originally identified as a pro-inflammatory mediator [600] however, recent work has suggested additional functions in a variety of other settings, such as reproduction and atherosclerosis [601-603]. Structurally, PAF has an intact glycerophospholipid structure containing PC, an acetyl residue and an ether-bonded fatty alcohol all of which contribute to its activity [604]. PAF binds to the PAF receptor which has been found to be expressed in numerous tissues and cells, among them platelets, neutrophils, macrophages, and eosinophils [605]. The PAF receptor belongs to the G-protein coupled receptor (GPCR) superfamily and is composed of seven transmembrane helices. Signalling through this receptor leads to the activation of second messengers (calcium, cyclic AMP, inositol 1,4,5-trisphosphate and diacylglycerol) and induces the activation of kinases, such as MAPK and protein C kinase, and phospholipases, such as phospholipase C $\beta$  (reviewed in [601]).

Several physiological and pathophysiological roles have been found for PAF and its receptor. For example, PAF receptor KO mice have been shown to display drastically reduced systemic anaphylactic responses when actively sensitised and challenged with Ag [606]. Moreover, a study investigating the role of PAF in lung inflammation discovered that PAF receptor KO mice developed significantly milder symptoms than WT mice [607]. PAF has also been shown to play a role in endotoxic shock [608] and is considered to reflect a secondary reaction to endotoxin/LPS which leads to the release of pro-inflammatory cytokines (TNF $\alpha$ , IL6, IL1) and other mediators (PAF). LPS has also been found to directly activate the PAF receptor, however PAF receptor KO mice display the same sensitivity to LPS as WT mice. As macrophages from WT and PAF receptor KO mice are capable of producing the same amounts of pro-inflammatory cytokines, it is believed that PAF is not essential for endotoxic shock but rather might be exacerbating its effects [606].

ES-62 and PAF share some structural homology as both contain PC and PC has been implicated in the binding to PAF receptor (Figure 4.4) [609, 610]. Indeed PC-containing structures, such as cell wall components of Gram-positive bacteria [611], have been reported to enter cells by subverting the PAF receptor and as signalling via the PAF receptor causes various inflammatory responses the PAF receptor may be one of the target receptors for ES-62. Thus it may be possible that ES-62 binds this receptor and interferes with inflammatory responses either by signalling through the receptor or by inhibiting PAF or even LPS binding/signalling.

#### **4.1.4 ES-62 can modulate DC functions through TLR4**

As discussed earlier ES-62 is a PC-containing molecule secreted by the rodent filarial nematode *Acanthocheilenema viteae*. Homologues of the protein can be found in other species of filarial nematodes and hence PC-ES-62 can be considered as a conserved molecule which plays an important role in the parasite's life. Indeed it has been postulated that ES-62 has immunomodulatory functions and is thought to be contributing to promoting the long-lasting survival of the parasite within its host. Consistent with this, in recent *in vivo* and *in vitro* studies ES-62 has been shown to exhibit immunomodulatory properties on cells of the innate immune system such as DCs and macrophages [402, 404, 414, 416-419, 422, 425, 426, 430, 612-616]. Thus ES-62-treated DCs exhibit decreased production of IL12 upon LPS stimulation. Furthermore if in contact with T cells, such ES-62-treated DCs promote Th2 development and prevent the induction of Th1-mediated pathology.

Recent studies conducted in this lab have investigated the possibility that ES-62 mediates its effects via modulation of TLR signalling. Initial studies examined whether exposure to ES-62 altered the LPS mediated stimulation of DCs by interfering with the LPS recognition complex which consists of CD14, TLR4 and MD-2 [403]. It was determined that ES-62 neither down-regulated the expression of components of the LPS receptor complex nor disrupted the interaction between LPS and the LPS-binding protein (LBP). Hence further studies investigating the usage of TLRs and MyD88 by ES-62 were carried out, using bmDCs from TLR4

and MyD88 KO mice which showed that this receptor, as well as the adaptor molecule had important functions in ES-62 signalling [402, 403]. For example, in MyD88 KO mice, ES-62 treatment of DCs resulted in a rather immature phenotype, characterised by dramatically reduced expression of CD40 and the ablation of IL-12p40 and TNF $\alpha$  production [403]. Similarly, results from studies in DCs and macrophages from TLR4 KO mice indicated that ES-62 required the receptor for the production of pro-inflammatory cytokines and co-stimulatory molecule expression [403]. Furthermore it was shown that ES-62 can interfere with signalling through other TLRs via TLR4. To further analyse the role of TLR4 in ES-62 signalling, DCs from C3H/HeJ mice were used. This mouse strain has a point mutation in the TIR domain of TLR4 which prevents its dimerisation and thus is thought to disrupt downstream signal transduction. Surprisingly, it was demonstrated that most of the immunomodulatory functions of ES-62 were intact in these mice [402, 403]. This indicated that ES-62 may need TLR4 to be present but not fully functional to exert its functions, suggesting that either a co-receptor exists through which ES-62 can signal or that ES-62 binding to TLR4 restores the function of the TLR, for example by cross-linking it. In summary, the results of these investigations have demonstrated that ES-62 acts via a TLR4-MyD88-dependent pathway which does not necessarily require TLR4 to be fully functional.



## 4.2 Aims

ES-62 exhibits diverse immunomodulatory properties on cells of the innate immune system, such as DCs and macrophages. Various studies have investigated the role of TLR4 and MyD88 in this context and the results indicated that ES-62 signalling involves both TLR4 and MyD88, however the exact mechanism has not been defined.

Interestingly, the literature suggests that TLR4 KO/MyD88 KO mice polarise towards a Th2 phenotype, a proposal consistent with the finding that LPS drives Th1 responses by signalling through the TLR4 receptor. Thus, although at first it might appear to be surprising that ES-62 can bind TLR4 and use it to drive a Th2 response this may simply reflect the possibility that ES-62 can subvert/rewire TLR4 signalling.

To date, studies investigating the immunomodulatory effects of ES-62 on DCs from TLR4 and MyD88 KO mice have only described changes in the phenotype and cytokine profile of these cells and have not investigated their ability to present Ag and/or activate T cells. DCs play a major role in presenting Ag to T cells and they initiate their differentiation into Th1 or Th2 cells. ES-62 is thought to modulate this priming process possibly by changing the DC phenotype in terms of the activation status or the receptor expression profile of targeted DCs.

Interestingly, as shown in chapter 3 of this thesis ES-62 can have immunomodulating effects in cells that do not express TLR4, such as plasmacytoid dendritic cells (pDC). This finding indicated that ES-62 must subvert alternative receptors to fully exhibit its functions or indeed to modulate cells that naturally do not express TLR4. As recent studies have demonstrated cross-talk between C-type lectin receptors (CLR), such as Dectin-1, and TLRs, the ability of ES-62 to bind the mannose receptor (MR) which belongs to the class of CLRs was tested. Indeed, in these studies it was determined that ES-62 can bind the MR, however no further studies investigating the ability of ES-62 to modulate DC function through this receptor were carried out. Another receptor that was tested was the PAF receptor.

As PAF also contains PC, a component of ES-62 associated with some of its immunomodulatory functions, it was proposed that ES-62 may also bind this receptor, which was confirmed recently (personal communication, Elaine Tuomamen, St. Jude Children's Research Hospital, Memphis, Tennessee). Thus, as ES-62 is able to bind more than one receptor, it may well be possible that it can also mediate its functions through one or more of these receptors, especially if TLR4 is not expressed on the cell surface. Alternatively, one or more of these receptors may act as a co-receptor in ES-62 mediated signalling. Therefore it was decided to further investigate the ability of ES-62 to mediate its DC-driven functions through the mannose and PAF receptor. Specifically, it was decided to extend the initial studies and to investigate whether:

1. ES-62 or LPS-treated DCs from TLR4 and MyD88 KO mice would be able to prime naïve T cells
2. the cytokine profile of the T cells primed by ES-62- (Th2) or LPS- (Th1) matured DCs would change when using DCs derived from either MyD88 or TLR4 KO mice
3. Increasing Ag-load could affect ES-62 mediated polarisation of the response and hence could facilitate a Th1 phenotype regardless of DC maturation
4. the MR acted as a co-receptor for ES-62, initiating crosstalk with TLR4 to transduce ES-62 mediated signalling
5. ES-62 could mediate its immunomodulatory properties through the PAF receptor

## 4.3 Results

### 4.3.1 Are TLR4 and MyD88 required for ES-62-mediated modulation of T cell responses?

Ligation of TLRs on DCs initiates a signalling cascade leading to the activation of NF- $\kappa$ B and hence the production of pro-inflammatory cytokines such as TNF $\alpha$  and IL12. Thus for example in the case of LPS, this cascade involves mainly TLR4 and the adaptor molecule MyD88. As shown previously [402, 403] LPS is not able to induce DC maturation, as characterised by a lack of co-stimulatory molecule up-regulation and impaired pro-inflammatory cytokine production (IL12, TNF $\alpha$ ) in cells from TLR4 (Figure 4.5) or MyD88 (Figure 4.6) KO mice. These defective responses are in turn thought to result in the ablation of Th1 responses in TLR4 KO mice [617]. By contrast, MyD88 KO mice, however, are believed to be able to mount some Th1 responses due to TLR4-dependent, MyD88-independent signalling pathways. ES-62 has also been shown to depend on the TLR4-MyD88 signalling cascade not only for its initial maturation of DCs but also for its inhibition of TLR2 mediated pro-inflammatory signals [402, 403]. However, as it was shown that TLR4 does not need to be functional in this process [402, 403], it was suggested that ES-62 might interact with other, yet unidentified, receptors which either use the same signalling cascade (MyD88) or interfere with it thus blocking e.g. LPS signalling. Consistent with this suggestion is the discovery that ES-62 can have some effects on pDCs (see chapter 3) which do not express TLR4.

It was therefore decided to explore this further by determining if TLR4/MyD88 signalling is required for the ES-62-mediated polarisation of T cell responses towards a Th2 phenotype which has previously been demonstrated by this lab [146]. Thus the effects of ES-62- or LPS-treated bmDCs from either wild type C57BL/6 mice, MyD88- or TLR4-KO mice (genetic background of both KO strains is the C57BL/6 mouse strain) on priming of T cells from OT II mice (OVA-TCR transgenic mice on C57BL/6 background) were compared.

#### **4.3.1.1 The effects of ES-62- or LPS-matured treated bmDCs from TLR4 KO mice on T cell responses**

Consistent with the literature LPS-treated DCs were unable to mount a significant pro-inflammatory or Th1 response by OVA-specific T cells as reflected by the absence of Ag-specific TNF $\alpha$ , IL6, IL12p40 and p70, IFN $\gamma$  and IL2 responses (Figure 4.7). Furthermore, no Th2 cytokines could be detected in the TLR4 KO group whereas WT mice, albeit Ag-independently, appeared to be able to produce some IL4 and IL5 (Figure 4.7). The reason for this might be that whilst the majority of the Ag-specific population are Th1-polarised, some unpolarised T cells do secrete a very small amount of IL4 and IL5 which simply represents their activation rather than their polarisation status. Moreover, whilst IL10 and to a much lesser extent, TGF $\beta$  were produced spontaneously in these cultures, such release was suppressed in an Ag-dependent manner perhaps suggesting that these cytokines were produced by the DCs and DC-T cell contact suppressed this induction (Figure 4.7). Thus the lack of Th1 and Th2 responses might suggest that LPS-DCs from TLR4 KO mice are unable to activate T cells at all.

It has similarly been shown that stimulation of bmDCs from TLR4 KO mice with ES-62 does not result in their maturation as far as surface markers and pro-inflammatory cytokine production are concerned (Figure 4.8). Thus, when analysing the cytokine profile from co-cultures containing ES-62-bmDCs from TLR4 KO mice, it was not at all surprising to discover that these DCs also failed to induce a pro-inflammatory and/or Th1 response. However, ES-62-bmDCs from TLR4 KO mice were also unable to induce a proper Th2 response. Thus, although IL5 levels were quite similar to those found in ES-62-treated WT mice, IL4 production was completely abolished (Figure 4.8). As one of the potential mechanisms through which ES-62 could promote anti-inflammatory effects is the promotion of Tregs, the production of Treg cytokines IL10 and TGF $\beta$  was also analysed. Here ES-62-matured WT DCs were able to prime Ag-specific IL10, but not TGF $\beta$  responses. However, ES-62-treated bmDCs from TLR4 KO mice were unable to induce such IL10 responses in co-cultured T cells and although there

was a hint of an Ag-specific induction of TGF $\beta$  in these cultures, the levels observed were barely detectable (Figure 4.8).

Collectively these data showed that neither LPS nor ES-62-treated bmDCs from TLR4 KO mice were able to induce a Th1 or Th2 response. However, quite surprisingly, it was shown that such LPS-treated DCs could induce production of regulatory cytokines as long as almost no Ag was present. However whether this is due to impurities in the LPS signalling via TLR2 [618] or due to an entirely different receptor and signalling mechanism is as yet unknown.

#### **4.3.1.2 The effects of ES-62- or LPS-treated bmDCs from MyD88 KO mice on T cell responses**

Previous investigations using bmDCs from MyD88 KO mice revealed that these DC could still partially mature in response to stimulation with LPS and ES-62, possibly by using MyD88-independent signalling pathways. This maturation was characterised by a very slight up-regulation of the co-stimulatory molecules CD54, CD80 and CD86 and, to a lesser extent, CD40 (Figure 4.6) however no pro-inflammatory cytokines were produced. Thus it was decided to investigate whether these semi-mature bmDCs would be able to prime and/or polarise T cells.

Reflecting the ability of LPS to exhibit some of its functions in a MyD88-independent manner it has been reported that MyD88 KO mice still have some capacity to induce a Th1 response. Consistent with this, when pro-inflammatory and Th1 cytokines were analysed in cultures in which T cells were primed with LPS-matured MyD88 KO DCs, whilst TNF $\alpha$ , IL6 and IL12p40 production were either abolished or significantly reduced in the MyD88 KO co-cultures (compared to the WT co-cultures), surprisingly an Ag-dependent increase in IL12p70 production could be seen, and at significantly higher levels than those found in WT cultures. As IL12p70 is important for IFN $\gamma$  production and thus involved in Th1 polarisation it was reassuring to find a similar Ag-dependent increase in IFN $\gamma$  levels indicating that LPS-maturation of bmDCs from MyD88 KO mice can indeed lead to Th1 polarisation in T cells (Figure 4.9). To fully assess the ability of these T cells to

produce cytokines, IL4 and IL5 were also analysed, however no production of the two Th2 cytokines could be detected (Figure 4.9). To investigate whether LPS-matured bmDCs from MyD88 KO mice also had the ability to induce Treg-associated cytokine production, TGF $\beta$  and IL10 levels were measured. No significant difference between IL10 levels from WT and MyD88 KO mice could be detected whilst TGF $\beta$  production was increased. However as this increase was Ag-independent it was not clear whether the semi-mature DCs were producing TGF $\beta$  themselves or inducing a higher production of this cytokine by T cells.

To determine whether ES-62 was still able to exhibit its Th2-polarising effects through MyD88 KO bmDCs, supernatants from these cultures were analysed for the levels of pro-inflammatory, Th1 and Th2 cytokines. Similarly, to the LPS-matured MyD88 KO bmDCs, ES-62-matured MyD88 KO DCs did not induce production of the pro-inflammatory cytokines TNF $\alpha$ , IL6 and IL12p40. However, these DCs did recapitulate, albeit at low levels, the enhanced IL12p70 response observed with LPS-matured cells. Nevertheless, IFN $\gamma$  levels were also significantly higher in ES-62-matured MyD88 KO bmDC-T cell cultures at moderate Ag levels whereas at high Ag levels no difference to WT cultures could be found (Figure 4.10). To analyse whether ES-62-matured MyD88 KO mice had retained their ability to polarise T cells towards a Th2 phenotype, IL4 and IL5 production were measured. Interestingly, in contrast to WT cultures, hardly any production of these cytokines could be found until the highest Ag concentrations tested indicating a strong inhibition of the ability of ES-62 to drive a Th2 response in absence of MyD88 signalling (Figure 4.10). Nevertheless these results suggested that ES-62-matured bmDCs can still drive Th2 development in the absence of MyD88 signalling. Finally, as ES-62-matured MyD88 KO bmDCs displayed an even less mature phenotype than their WT counterparts, and as such a phenotype is regarded as driving a Treg response, it was decided to investigate whether these KO bmDCs were able to drive a Treg response. Interestingly, while IL10 levels remained the same as in WT cultures, TGF $\beta$  production, albeit in an Ag-independent manner, was increased (Figure 4.10). This suggests that similarly to LPS-matured bmDCs, either these semi-mature cells promote increased secretion

of this regulatory cytokine from T cells or upon T cell contact start to produce it on their own.

Collectively, the data from these co-cultures revealed MyD88 KO DCs matured with ES-62 and LPS still retained some ability to differentially polarise T cells. Interestingly, although, ES-62 appears to have partially lost its ability to drive a Th2 response, it retained its ability to drive IL10 production. As the ES-62-treated co-cultures also showed some Th1 cytokine production (IFN $\gamma$ ) it is possible that anti-Th1 actions of ES-62 reflect modulation of DC function through MyD88 dependent signalling, thus resulting in slightly increased levels of Th1 cytokines in co-cultures primed by ES-62 exposed MyD88 KO DCs.

#### **4.3.2 The effects of ES-62 and LPS treatment on bmDCs derived from mannose receptor KO mice**

Investigations of cells from TLR4 mutant and KO mice have shown that the presence of TLR4, but not its ability to function, is required for ES-62 to mediate most of its effects on bmDCs. Thus it has been speculated that one or more co-receptors might be involved in ES-62 signalling and as the MR has recently been shown to bind ES-62 (personal communication, Siamon Gordon, University of Oxford) it was decided to investigate whether this receptor was indeed involved in ES-62 mediated immunomodulation of bmDC responses.

Analyses of cytokine release from ES-62- and LPS-treated MR KO bmDC showed very few differences to those obtained with WT bmDCs. Thus, LPS induced significantly increased production of IL12p40, TNF $\alpha$  and IL6 whereas little or no production of these cytokines was found in ES-62 cultures (Figure 4.11). IL6 and IL12p40 production was found to be reduced in untreated and ES-62-treated MR KO cultures compared to WT cultures whereas hardly any difference could be seen upon LPS stimulation. In case of TNF $\alpha$  the reverse was true as LPS-treated MR KO cultures showed reduced production of this cytokine compared to WT cultures (Figure 4.11). Furthermore with the exception of IL12p40 levels in WT cultures, ES-62 pre-treatment rather surprisingly did not result in a reduced production of pro-

inflammatory cytokines in these experiments. IL10 production was also analysed and, in general, revealed lower levels in the MR KO relative to WT cultures. Nevertheless, it was clear that maturation with ES-62 induced higher levels of IL10 than maturation with LPS and that pre-exposure to ES-62 enhanced the LPS response in both WT and KO cultures (Figure 4.11).

Thus collectively very few changes could be seen in these cultures indicating that the MR might not be crucial for ES-62 signalling although the inhibition of LPS-induced IL12p40 production appeared to be abolished in the MR KO cultures. However, as ES-62 binds this receptor it cannot be ruled out that the parasite protein uses this receptor either to mediate some functions not analysed here or possibly as a default receptor if other vital receptors such as TLR4 are unavailable.

#### **4.3.3 The effects of ES-62 and LPS treatment on bmDCs from PAF receptor KO mice**

Platelet activating factor and ES-62 share some structural similarity as both contain PC and therefore it has been speculated that ES-62 could signal through the PAF receptor. Indeed, recent studies carried out in the lab of Elaine Tuomamäki (St. Jude Children's Research Hospital, Memphis, Tennessee) showed that ES-62, via its PC moiety, was able to bind this receptor on rat brain capillary endothelial cells (rBCEC<sub>6</sub>) (Figure 4.12) and primary neurons from mouse embryos (Figure 4.12) thus revealing a new possibility for an alternative receptor or co-receptor for ES-62-mediated immunomodulation. Therefore it was decided to further investigate whether ES-62 was depending on this receptor to mediate its immunomodulatory actions by studying the effects of ES-62 on bmDCs derived from PAF receptor KO mice.

As with the MR KO cells, initial analyses did not reveal many differences between WT and PAF receptor KO bmDCs. In both cases, ES-62 was able to slightly induce the production of IL6, TNF $\alpha$  and IL12p40, whereas levels of IL12p70 were decreased in both, WT and PAF KO bmDCs, indicating that ES-62 did not induce production of active IL12 (Figure 4.13). Consistent with previous data, LPS



maturation of WT bmDCs induced a very high production of these pro-inflammatory cytokines, however this increase was only seen for IL6 and TNF $\alpha$  in PAF KO mice. By contrast, and rather surprisingly, IL12p40 and IL12p70 did not show any significant increase upon LPS exposure in PAF KO bmDCs which may indicate that PAF signalling can be important for some of the LPS mediated effects. In agreement with this, increased levels of IL12p70 were found in pDCs which do not express TLR4 (see chapter 3) indicating that this cytokine can be produced by signalling through a different receptor, possibly the PAF receptor (Figure 4.13). Analyses of IL10 production also revealed a similar pattern between WT and PAF KO cultures, although in general, higher levels of this cytokine were found in cells from the PAF KO mice. To determine whether ES-62 could prevent production of pro-inflammatory cytokines and enhance production of IL10, cultures were pre-exposed to the parasite product before maturing them with LPS. Interestingly while levels of IL6 and IL12p70 decreased in WT bmDCs, IL12p70 levels remained almost the same in PAF KO bmDC cultures (Figure 4.13). However as LPS did not induce a significant increase in production of this cytokine in PAF-receptor KO DCs, no inhibition by ES-62 was expected. TNF $\alpha$  production upon such pre-exposure to ES-62 was unaffected whereas IL6 production was entirely abolished in both cultures indicating that the production and ES-62 mediated inhibition of these two cytokines did not depend on signalling through the PAF receptor. Interestingly analysis of IL10 production revealed that in contrast to WT bmDCs, ES-62 pre-exposure of PAF KO bmDCs did not result in increased production of this cytokine.

#### **4.3.4 The effects of ES-62- or LPS-treated bmDCs from PAF receptor KO mice on T cell responses**

Although analysis of cytokine production in response to ES-62 and LPS stimulation only revealed very few differences between WT and PAF receptor KO bmDC cultures, as PAF and ES-62 both express PC, it was decided to further investigate whether ES-62-matured bmDCs from PAF KO mice and from WT mice differentially modulated T cell polarisation. Furthermore, as some LPS mediated effects, such as IL12p40 and p70 production, were inhibited in PAF receptor KO

bmDCs, it was also decided to further investigate whether LPS-matured PAF receptor KO DCs could still drive a Th1 response in these cultures and if so, whether pre-exposure to ES-62 could modulate this response. Interestingly, and also quite surprisingly, one of the first discoveries was that co-cultures containing WT bmDC showed a slightly different cytokine production profile than that seen in previous cultures. One explanation for this could be that both WT mice and their KO littermates came from a different research facility and were exposed to a slightly different environment. In addition, and in contrast to previous experiments, bmDCs were not developed directly after harvest of bm but rather the progenitor cells were frozen for transport and stored in liquid nitrogen until use. Thus it is possible that due to slightly different handling procedures of the animals and due to the storage of the cells bmDCs behaved differently in culture.

Analyses of pro-inflammatory cytokine production revealed that co-cultures containing ES-62-matured PAF receptor KO bmDCs, but not WT bmDCs, produced highest levels of TNF $\alpha$ , IL6, IL12p40 and IL12p70 if no Ag was added to the cultures but that production of these cytokines was strongly decreased even at low Ag concentrations (Figure 4.14). By contrast, LPS maturation of PAF receptor KO DCs resulted in a similar pro-inflammatory cytokine production pattern to that found in WT co-cultures. Furthermore the effects observed in ES-62-matured DC co-cultures were lost if the ES-62-treated bmDCs were subsequently matured with LPS (Figure 4.14). Interestingly, no significant differences could be detected between the Th1 profile found in PAF receptor KO and WT co-cultures and ES-62-pre-treatment of bmDCs from both WT and KO cultures did not alter LPS-mediated effects. However, it should be noted that in these experiments, neither WT nor PAF receptor KO DCs matured with either LPS or ES-62 were effective at driving Th1 responses.

To investigate whether ES-62 still had the ability to drive a Th2 response via PAF receptor KO DCs, IL4, IL5 and IL17E (IL25) production were tested. In WT co-cultures, ES-62-matured bmDCs appeared to be able to drive Ag-independent production of Th2 cytokines at low levels that was lost at high Ag concentrations (Figure 4.15). By contrast, priming by ES-62-treated PAF receptor KO bmDCs was

not subject to this Ag-dependent desensitisation as no decrease of IL4, IL5 and IL17E could be found. Furthermore, LPS maturation of bmDC from PAF receptor KO, but not WT, mice also resulted in Ag-independent production of these three Th2 cytokines. Interestingly, pre-exposure to ES-62 and subsequent maturation with LPS of PAF receptor KO bmDCs induced an Ag-dependent decrease of all three cytokines in WT and of IL5 and IL25 in KO DC co-cultures. As these results were quite puzzling it was decided to assess the levels of cytokines associated with Tregs in these cultures. Similarly to the Th2 cytokine profile, Treg cytokine production also revealed differences between PAF receptor KO and WT co-cultures. Firstly while no TGF $\beta$  was produced in LPS-matured WT bmDC-T cell cultures, levels in PAF KO co-cultures were increased strongly in an Ag-dependent manner. Secondly, in contrast to ES-62-matured WT DC co-cultures where low TGF $\beta$  and IL10 levels were decreased in an Ag-dependent manner, PAF receptor KO bmDC-T cell co-cultures revealed a strong Ag-dependent increase in production (Figure 4.15). Interestingly, ES-62 pre-exposure and subsequent LPS maturation did not result in a further increase in the Treg-promoting cytokines in PAF receptor KO cultures.

Collectively, it seems that both ES-62 and LPS signalling is affected in bmDC-derived from PAF receptor KO mice. T cells activated by ES-62-treated PAF receptor KO bmDCs appear to be unable to induce an inflammatory response even at very high Ag concentrations but can still promote a Th2/Treg phenotype. Surprisingly, the same can be said for T cells co-cultured with LPS-matured DCs which probably indicates that (secondary) signalling through the PAF receptor contributes to an LPS response. Interestingly, ES-62 pre-treated LPS-matured DCs mostly responded like WT DCs indicating that although the PAF receptor seems to be important for ES-62 signalling it is not crucial for the parasite product to exhibit all of its immunomodulatory effects.

## **4.4 Discussion**

### **4.4.1 TLR4 plays a role in ES-62–modulation of bmDC-driven priming and polarisation of T cell effector responses**

It has been shown in previous studies [402, 403] that expression of TLR4 is necessary for induction of bmDC maturation upon ES-62 and LPS exposure. Furthermore it has also been shown that, in the case of ES-62, TLR4 does not need to be functional for this process [402, 403]. ES-62 either acts to restore the receptors function perhaps for example by physically crosslinking and hence dimerising TLR4, or alternatively simply uses TLR4 to attach to the cell and signals through a different pathway, possibly by using a second co-receptor. However as shown in this thesis, some immune system cells, such as pDCs, do not express TLR4, but seem to be responsive to ES-62, suggesting that ES-62 can mediate at least some of its effects through an, as yet, unidentified receptor, independently of TLR4.

Nevertheless, and perhaps consistent with the proposal that ES-62 subverts TLR4 signalling, it is believed that TLR4 KO mice are predisposed to Th2 responses. Indeed a recent study of the effect of very low dose LPS treatment in short and long term models of asthma showed that whilst LPS attenuated the allergic response in WT mice, TLR4 KO mice showed an exacerbation of disease characterised by significantly increased Th2 cytokine production and an increase in the number of eosinophils [619]. Thus, this data provide support for the idea that ES-62-mediated immunomodulation could reflect interference with TLR4 signalling. Furthermore as TLR4 KO are already predisposed to promoting Th2 responses, they may suggest that ES-62 might be able to increase this response, possibly by using a different receptor or co-receptor. Interestingly however, the data gathered from co-cultures primed by ES-62-matured bmDCs from TLR4 KO mice revealed that these bm DCs were unable to polarise T cells (Figure 4.7 and 4.8). Thus it appears that whilst ES-62 does not need a functional TLR4 receptor to exert its functions, it might either require certain structures on TLR4 to bind or signalling pathways downstream of TLR4 which were inaccessible in the TLR4 KO bmDCs. Furthermore ES-62 might require the presence of a specific other receptor

or co-receptor in the absence of TLR4 and it is possible that such a receptor or co-receptor is not expressed on cells normally expressing TLR4 or perhaps was “knocked out” with TLR4, respectively.

As expected, LPS maturation of these bmDCs did not result in Th1 polarised T cells as usually seen in WT bmDC-T cell cultures. Interestingly these LPS-matured DCs still caused TGF $\beta$  and IL10 production possibly by driving some late maturation of these DCs through TLR2 resulting in semi-mature DCs which have been implicated in the production of Tregs and/or tolerised/anti-inflammatory responses.

Thus in summary the data from these co-cultures indicate that, at least in bmDCs, the presence of TLR4 is necessary for ES-62 to mediate its functions. However, it cannot be ruled out that DCs, or other cells derived from different origins, express receptors which ES-62 can bind to and through which it can signal.

#### **4.4.2 ES-62 mediates its anti-inflammatory and/or pro-Th2 effects by signalling through MyD88**

ES-62 and LPS can both induce partial maturation of bmDCs from MyD88 KO mice (Figure 4.5). In the case of LPS, it is believed that such signalling is a consequence of the “MyD88 independent pathway” [620] which although it might result in less pro-inflammatory cytokine production, can still induce Th1 polarisation of T cells. Whether the ES-62-induced modulation of bmDC maturation required to initiate the ES-62-associated anti-inflammatory response is dependent on MyD88 signalling has not been investigated previously. Thus, it was very interesting to find that whilst the production of Th2 cytokines required higher concentrations of Ag, IFN $\gamma$  production was significantly increased when ES-62-matured MyD88 KO bmDCs were used to prime T cells. However, similarly to co-cultures containing ES-62-treated WT DCs, production of pro-inflammatory cytokines was inhibited and thus it is possible that ES-62 mediates the inhibition of pro-inflammatory cytokines independently of the induction of Th2 response. In addition it is possible that the increased production of the Th1 cytokine IFN $\gamma$  is due to the inhibition of Th2

polarisation (Figure 4.10). Consistent with the idea that ES-62 uses different signalling mechanisms and/or receptors for different immunomodulatory functions, the levels of both cytokines (IL10, TGF $\beta$ ) associated with the polarisation towards Treg responses were unaffected, indicating that the DC phenotype required for their induction was MyD88-independent. Collectively, therefore, it appears that ES-62 acts to alter Th1 and Th2 responses by inducing a DC phenotype resulting from signalling through MyD88 whereas the regulatory functions associated with the parasite product might be regulated in a different manner. Whether this latter process is mediated via a different signalling pathway downstream of TLR4, or through an entirely different receptor and signalling pathway, has not yet been determined.

#### **4.4.3 Could the mannose receptor be a co-receptor for ES-62?**

The mannose receptor (MR) belongs to the family of C-type lectin receptors that mediates endocytosis in DCs. In the mouse, the MR has been shown to be expressed in immature peripheral DCs but not in DCs found in lymphoid organs [621]. As it binds to ligands containing mannose, fructose, N-acetylglucosamine and glucose, and as some of these structures are found in ES-62, it was hypothesised that ES-62 could bind and possibly mediate its functions through this receptor. Indeed, the MR has recently been shown to bind ES-62 (personal communication, Siamon Gordon, University of Oxford) and thus its potential as a receptor for ES-62 on bmDCs was investigated. However the data obtained from these studies did not reveal any striking differences between MR KO bmDCs and WT DCs with regard to their response to ES-62, indicating that, at least in bmDCs, this receptor was not crucial for ES-62 mediated DC maturation (Figure 4.11). However, as ES-62 binds this receptor it might still be possible that the parasite protein mediates some as yet untested T cell polarising functions through the MR. Furthermore, it is possible that although ES-62 can signal through this receptor, it may be redundant when other receptors, such as TLR4, are expressed on the cell surface.

#### **4.4.4 ES-62 and LPS mediate some of their immunomodulatory effects through the PAF receptor**

Another receptor which has recently been shown to bind ES-62 is the PAF receptor, an interesting finding given that both PAF and ES-62 contain PC. Furthermore, a recent study determined that PC-containing Gram-positive bacterial cell walls could enter endothelial cells, cardiomyocytes and neurons via the PAF receptor [611]. Thus it was hypothesised that PAF receptor could also be a receptor used by ES-62 to enter the cells and to mediate its functions. This hypothesis has recently been confirmed using WT and PAF receptor KO rat brain capillary endothelial cells (rBCEC<sub>6</sub>) and primary neurons from mouse embryos (personal communication, Elaine Tuomamen, St. Jude Children's Research Hospital, Memphis, Tennessee). In these studies it was not only shown that ES-62 could bind the receptor and be internalized, it was also shown using PC-free-ES-62 that the PC moiety was of crucial importance for this process. Interestingly, although not all of the ES-62 co-localised with the PAF-receptor on primary neurons from mouse embryos, most did, indicating that ES-62's interaction with the neurons was largely via the PAF receptor (Figure 4.12). Thus these data suggested that, in cells which do not express TLR4, the PAF receptor could be an alternative receptor for ES-62.

Initial studies with PAF-receptor KO bmDCs did not reveal major differences to WT bmDCs, with regard to their maturation phenotype following exposure to ES-62 (Figure 4.13). However as these cells express a functional TLR4 receptor, which might be responsible for the cytokine production analysed, these data only confirmed that PAF receptor expression was not crucial for ES-62-mediated DC maturation. Interestingly and quite surprisingly, when responses to LPS-induced maturation of PAF receptor KO DCs were tested, a slight inhibition in the production of IL12p40 and p70 could be seen indicating that the PAF receptor might be important for some of the LPS mediated pro-inflammatory effects and that LPS signalling via this receptor, either directly or indirectly, has agonistic effects on pro-inflammatory cytokine production. Consistent with this, PAF has been shown to be released by cells in response to LPS and such release is known to cause

systemic effects (endotoxic shock) *in vivo* by acting synergistically with other pro-inflammatory cytokines [622]. Thus a lack of PAF receptor would prevent these synergistic effects and might explain the slight decrease in pro-inflammatory cytokine production observed (Figure 4.16).

To further determine whether PAF receptor-mediated signalling had an effect on effector T cell polarisation, PAF receptor KO bmDCs matured with ES-62 or LPS were tested in the co-culture system. Although the WT data obtained from these co-cultures was not ideal, these studies revealed that ES-62 does not depend on PAF receptor expression to drive Th2 polarisation. Indeed, as the production of Th2 cytokines was not decreased at high Ag concentrations, whereas all pro-inflammatory cytokines were, it could be argued that the Th2/anti-inflammatory effects of ES-62 were if anything rather enhanced when T cells were primed by ES-62-matured PAF receptor KO bmDCs (Figure 4.14 and 4.15). This could indicate that ES-62 might bind this receptor to block its pro-inflammatory effects (Figure 4.17). Interestingly, and perhaps consistent with this, PAF receptor KO bmDCs matured by LPS were unable to mount a proper Th1/pro-inflammatory response but instead displayed Ag-independent production of Th2 cytokines. LPS has been shown to directly activate the PAF receptor [623, 624] and thus could perhaps amplify its Th1 effects by signalling through this receptor; however LPS signalling through TLR4 has also been shown to induce PAF production which in turn binds the PAF receptor and initiates a signalling cascade promoting inflammation. Indeed PAF is produced in response to LPS in various cells and organs [622] and thus interfering with this receptor would be an ideal mechanism for ES-62 to inhibit some pro-inflammatory effects and drive development of Th2 cells (Figure 4.18).

However one of the problems with the above discussed experiments was that bmDCs express TLR4 which is used by ES-62 to mediate some of its effects. Thus to further investigate the role of PAF receptor in ES-62-mediated immunomodulation it would be necessary to use primary cells that naturally do not express TLR4, such as pDCs. This would then give an insight whether ES-62 can affect such cells by using only the PAF receptor and also whether signalling

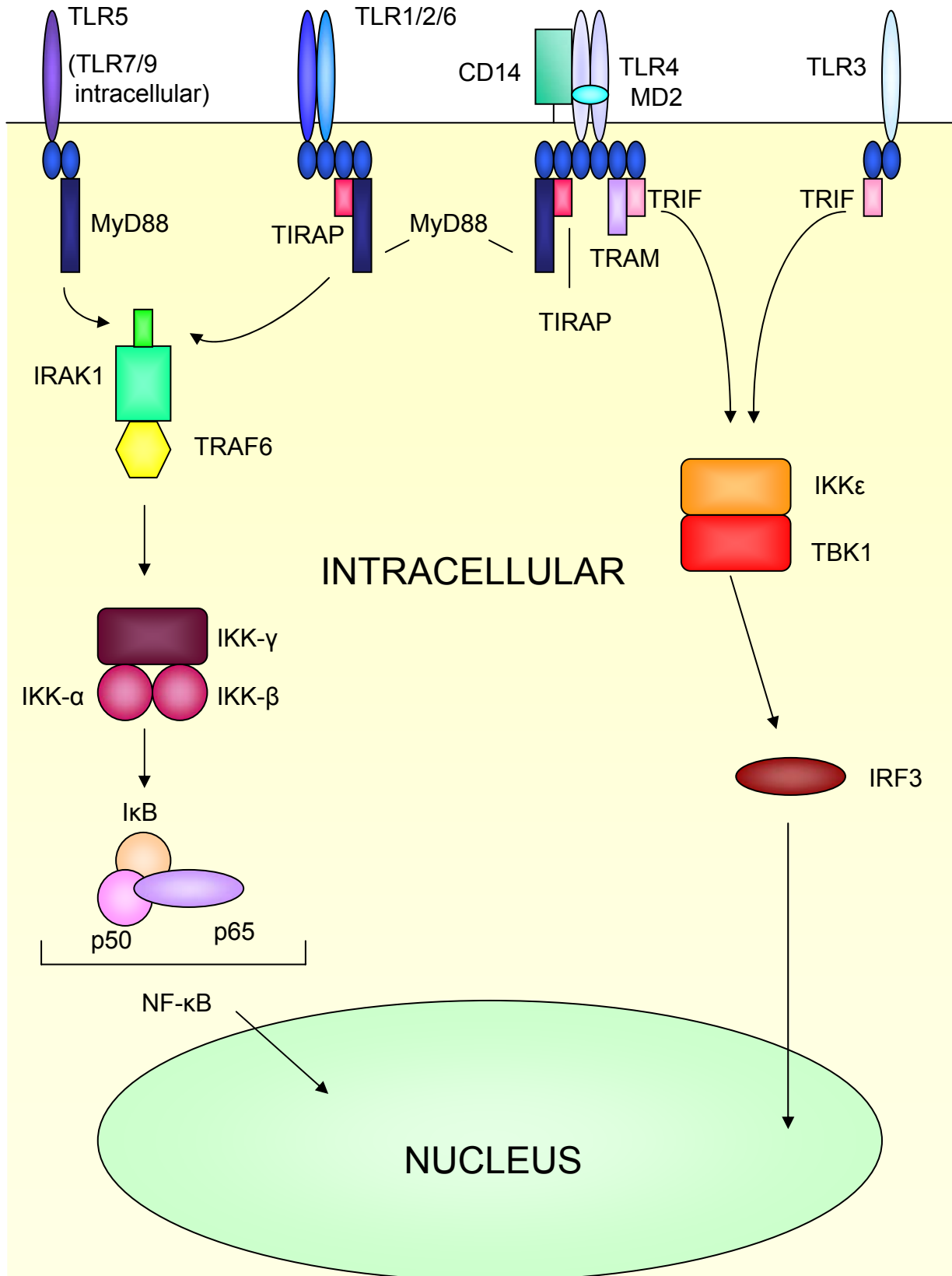


through the PAF receptor is crucial for ES-62 mediated immunomodulation in the absence of TLR4.

### **Figure 4.1 TLR mediated signalling**

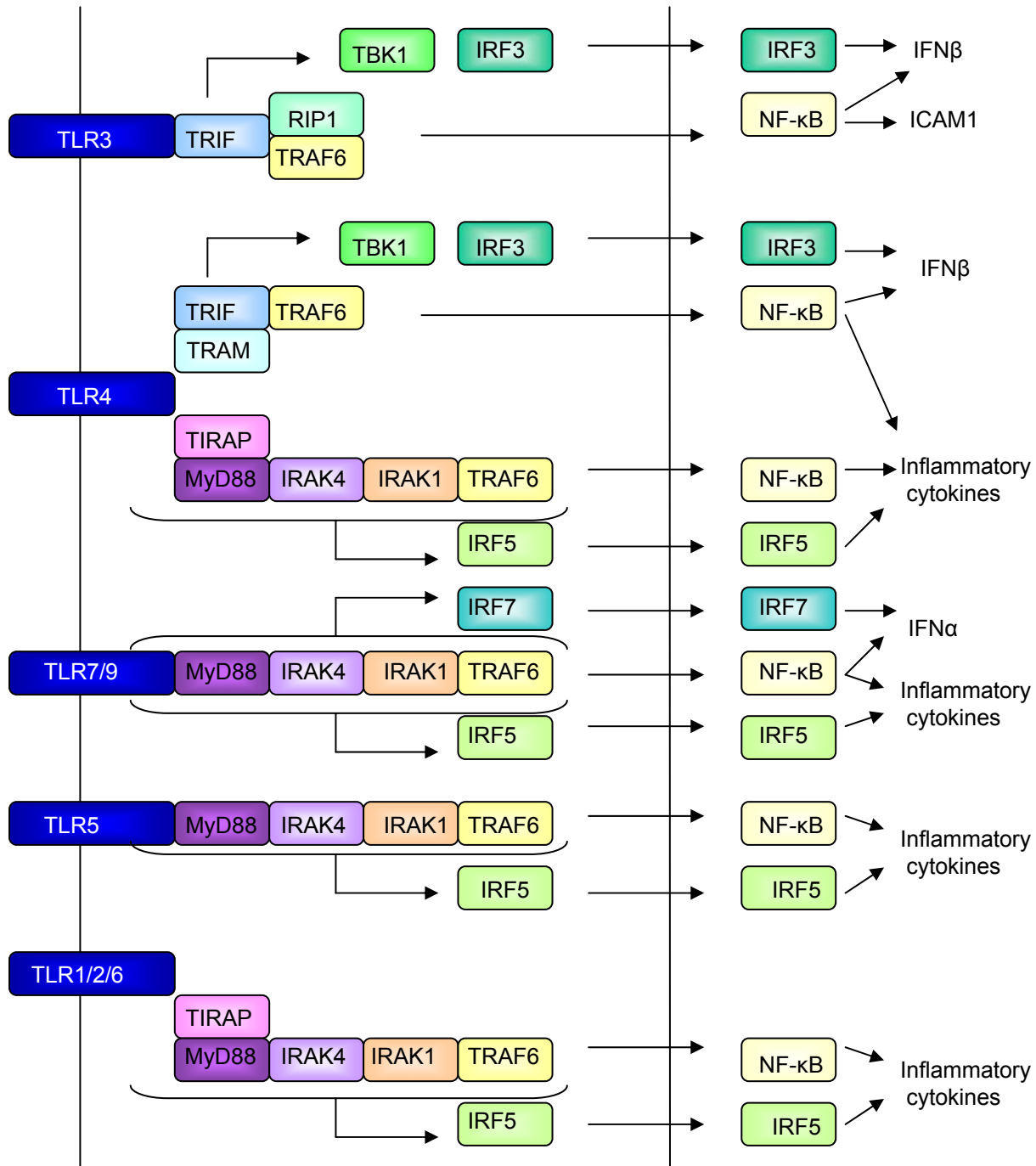
Four different Toll-interleukin 1 receptor (TIR) domain-containing adaptor molecules mediate TLR signalling pathways. Myeloid differentiation primary-response protein 88 (MyD88) has been shown to be essential for signalling via all TLRs with the exception of TLR3 which does not utilise MyD88 as an adapter molecule and TLR4, which can signal MyD88-dependent and independently. TIR-domain-containing adaptor protein (TIRAP) is essential for MyD88-dependent signalling through TLR2 and TLR4. Downstream effects of such MyD88 signalling lead to the activation of NF- $\kappa$ B, its translocation into the nucleus and to the production of pro-inflammatory cytokines. Signalling in a MyD88-independent way through TLR3 and TLR4 involves the adapter molecule TRIF (TIR-domain-containing adaptor protein inducing Interferon  $\beta$ ). In addition to TRIF, TLR4 signalling also depends on the recruitment of another adapter molecule namely TRAM (TRIF related adaptor protein) to initiate signalling via this pathway. Downstream effects of TRIF and TRIF/TRAM signalling lead to the activation of IRF3 and its translocation to the nucleus where it activates transcription of type I interferons.

EXTRACELLULAR



**Figure 4.2 The production of pro-inflammatory cytokines is mediated through different TLR signalling pathways**

With the exception of TLR3, all TLRs share the MyD88-dependent signalling pathway that leads to the activation of NF- $\kappa$ B or to the association of MyD88 and TRAF6 with IRF5. Upon activation, both NF- $\kappa$ B and IRF5 translocate to the nucleus where they initiate transcription of genes encoding for inflammatory cytokines. Signalling through TLR7 and 9 can also lead to the association of MyD88, IL-1R associated kinase 1 (IRAK1) and TNF-receptor-associated factor 6 (TRAF6) with IRF7 which induces IFN $\alpha$  gene transcription after translocating into the nucleus. TLR3 and TLR4 can function in a MyD88-independent signalling pathway which is mediated by the activation of IRF3 or by utilising RIP1 and TRAF6 to activate NF- $\kappa$ B leading to production of IFN $\beta$  and ICAM1. However production of inflammatory cytokines such as IL12, IL6 and TNF $\alpha$  seems to be suppressed.

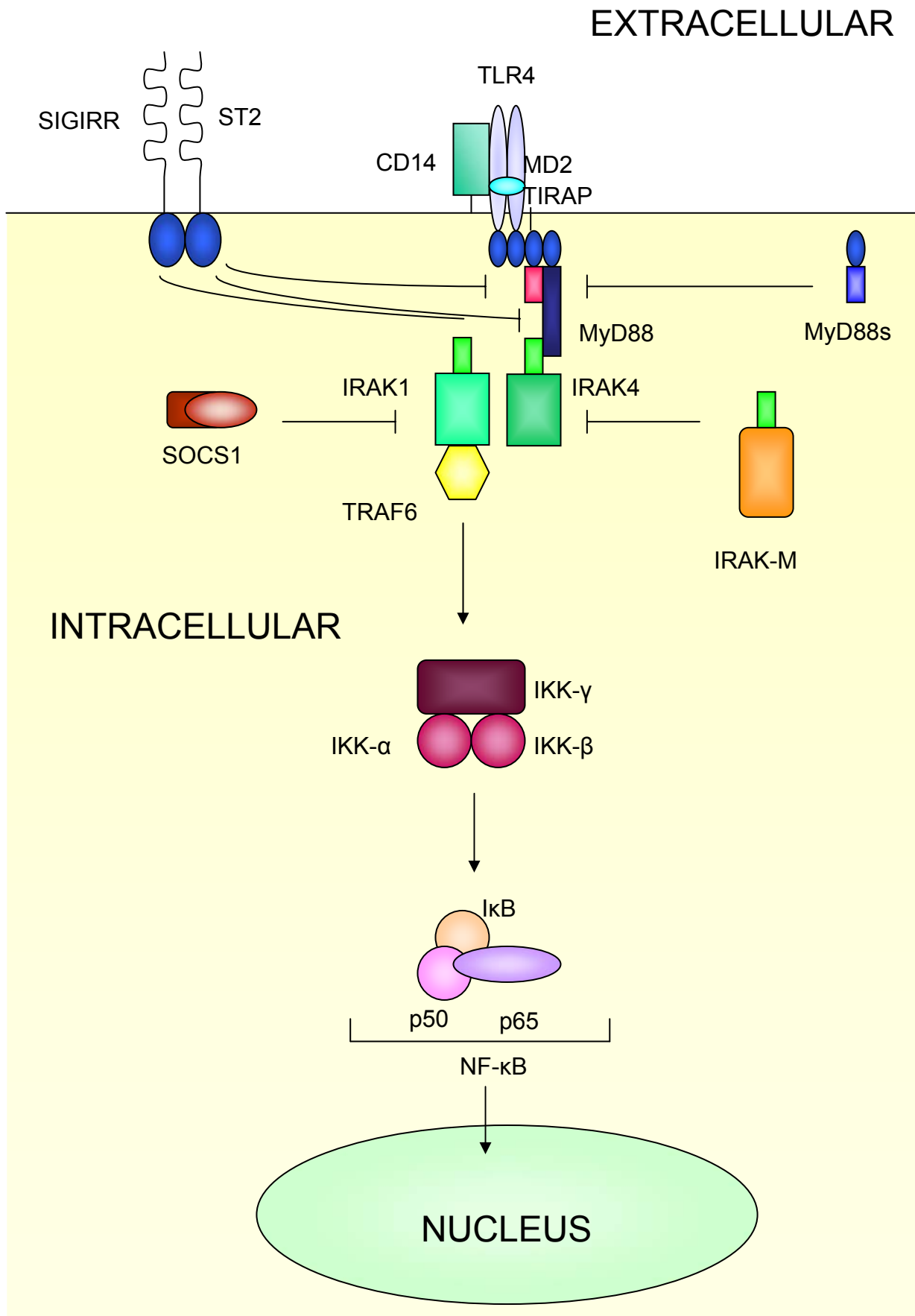


Cytoplasm

Nucleus

### **Figure 4.3 Molecules involved in inhibiting TLR dependent signalling pathways**

Several molecules have been reported to be involved in the regulation of negative signalling in TLR signalling pathways. For example whilst IRAK1-M inhibits dissociation of the IRAK1-IRAK4 complex from the receptor, SOCS1 is thought to block the activity of IRAK1 and MyD88s inhibits binding of IRAK4 to MyD88. ST2 suppresses TLR signalling by sequestration of MYD88 and TIRAP through its TIR domain. SIGIRR has been shown to interact with IRAK and TRAF6 however its exact function in TLR suppression has not been determined yet.

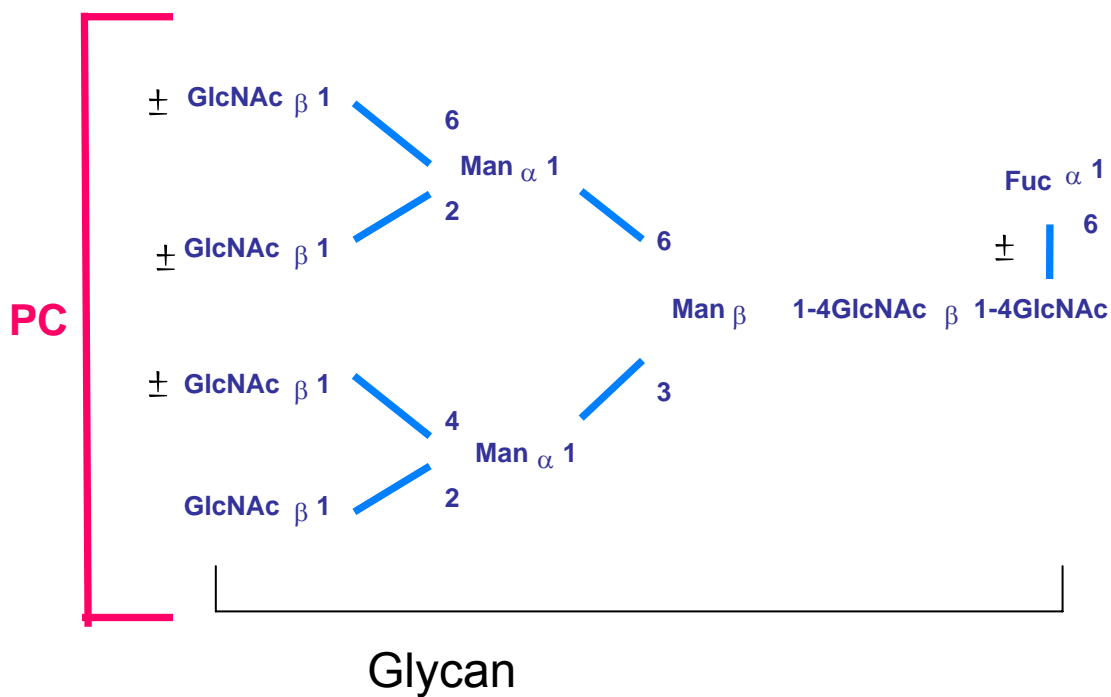


#### Figure 4.4 ES-62 and PAF both contain PC

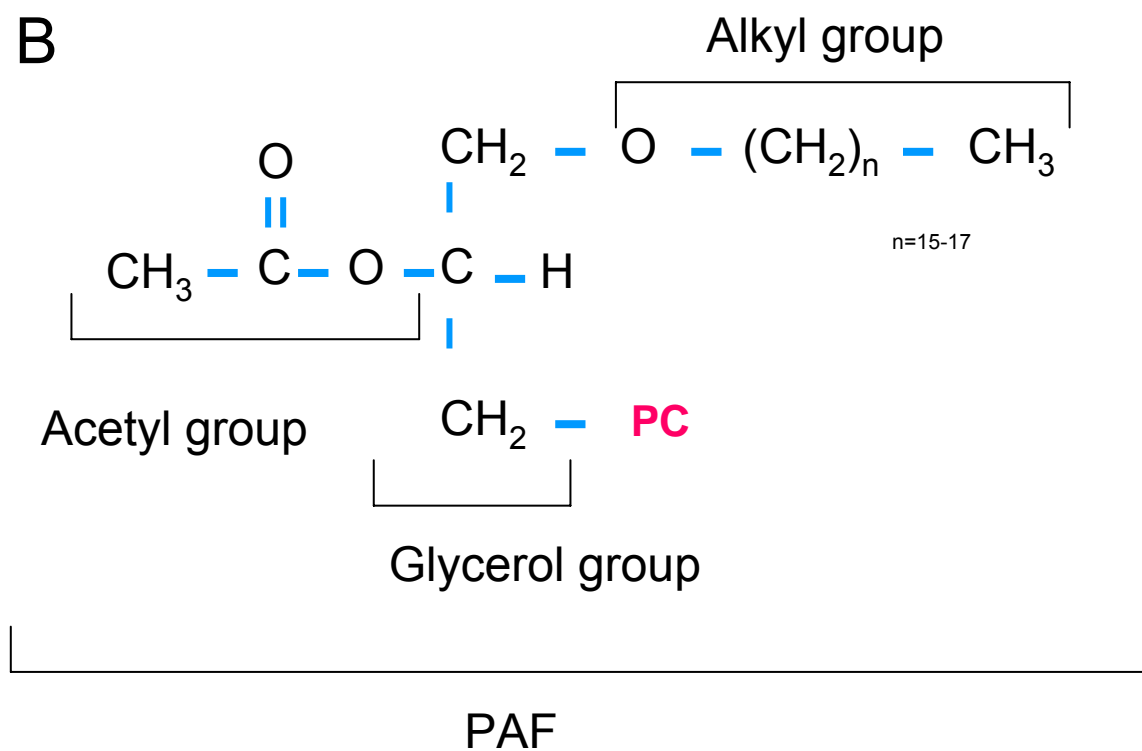
ES-62 and PAF both contain PC. In case of ES-62 (**A**) PC is attached to one or more of the three N-type glycans characterised on the molecule. In case of PAF (**B**) PC is attached to position 3 of the glycerol group of the molecule (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine).



A



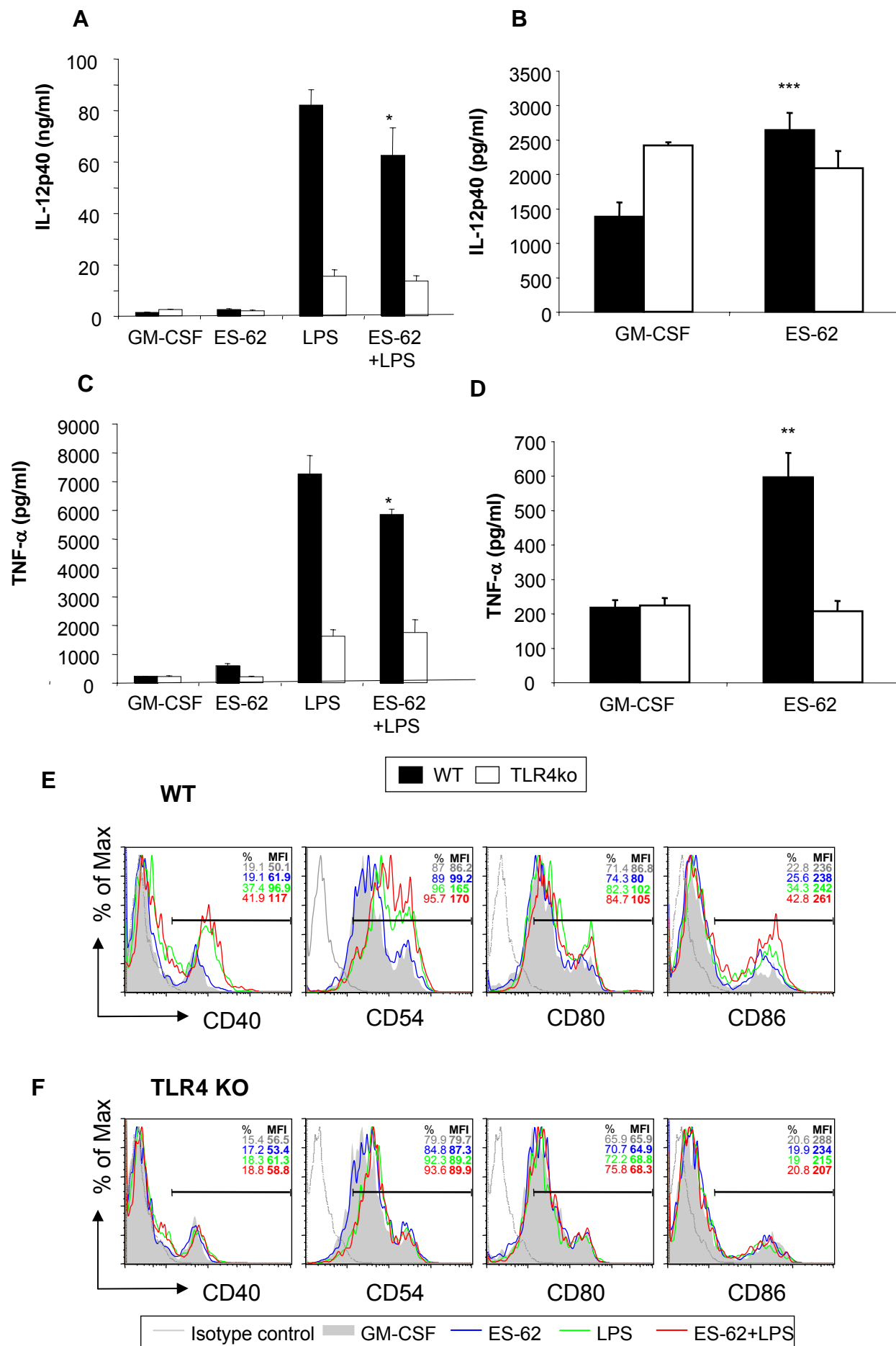
B



**Figure 4.5 Effects of ES-62-induced maturation on pro-inflammatory cytokine production and co-stimulatory molecule expression by TLR4 KO DC**

Bone marrow-derived dendritic cells were grown from wild type and TLR4 KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either matured with GM-CSF or ES-62 (2  $\mu$ g/ml) for 24 h on d 6 and subsequently in the absence or presence of LPS (1  $\mu$ g/ml) for an additional 24 h on d 7. On d 8, IL-12p40 (**A+B**) and TNF- $\alpha$  (**C+D**) in culture supernatants were measured by ELISA. Figures **B** and **D** display the responses of the ES-62 and GM-CSF groups on an expanded scale. Data are presented as means  $\pm$  SD and are representative of 3 independent experiments. \*\*\* $p < 0.005$ , \*\* $p < 0.01$ , \* $p < 0.05$  ES-62+LPS compared to LPS, ES-62 compared to GM-CSF.

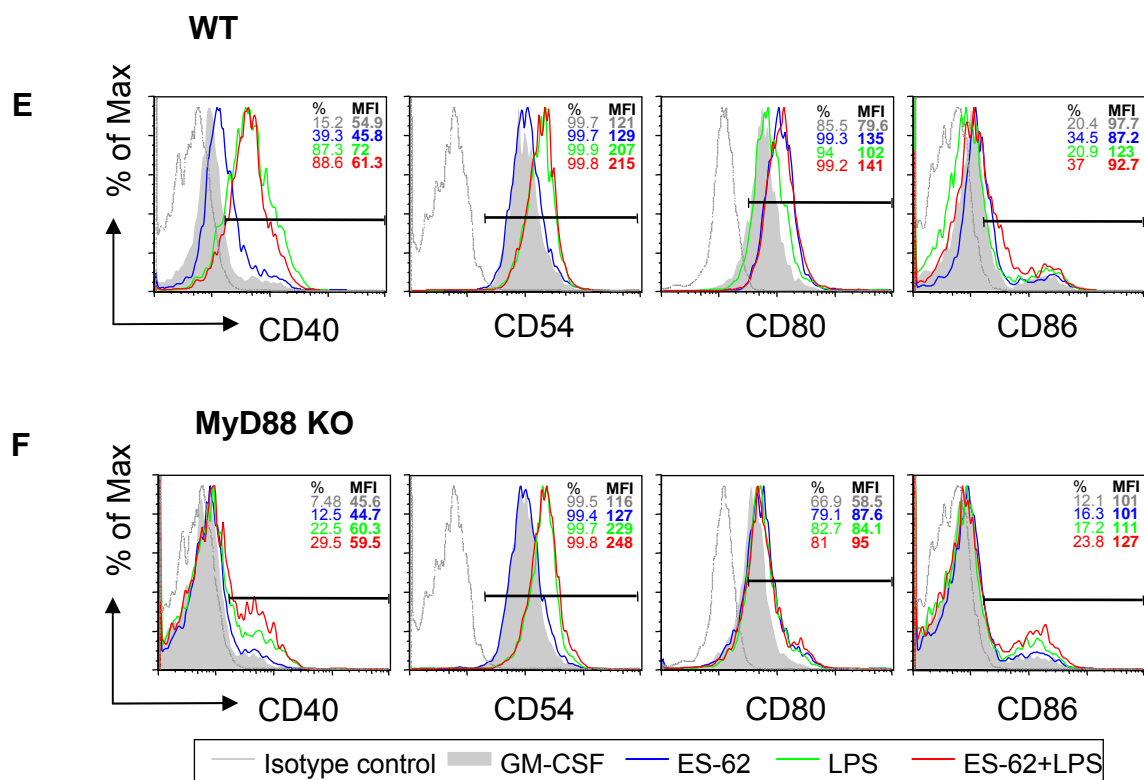
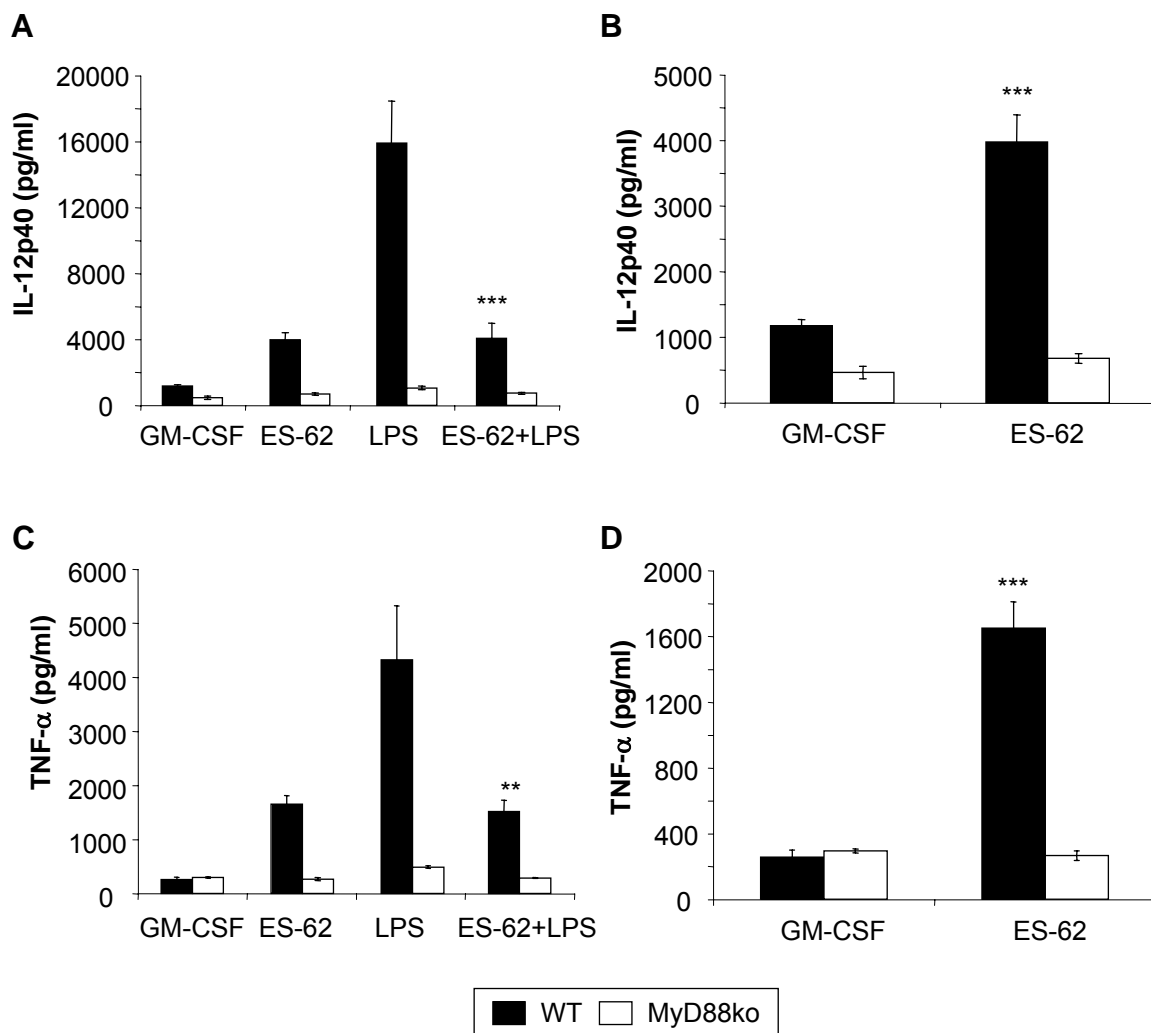
Furthermore on d 8 DCs from the cultures were harvested and the CD11c<sup>+</sup> population was subsequently analysed for co-stimulatory molecule expression by flow cytometry (**E** = WT, **F** = TLR4 KO). Data are presented as histograms of marker positive cells relative to isotype controls. Located within each histogram is the percentage of positive staining cells and corresponding MFI contained under the marker. Data is representative of 3 independent experiments.



**Figure 4.6 Effects of ES-62 treatment on pro-inflammatory cytokine production and co-stimulatory molecule expression by MyD88 KO DC**

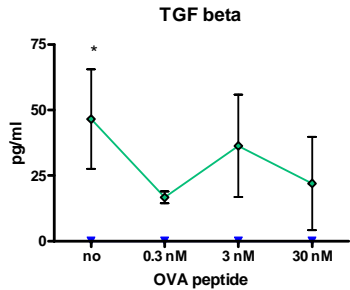
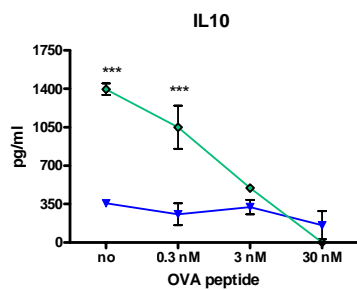
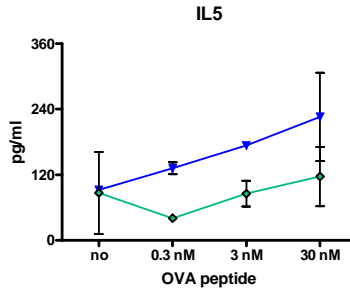
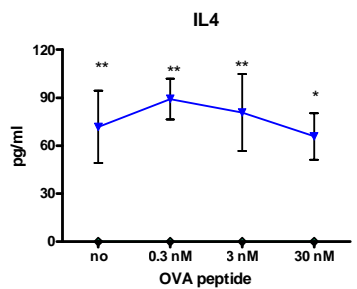
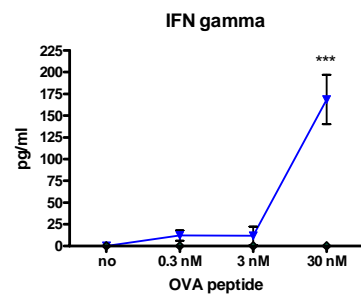
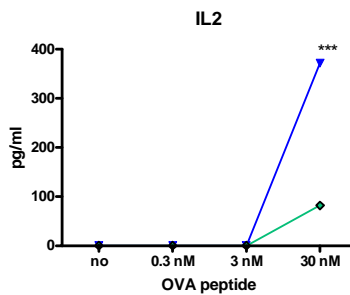
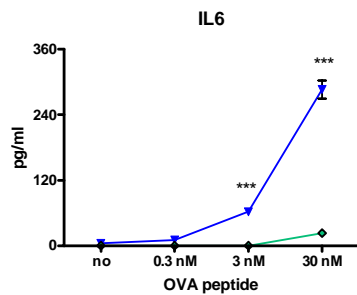
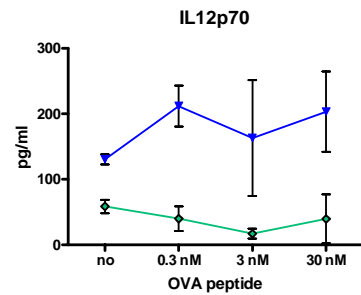
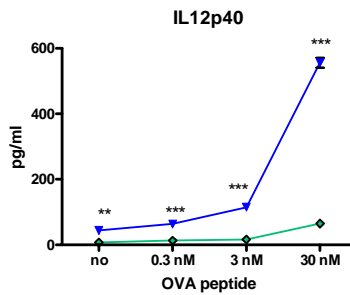
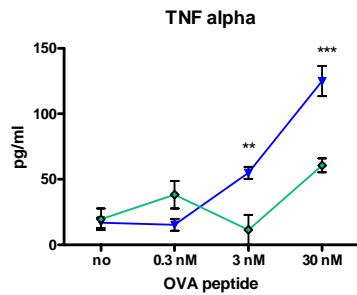
Bone marrow-derived dendritic cells were grown from wild type and MyD88 KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either matured with GM-CSF or ES-62 (2 µg/ml) for 24 h on d 6 and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, IL-12p40 (**A+B**) and TNF-α (**C+D**) in culture supernatants were measured by ELISA. Figures **B** and **D** display the responses of the ES-62 and GM-CSF groups on an expanded scale. Data are presented as means ± SD and are representative of 3 independent experiments. \*\*\*p<0.005, \*\*p<0.01 ES-62+LPS compared to LPS, ES-62 compared to GM-CSF.

Furthermore on d 8 DCs from the cultures were harvested and the CD11c<sup>+</sup> population was subsequently analysed for co-stimulatory molecule expression by flow cytometry (**E** = WT, **F** = MyD88 KO). Data are presented as histograms of marker positive cells relative to isotype controls. Located within each histogram is the percentage of positive staining cells and corresponding MFI contained under the marker. Data is representative of 3 independent experiments.



#### **Figure 4.7 The effects of LPS-matured TLR4 KO bmDCs on T cell polarisation**

Bone marrow-derived dendritic cells were grown from wild type and TLR4 KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then matured with LPS (1 µg/ml) for 24 h on d 7. On d 8, bmDC (2 x 10<sup>4</sup> cells/well) were pulsed with OVA peptide and co-cultured with OTII Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells (2 x10<sup>5</sup> cells/well) for 72 h before IL2, IL4, IL5, IL6, IL10, IL12p40, IL12p70, TGFβ, IFN-γ and TNFα production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key. \*\*\*p<0.005, \*\*p<0.01, \* p<0.05 TLR4 KO compared to relevant WT group.

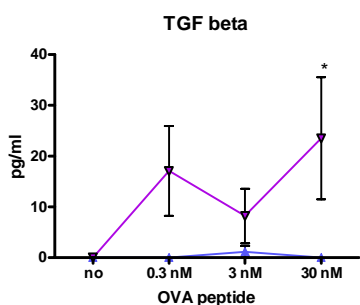
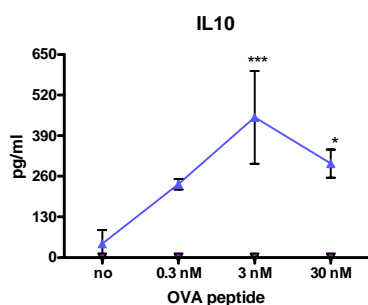
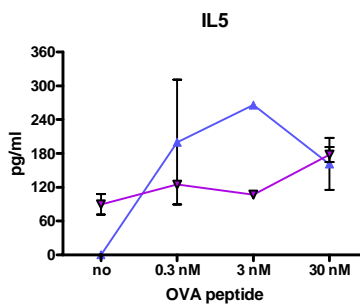
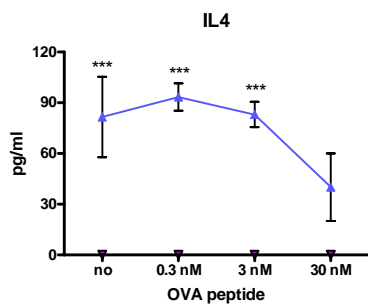
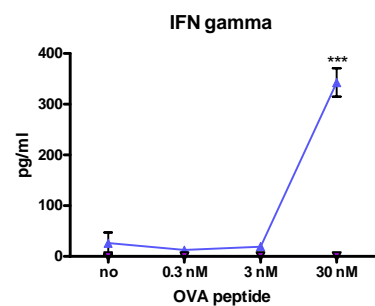
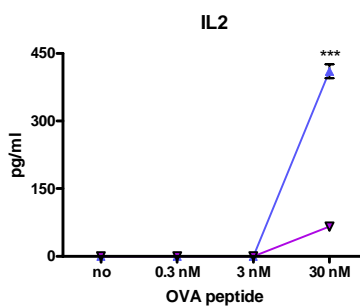
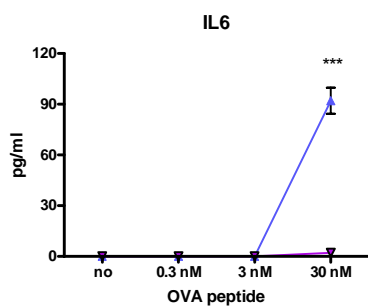
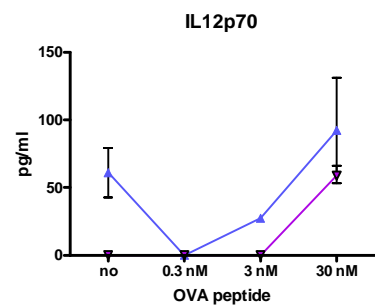
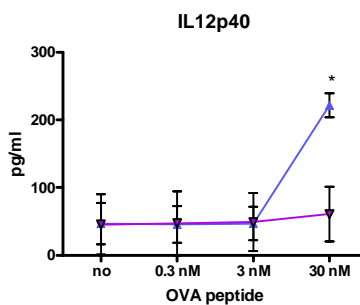
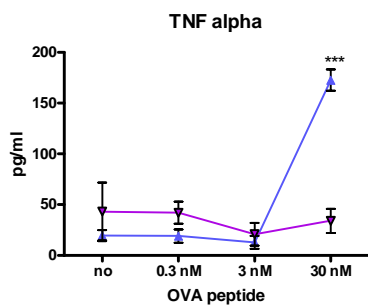


—▼— WT LPS —◆— TLR4 KO LPS

**Figure 4.8 The effects of ES-62-matured TLR4 KO bmDCs on T cell polarisation**

Bone marrow-derived dendritic cells were grown from wild type and TLR4 KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then matured with ES-62 (2 µg/ml) for 24 h on d 7. On d 8, bmDC ( $2 \times 10^4$  cells/well) were pulsed with OVA peptide and co-cultured with OTII Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) for 72 h before IL2, IL4, IL5, IL6, IL10, IL12p40, IL12p70, TGFβ, IFN-γ and TNFα production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key. \*\*\*p<0.005, \*\*p<0.01, \* p<0.05 TLR4 KO compared to relevant WT group.

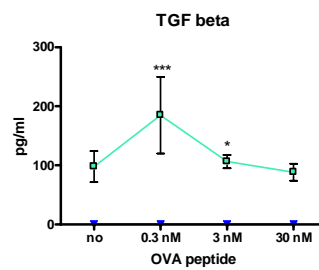
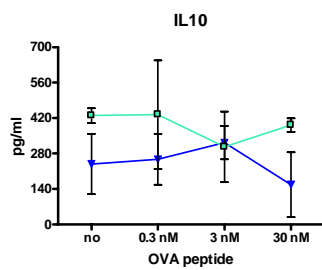
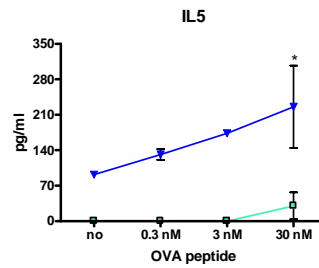
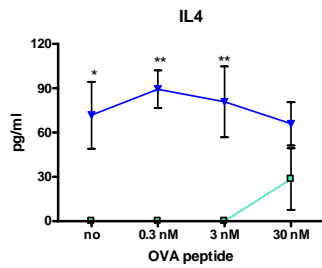
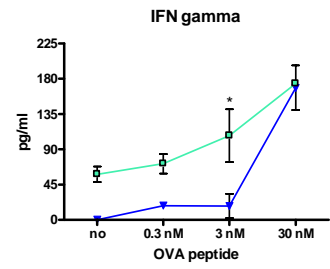
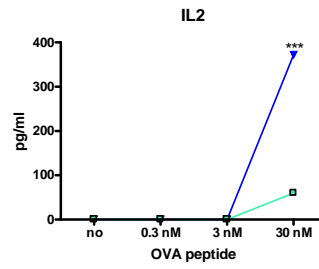
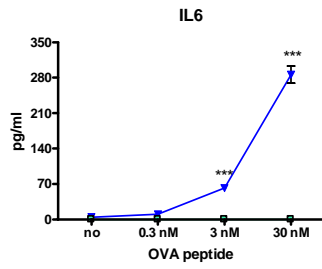
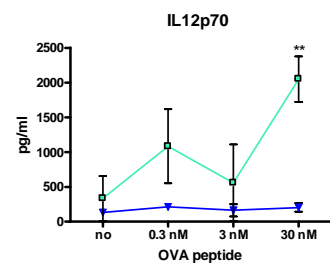
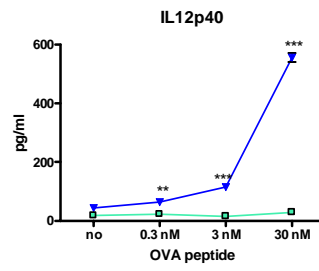
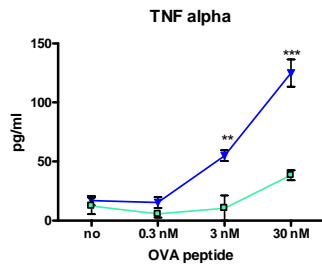




—▲— WT ES-62    —▼— TLR4 KO ES-62

**Figure 4.9 The effects of LPS-matured MyD88 KO bmDCs on T cell polarisation**

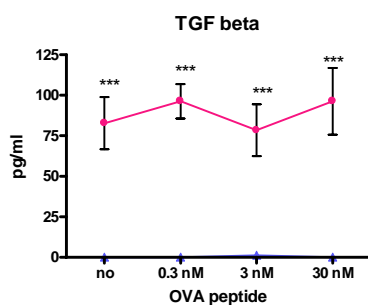
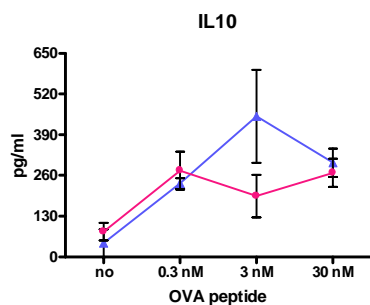
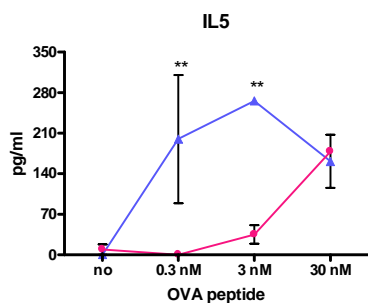
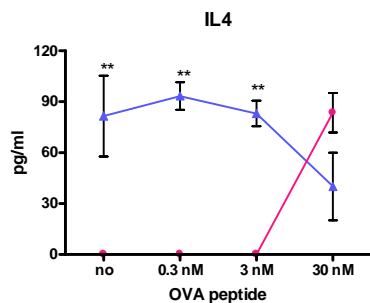
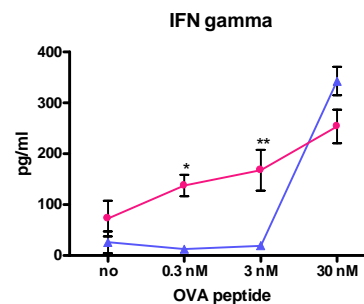
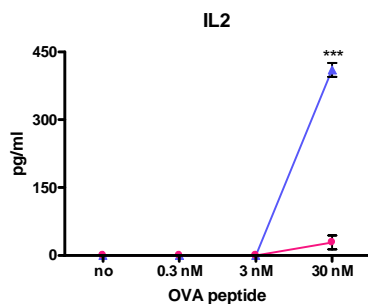
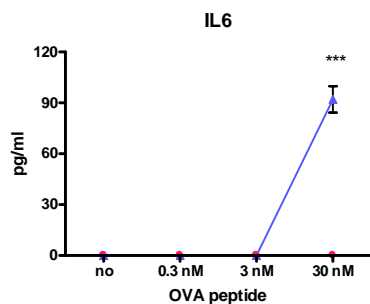
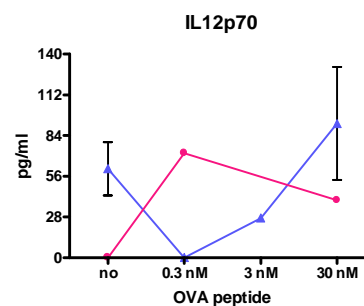
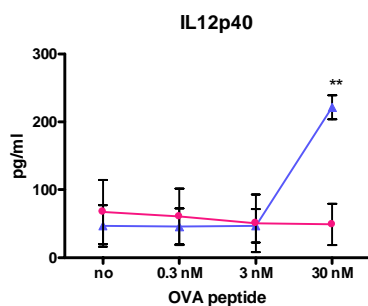
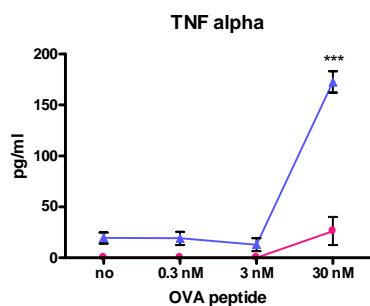
Bone marrow-derived dendritic cells were grown from wild type and MyD88 KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then matured with LPS (1 µg/ml) for 24 h on d 7. On d 8, bmDC ( $2 \times 10^4$  cells/well) were pulsed with OVA-peptide and co-cultured with OTII Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) for 72 h before IL2, IL4, IL5, IL6, IL10, IL12p40, IL12p70, TGFβ, IFN-γ and TNFα production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key. \*\*\*p<0.005, \*\*p<0.01, \* p<0.05 TLR4 KO compared to relevant WT group.



—▼— WT LPS    —■— MyD88 KO LPS

**Figure 4.10 The effects of ES-62-matured MyD88 KO bmDCs on T cell polarisation**

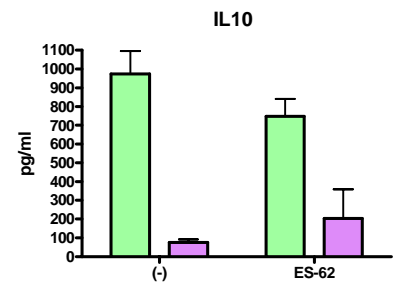
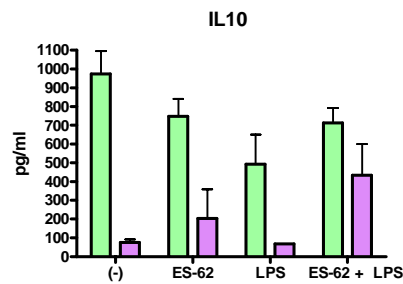
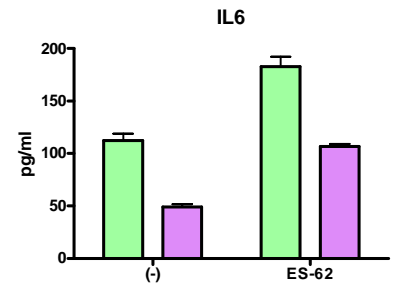
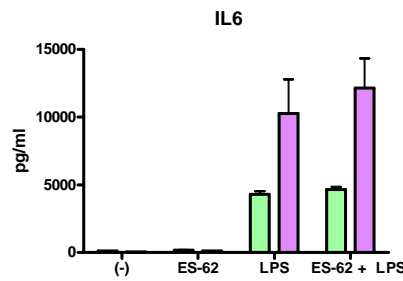
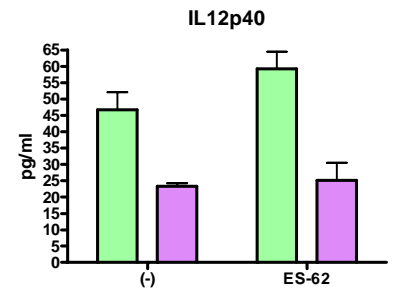
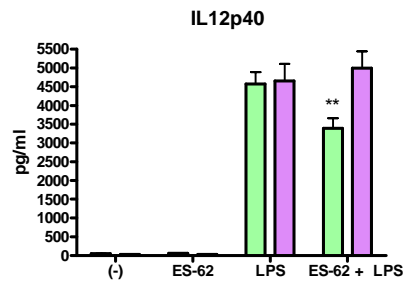
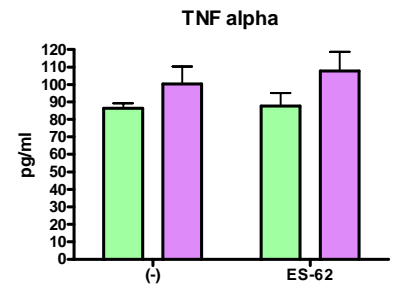
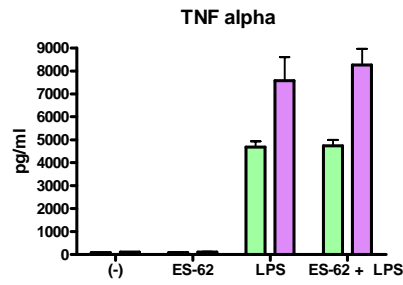
Bone marrow-derived dendritic cells were grown from wild type and MyD88 KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then matured with ES-62 (2 µg/ml) for 24 h on d 7. On d 8, bmDC ( $2 \times 10^4$  cells/well) were pulsed with OVA peptide and co-cultured with OTII Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) for 72 h before IL2, IL4, IL5, IL6, IL10, IL12p40, IL12p70, TGFβ, IFN-γ and TNFα production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key. \*\*\*p<0.005, \*\*p<0.01, \* p<0.05 TLR4 KO compared to relevant WT group.



—▲— WT ES-62    —●— MyD88 KO ES-62

**Figure 4.11 Effects of ES-62 treatment on pro-inflammatory cytokine production by MR KO DC**

Bone marrow-derived dendritic cells were grown from wild type and MR KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either matured with GM-CSF or ES-62 (2 µg/ml) for 24 h on d 6 and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, TNF- $\alpha$ , IL-12p40, IL6 and IL10 in culture supernatants were measured by ELISA. Data are presented as means  $\pm$  SD. \*\*p<0.01 ES-62+LPS compared to LPS.



WT Mannose KO

### Figure 4.12 Interaction of ES-62 and PC-free ES-62 with the PAF receptor

Immunofluorescent staining for ES-62 and PC-free ES-62 and its co-localisation with the PAF receptor.

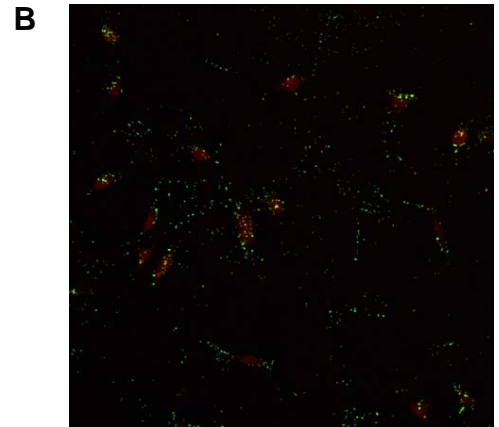
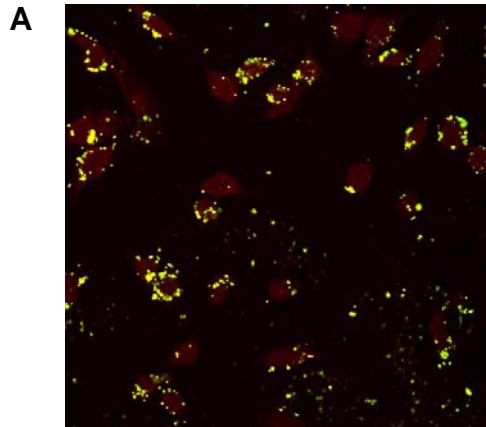
Rat brain capillary endothelial cells (rBCEC<sub>6</sub>) (**A+B**) or primary wild type (**C+D**) and PAF receptor KO (**E+F**) neurons from mouse embryos were stimulated with TNF $\alpha$  to up-regulate PAF receptor expression and then incubated for 2 h with green fluorescent beads coated with either ES-62 or PC-free ES-62 (produced by *Pichia*). The cells were then washed, fixed and stained for PAF receptor (red; conjugated anti-PAF receptor-rhodamin). Pictures were kindly provided by Elaine Tuomamen (St. Jude Children's Research Hospital, Memphis, Tennessee).



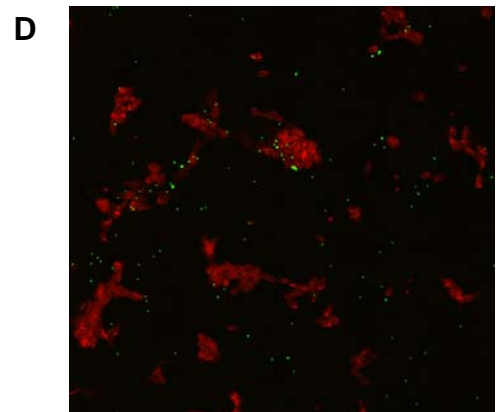
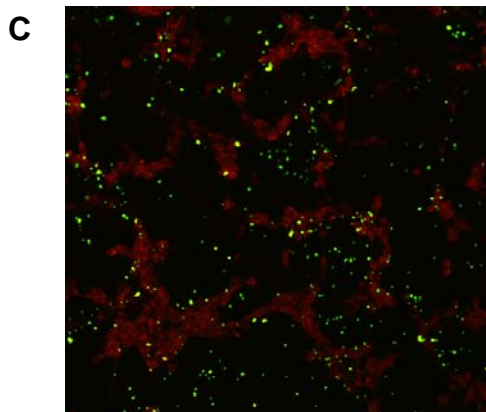
**ES-62**

**PC free ES-62**

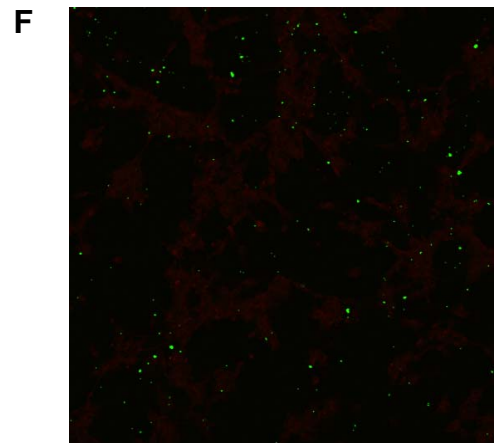
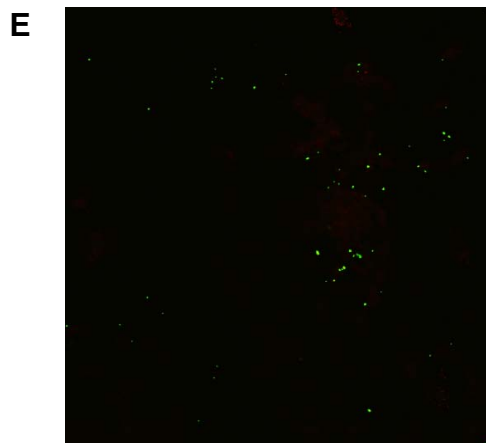
**RBCEC6**



**WT primary neurons**



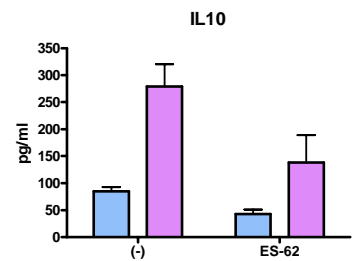
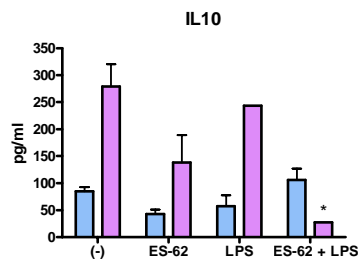
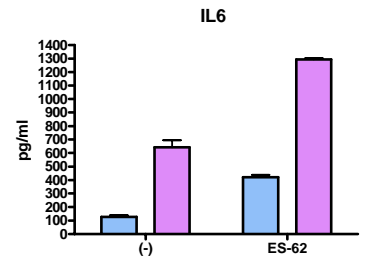
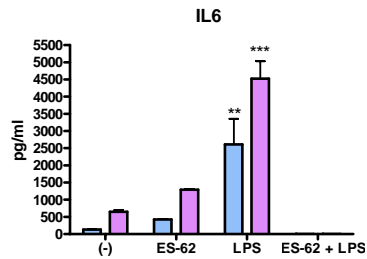
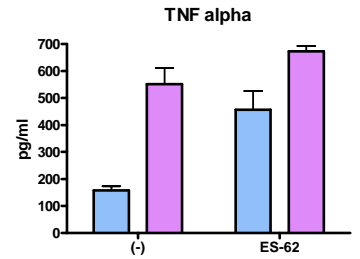
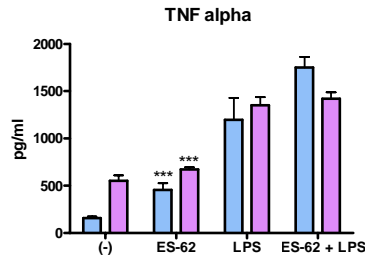
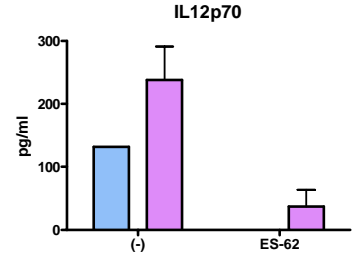
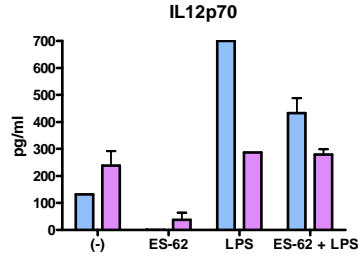
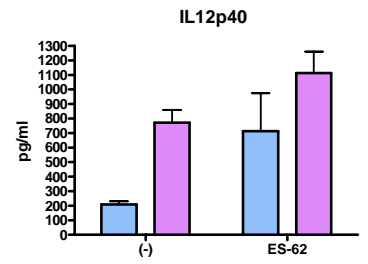
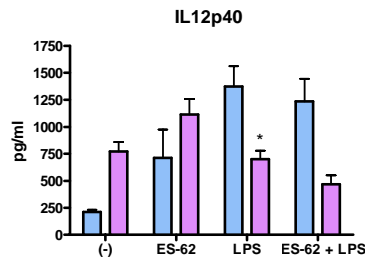
**PAFr<sup>-/-</sup> primary neurons**



 PAF receptor  ES-62/ PC free ES-62 beads  co-localisation

**Figure 4.13 Effects of ES-62 treatment on pro-inflammatory cytokine production by PAF receptor KO DC**

Bone marrow-derived dendritic cells were grown from wild type and PAF receptor KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then either matured with GM-CSF or ES-62 (2 µg/ml) for 24 h on d 6 and subsequently in the absence or presence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, TNF- $\alpha$ , IL-12p40 and p70, IL6 and IL10 in culture supernatants were measured by ELISA. Data are presented as means + SD. \*p<0.05, \*\*p<0.01 ES-62+LPS compared to LPS, \*p<0.05, \*\*p<0.01, \*\*\*p<0.005 ES-62 compared to LPS.



WT

PAF -/-

**Figure 4.14 The effects of ES-62- and/or LPS-matured PAF receptor KO  
bmDCs on Th1 cell polarisation**

Bone marrow-derived dendritic cells were grown from wild type and PAF receptor KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then matured with or without ES-62 (2 µg/ml) on d 6 for 24 h and subsequently in the presence or absence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, bmDC ( $2 \times 10^4$  cells/well) were pulsed with OVA peptide and co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) for 72 h before IL2, IL6, IL12p40, IL12p70, IFN- $\gamma$  and TNF $\alpha$  production was measured from culture supernatants by ELISA. Data are presented as means  $\pm$  SD. The various combinations of DC maturational stimuli are indicated in the key.

Significance: \*p<0.05, \*\*p<0.01, \*\*\*p<0.005

PAF -/-:

IL12p40: \*\*\*, \* ES-62 vs LPS, \*\*\*, \*\*ES-62 vs ES-62+LPS

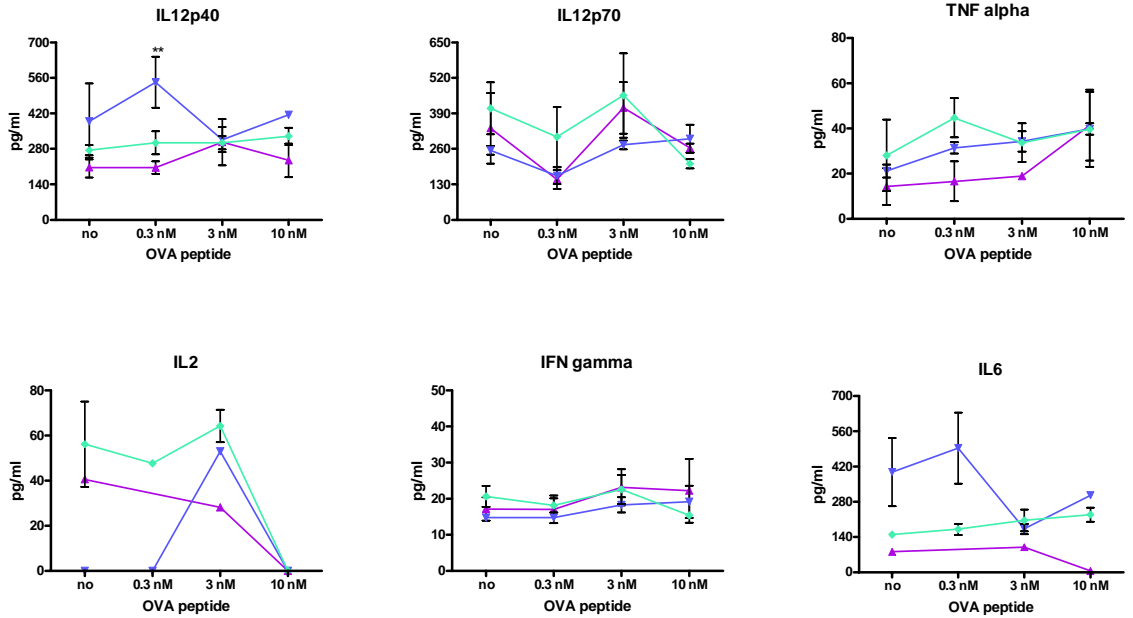
TNF $\alpha$ : \*ES-62 vs LPS, \*\*, \* ES-62 vs ES-62+LPS

IL6: \*\*\* ES-62 vs LPS, \* ES-62 vs ES-62+LPS

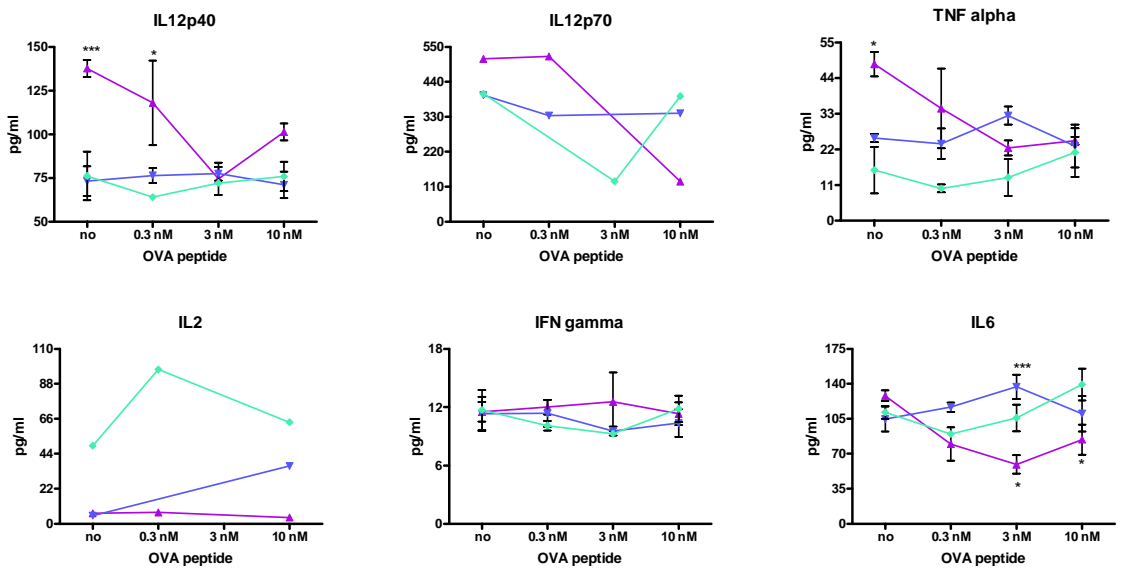
WT:

IL12p40: \*\* ES-62 vs LPS

## WT bmDC –T cell cultures



## PAF -/- bmDC –T cell cultures

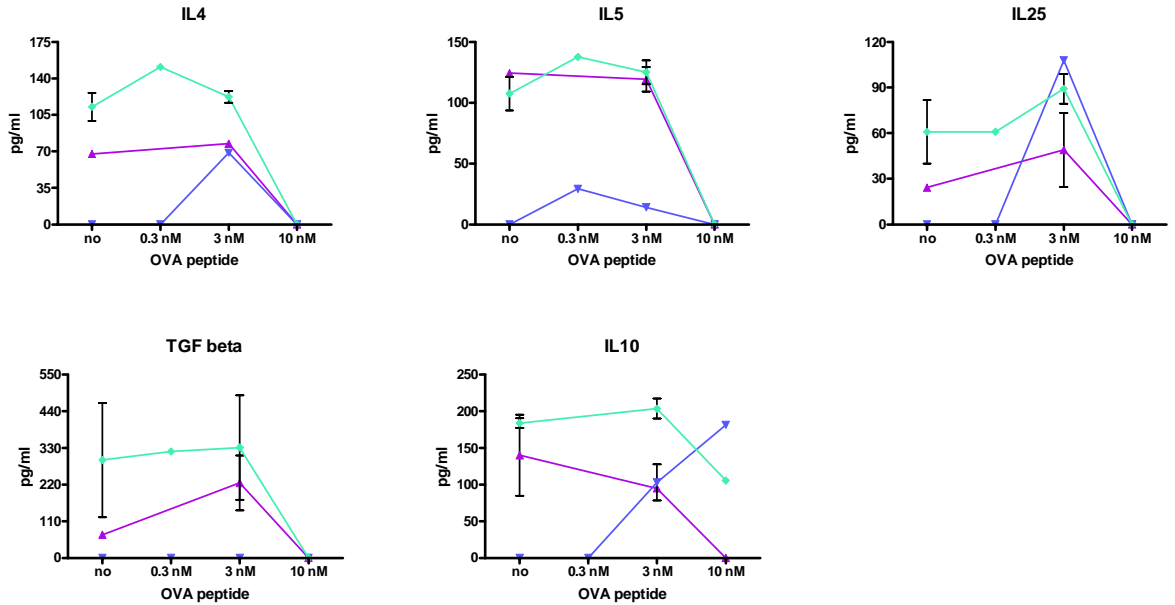


▲ ES-62   
 ▼ LPS   
 ◆ ES-62 + LPS

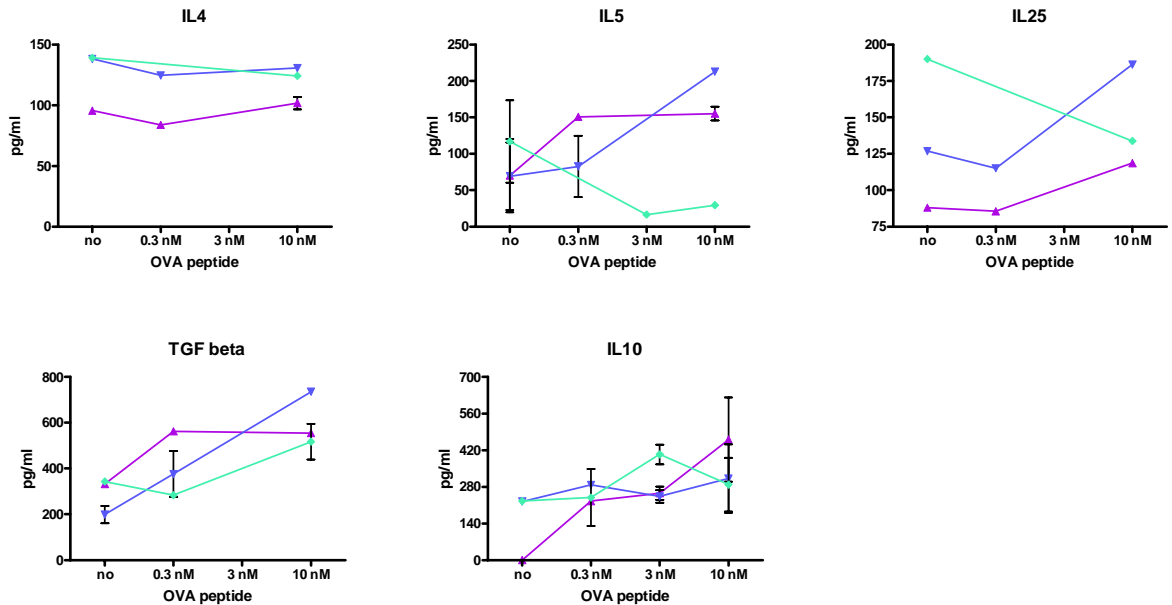
**Figure 4.15 The effects of ES-62- and/or LPS-matured PAF receptor KO  
bmDCs on Th2/Treg cell polarisation**

Bone marrow-derived dendritic cells were grown from wild type and PAF receptor KO mice *in vitro* in the presence of GM-CSF for 6 d. These bmDC were then matured with or without ES-62 (2 µg/ml) on d 6 for 24 h and subsequently in the presence or absence of LPS (1 µg/ml) for an additional 24 h on d 7. On d 8, bmDC ( $2 \times 10^4$  cells/well) were pulsed with OVA peptide and co-cultured with DO.11.10 Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells ( $2 \times 10^5$  cells/well) for 72 h before IL4, IL5, IL17E, IL10 and TGFβ production was measured from culture supernatants by ELISA. Data are presented as means ± SD. The various combinations of DC maturational stimuli are indicated in the key.

## WT bmDC –T cell cultures



## PAF <sup>-/-</sup> bmDC –T cell cultures

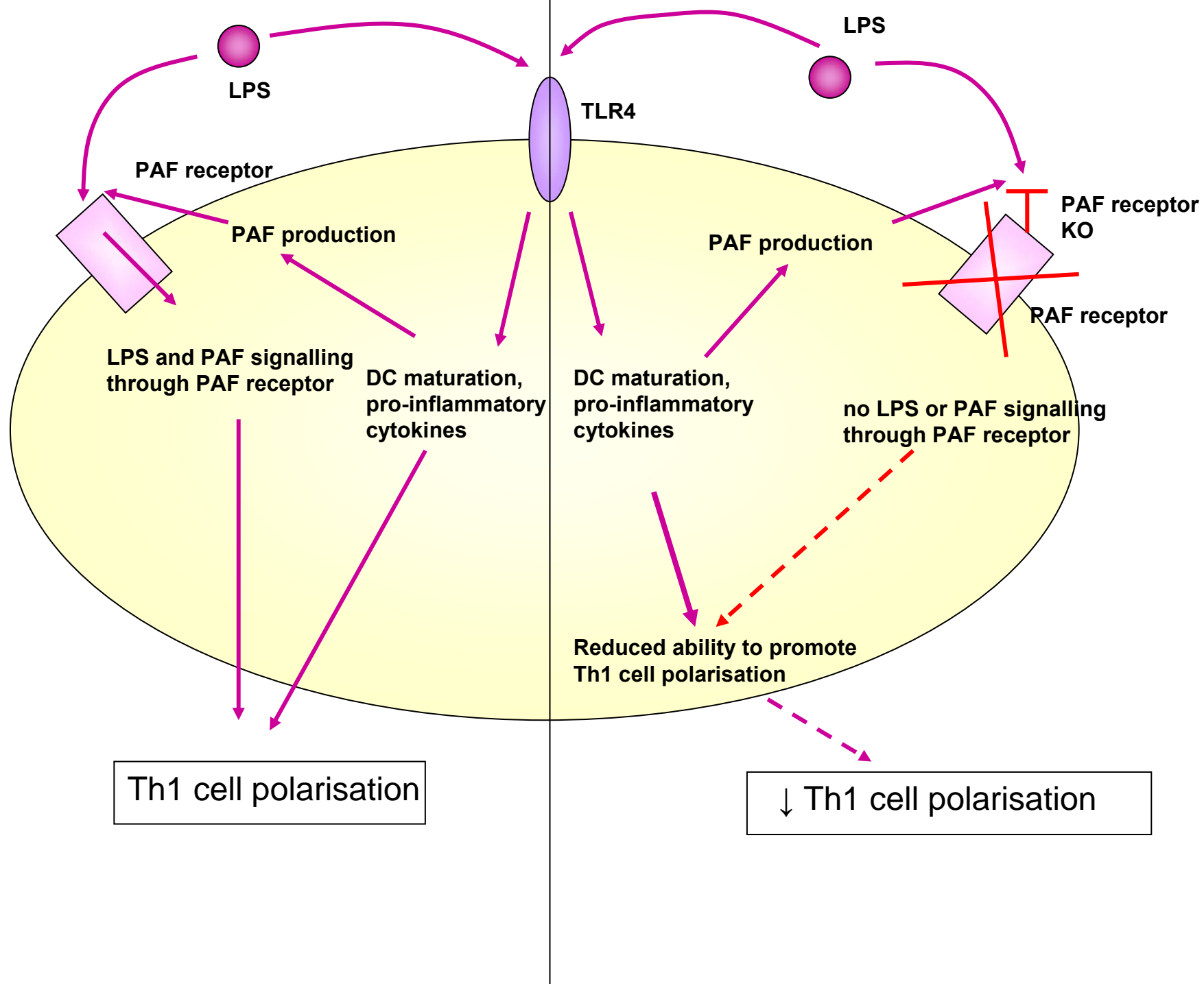


▲ ES-62   
 ▼ LPS   
 ◆ ES-62 + LPS

**Figure 4.16 Potential LPS signalling leading to Th1 cell polarization involving TLR4 and the PAF receptor**

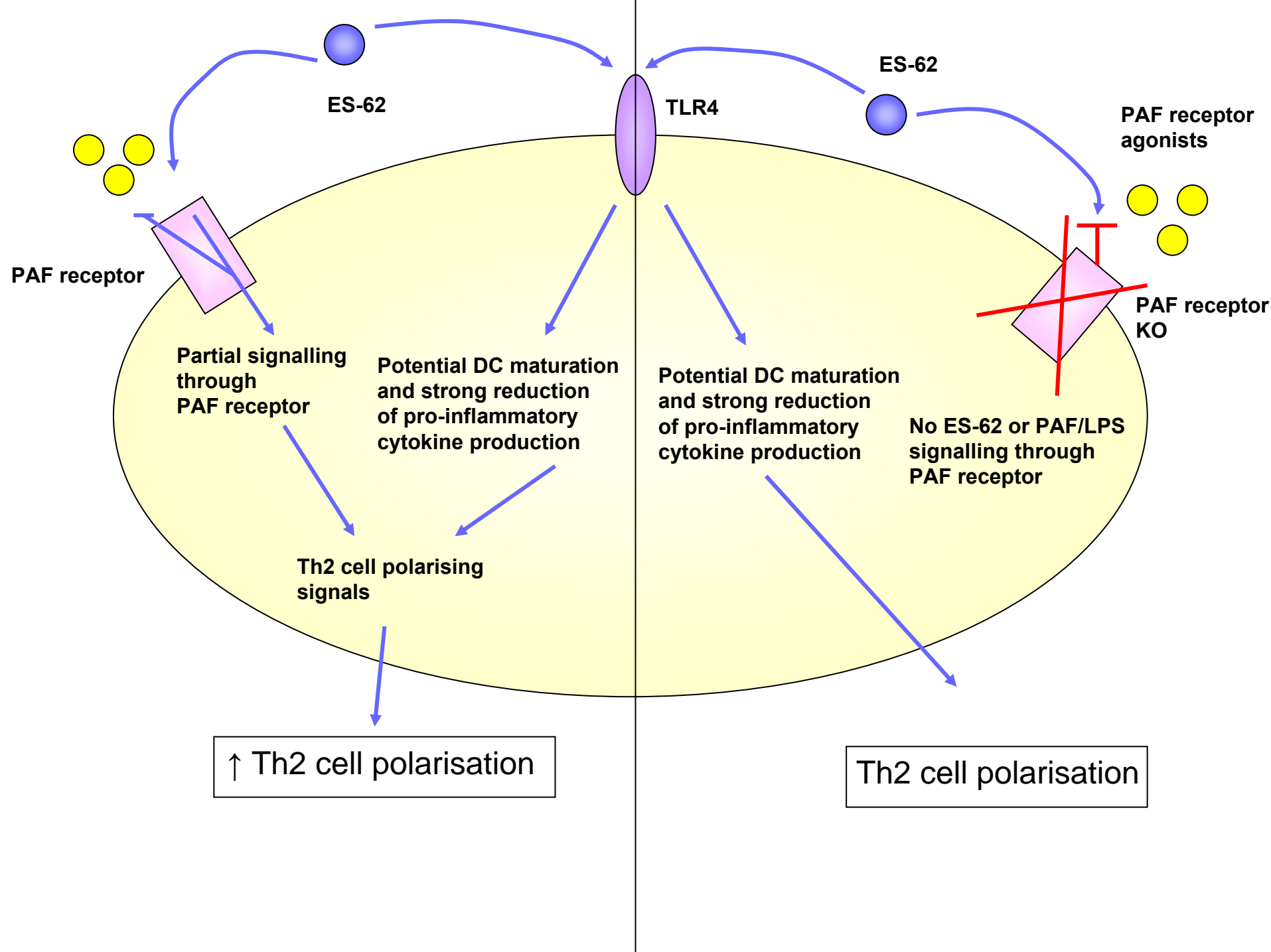
To initiate DC maturation LPS binds TLR4 and activates a signalling cascade which leads to the up-regulation of surface marker and pro-inflammatory cytokine production and to the release of PAF. PAF can then bind the PAF receptor to enhance pro-inflammatory signalling. Furthermore, LPS has also been shown to directly bind the PAF receptor. Collectively, activation of the PAF receptor and its intracellular signal transduction pathways may therefore provide further pro-inflammatory signals and thus be of crucial importance in polarizing T cells towards a Th1 phenotype. Putatively, in PAF receptor KO mice there is a lack of those additional pro-inflammatory signals from the PAF receptor and thus although the DC is fully matured due to LPS-signalling through TLR4, it cannot polarize T cells towards a Th1 phenotype.





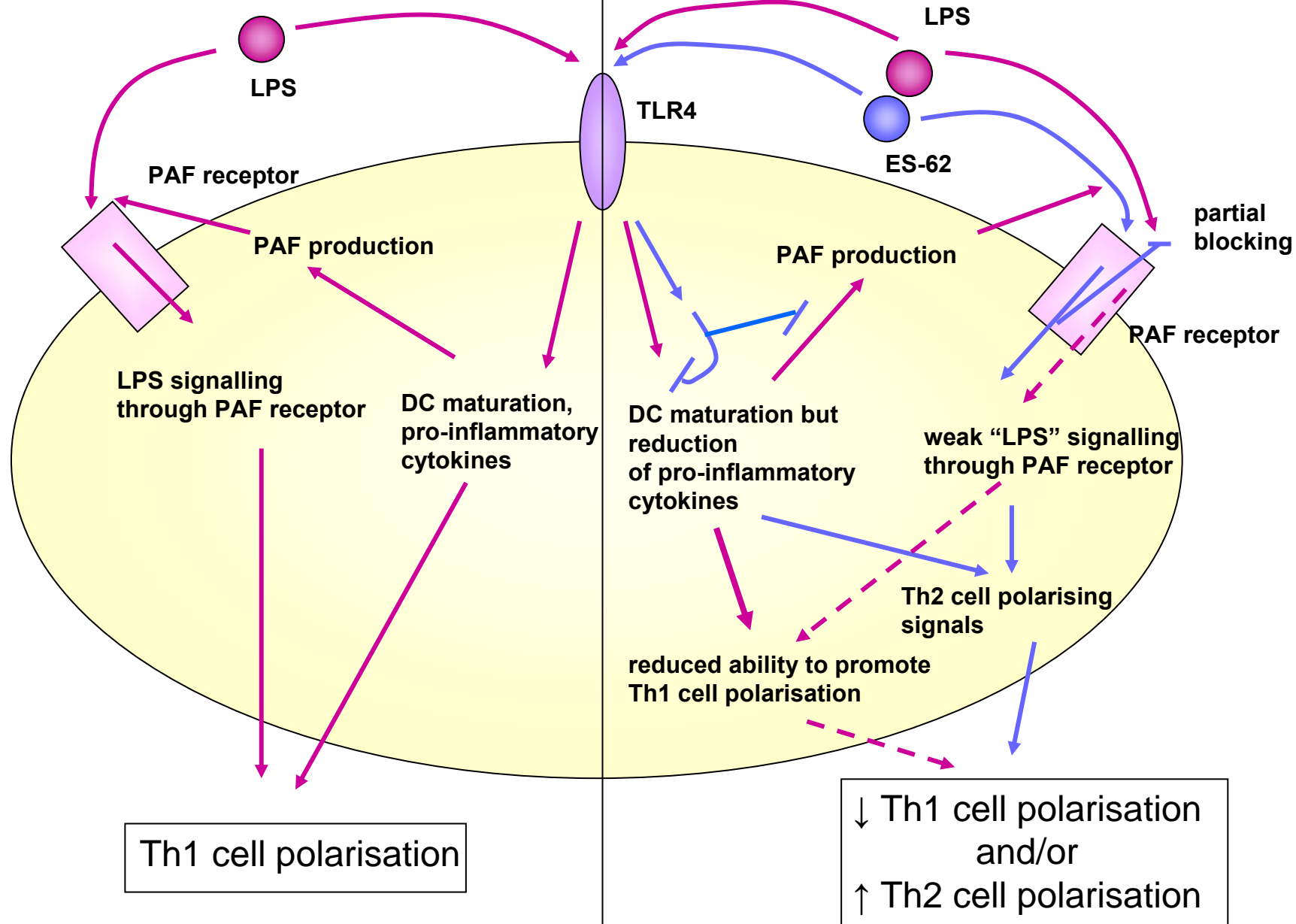
**Figure 4.17 Potential ES-62 signalling leading to Th2 cell polarization involving TLR4 and the PAF receptor**

ES-62-signalling through TLR4 leads to the generation of partially mature DCs. ES-62 has been shown to bind the PAF receptor and thus could putatively either prevent binding of any other agonist (PAF, LPS), possibly without activating the receptor, or interfere with downstream PAF signalling pathways. Thus as a result there would be a lack or a reduction of the pro-inflammatory signals normally provided by signalling through the PAF receptor and hence, these partially mature DCs might provide signals leading to the development of polarised Th2 effector T cells. In PAF receptor KO cells ES-62 would only be able to partially mature DCs through TLR4. However a lack of the PAF receptor might release the negative effect of LPS/PAF on the ability of ES-62 to induce Th2 polarisation



**Figure 4.18 Putative signalling mechanisms employed by ES-62 involving TLR4 and the PAF receptor leading to an inhibition of LPS-mediated Th1 cell polarization**

As described in Figure legend 4.16, LPS-signalling through TLR4 leads to the activation of DCs and to signalling through the PAF receptor which results in polarization of T cells towards a Th1 phenotype. ES-62 has been shown to inhibit some of the LPS-induced TLR4-mediated pro-inflammatory cytokine production, resulting in the generation of partially-mature DCs. Furthermore, ES-62 has been shown to bind the PAF receptor and thus could putatively either prevent binding of PAF/LPS and/or interfere with downstream signalling pathways. Thus as a result there could be a lack of additional pro-inflammatory signals normally provided by signalling through the PAF receptor and hence, reduced generation of Th1 polarising signals.



## **Chapter 5**

### **Characterisation of the immunomodulatory effects of ES-62 in animal models of asthma and arthritis**

## **5.1 Introduction**

### **5.1.1 Asthma**

Allergic asthma is a chronic inflammation of the airways which is associated with a dysregulation in Th2-associated immunity, triggered by the inhalation of environmental allergens. It is characterised clinically by bronchial hyper-responsiveness, periods of acute airway obstruction, mucus hypersecretion, high serum IgE levels, responses that eventually lead to structural abnormalities in the lungs [625-628]. The immune mechanisms underlying such pathology have not been fully delineated but inflammatory cell infiltrates including mast cells, eosinophils, macrophages and lymphocytes have been identified in affected lungs by procedures such as broncho-alveolar lavage. In addition, Th2 cytokines such as IL4, IL5, IL13 and IL17E have been detected and are believed to facilitate the Th2 immune response [629]. For instance, IL4 has been shown to induce eosinophil differentiation from bone marrow progenitors which then expand to form an IL4-producing cell population and thus amplify the Th2 immune response [630]. Furthermore IL5, together with chemokines such as eotaxin, has been shown to recruit eosinophils to the airways and to activate them [629]. IL4 is also necessary for the B cell isotype switching which leads to increased IgE production [631].

IgE has been shown to play a central role in the pathogenesis of asthma and also in the onset of an acute attack [632, 633]. IgE is mainly found bound to its high affinity receptor FCεRI, on the surface of mast cells, or to the low affinity receptor FCεRII on other leucocytes. Upon Ag encounter, Ag-IgE complexes are formed which cross-link the FCεRI receptor [634] and lead to degranulation [635] and thus to the release of histamine and other inflammatory mediators such as leukotrienes [629]. In addition to these well established roles for the classical Th2 signature effectors of IL4, IL5 and IgE, another cytokine, IL13, has been shown to play an important role in the pathogenesis of asthma by interacting with structural cells and DCs and by stimulating the production of TGFβ [636]. Consistent with this, IL13 has also been shown to be required for development of allergen-induced airway hyper-responsiveness in a mouse model of allergic airway inflammation [637].

Recent studies have demonstrated that IL25 (IL17E) is involved in mediating such Th2-associated allergic inflammation and indeed, it was shown that forced expression of IL25 (IL17E) resulted in increased airway eosinophilia, IgE, IL4, IL5 and IL13 production [638, 639]. Interestingly, IL17A production has also been found to occur in the lungs of ovalbumin-sensitised mice following airway allergen challenge [640]. IL17A appears to act here to regulate neutrophil recruitment into the bronchoalveolar space in response to allergens as its abrogation attenuates this process. However, abrogation of IL17A production also results in an increase in IL5 production in the lung and consequent eosinophil accumulation, suggesting that under these circumstances IL17A may perhaps exhibit a balancing or regulatory function [640].

#### **5.1.1.1 The role of DCs in asthma and allergic disease**

DCs can be found in every compartment of the lung [641-646] and can be broadly divided into two major populations of cDCs, the parenchymal lung DCs (PLDCs) and the epithelial DCs of the conducting airways (CADCs) [647]. CADCs form a dense network within the epithelial layer and continuously sample the airway for environmental Ags. In mice these cells are characterised by the expression of CD11c, CD11b, CD205, MHCII but not CD8 or CD4 and their short half life of about 36 h [645, 648, 649]. PLDCs are less well defined and are usually described as immature, myeloid-like DCs with a low expression of co-stimulatory molecules and a half life of around 10 d [648, 650]. Both of these subtypes of lung DCs express different sets of surface markers compared to DCs found in the spleen and in LNs and thus can probably be considered as 2 additional different DC subtypes. However, pDCs that closely resemble pDCs found in the periphery are also found and these are predominantly located in the interstitium of the lung [185].

In recent studies, it has been shown that, upon Ag encounter, respiratory tract DCs migrate to local lymph nodes where they preferentially prime Th2 responses [651]. In addition, it was demonstrated that this migration was accelerated under inflammatory conditions and that such DCs were capable of inducing both Th1 and Th2 responses [652]. However, respiratory tract DCs have also been reported to



have the capability of inducing immunotolerance [653-655]. One possible explanation for this apparent plasticity could be that DCs in the lung are fairly immature, perhaps due, at least in part, to the secretion of TGF $\beta$  by alveolar macrophages [656]. Moreover, due to their immature phenotype, contact with Ag under non-inflammatory conditions may not activate these cells enough to induce a T-effector response but rather may lead to the generation of T regulatory cells. Consistent with this, a recent study demonstrated that CD8<sup>+</sup>-mediastinal DCs from OVA-sensitised mice produced high levels of IL10 and generated Th2 cells which expressed both FoxP3 and GATA3 indicating that these DCs primed T cells with the capacity to function as regulatory cells [653]. Similarly, lung-derived pDCs have also been shown to be able to induce tolerance through the induction of Tregs. Furthermore as depletion of this cell type was found to induce a strong asthma-like pulmonary inflammation, it is believed that such pDCs may act to protect against Th2 sensitization in the lung [185]. In an analogous manner to that described above for lung-derived cDCs, the tolerogenic properties of lung pDCs might reflect their very immature phenotype [657-659] because when such pDCs become activated due to infection with viruses they switch from a tolerogenic to an immunogenic phenotype. This functional plasticity might therefore explain why an enhanced allergic response to harmless Ags can be seen under conditions of inflammation [660-662].

### **5.1.2 Rheumatoid arthritis**

Rheumatoid arthritis (RA) is a chronic autoimmune disease which affects about 1% of the population worldwide. The pathogenesis of RA has not been identified and is likely to be multifactorial. Clinically, RA is characterised by synovial inflammation, particularly of small joints, and subsequent cartilage and bone damage [663-666]. During the disease process, neoangiogenesis, cellular infiltration and hyperplasia resulting in aberrantly high production of inflammatory cytokines can be seen in the joints. Currently it is thought that the inflammation and tissue destruction results from complex cell-cell interactions in the synovium [667, 668] with the massive influx of T cells, B cells, fibroblast-like synoviocytes, macrophages (M $\Phi$ ) and DCs

orchestrated by a complex interplay of inflammatory cytokines, chemokines and matrix metalloproteases [669-671].

A range of pro-inflammatory cytokines such as TNF $\alpha$ , IL1, IL6, IL15, IL18 and GM-CSF [672, 673], mostly secreted by innate immune cells such as M $\Phi$ , DCs and monocytes but also by synovial fibroblasts in the inflamed joints, have been shown to play a major role in the pathogenesis of RA. TNF $\alpha$  was found to be one of the most important disease-driving cytokines with elevated levels of the TNF $\alpha$  usually being detected in the synovial fluid and serum of RA patients. Targeting of this cytokine with recombinant TNF receptor or anti-TNF $\alpha$  antibodies is one of the most successful anti-inflammatory treatments currently available [674]. Pertinent to this, a recent study demonstrated a role for TNF in the inhibition of naturally occurring Treg cells as evidenced by the enhanced production of Tregs, which are capable of suppressing effector T cells, by patients on TNF $\alpha$ -blocker therapy [675]. However treatment with TNF $\alpha$ -targeting drugs is not always successful, suggesting that although this cytokine plays an integral role in the inflammatory process, other cytokines or cellular processes might be crucial for the development of the disease.

#### **5.1.2.1 The role of DCs in RA and autoimmune disease**

Since autoimmune disease is defined as a loss of tolerance to self-Ag, DCs, as the major APCs, are thought to play a crucial role in the disease process. For example, it has been shown in various studies that DCs are able to prime MHC-restricted autoimmune responses in lymphoid organs and thereby promote the development of autoantibodies [454, 676, 677]. Furthermore DCs have been found in synovial tissue and synovial fluids where they are thought to take up and present (auto) Ag locally to T cells [678, 679] and also secrete various cytokines which determine the T effector cell outcome [680-688]. However the mechanisms underlying development of autoimmunity and the role(s) played by DCs are not entirely understood. Nevertheless, several studies suggest that DC-based central tolerance defects might contribute to this process. For instance inadequate interaction of DCs and thymocytes could lead to the release of autoreactive T cells from the thymus [689]. Alternatively, peripheral DCs could respond to altered self-

Ag<sub>s</sub>, such as citrullinated self-proteins which have been shown to play a pathogenic role in murine models of arthritis [690], and present them to specific T cells.

At a more molecular level, it has recently been shown that RelB, a molecule involved in the alternative NFκB signalling pathway which, amongst other functions, controls DC maturation, is of importance in the development of RA [691]. This is evidenced by the fact that several antirheumatic drugs have been demonstrated to reduce the number of RelB<sup>+</sup> DCs in the synovium [692] and that RelB-deficient mice display a decrease in severity of Ag-induced arthritis [693]. Furthermore, several environmental factors that impact on RA, such as nicotine and EBV infection, can influence RelB activity [694, 695] leading to disruption of NFκB pathways that promote pro-inflammatory cytokine secretion, impacting on the resultant T effector cell polarisation.

However, many questions in RA remain open. Neither the aetiology nor the pathogenesis of the systemic disease has been identified and even though it is generally believed that DCs play a crucial role in the disease process, it remains to be established whether specific DC subtypes are of importance.

#### **5.1.2.2 The role of T cells in rheumatoid arthritis**

Based on rodent studies, RA has generally been considered to be a Th1 mediated disease that is characterised by a lack of Th2 cytokine production and a relative dominance of IFNγ. However, this hypothesis has recently been challenged by several studies in murine models of inflammatory arthritis that have demonstrated that the pathogenic T cells were mainly producing IL17 and not IFNγ and thus suggesting that Th17 cells were driving the disease. For example, several investigations have shown that whilst collagen-induced arthritis (CIA) can still develop in IFNγ or IFN<sub>R</sub> deficient mice [696-698], it fails to do so in mice deficient for IL17 or IL23 [247], the latter of which is needed for a strong TH17 response [297]. Furthermore, whilst blocking of IL17 has also been shown to reduce the

severity of disease in CIA models, blocking of IFN $\gamma$  was found to exacerbate disease [530, 699, 700].

Various recent studies have elucidated some of the important roles for IL17/Th17 cells in driving disease in RA and in CIA models, including the activation of synovial fibroblasts to produce IL6 and IL8 [701], collagen destruction [702] and the increase in osteoclastogenesis [527]. However, although IL17 is the main cytokine produced by Th17 cells [287], Th17 cells also produce TNF $\alpha$  and GM-CSF [287], two pro-inflammatory cytokines which have also been shown to play important roles in the promotion of autoimmune arthritis. Indeed, recent studies demonstrated that IL17 exhibits effects that are both independent of, and synergistic with, TNF $\alpha$  in the induction of arthritis. For example, using TNF $\alpha$ -deficient mice it was demonstrated that whilst TNF was required for the induction of arthritis by IL-17 in naïve mice, this TNF $\alpha$  dependency of IL17-induced pathology is lost in mice with established arthritis [703]. These latter data have very interesting implications with regard to anti-TNF $\alpha$  therapies as they provide a possible explanation for why such treatments are not effective in all patients. Furthermore it suggests that additional reagents targeting IL17 could be of use in patients for whom blocking TNF $\alpha$  action does not stop disease progression.

### **5.1.3 The role of helminth infection in the suppression of allergic and autoimmune disease**

The incidence of allergic and autoimmune disease has been found to be considerably higher in the western world than in developing countries indicating that changes in environment and lifestyle might have an impact on the disease process. In the recently defined “Hygiene Hypothesis” it was suggested that a lack of appropriate priming of the immune response by infectious agents during childhood could be responsible for the increase in allergies [704, 705]. Though not stated in the hygiene hypothesis, a similar mechanism can be assumed for autoimmune disease as this type of disease is also rarely seen in third world countries [706]. The generally accepted mechanism underlying this theory is that exposure to parasites facilitates the development of an anti-inflammatory network

comprising of Tregs and DCs secreting Treg-promoting cytokines, such as IL10 and TGF $\beta$  which have been shown to counter-regulate Th1 and Th2 mediated inflammation. Indeed in support of this theory, it has been demonstrated in several studies that infection with parasites can not only prevent the induction of Th2-driven disease such as asthma but also Th1-mediated disease like RA, CIA (mouse model) or inflammatory bowel disease (IBD) [426, 707-712].

Parasitic helminths can infect humans for years or even decades. To achieve such long lasting infection these parasites have had to develop mechanisms to selectively suppress the host's immune system. It is therefore perhaps not surprising that as many helminths have developed the ability to modulate both Th1 and Th2 inflammatory responses to create a parasite survival-friendly environment, this has generated, as a by-product, the ability to also protect from allergies and autoimmune disease. One major mechanism by which such immunomodulation could be affected would be by the parasite facilitating the production of regulatory T cells by altering DC phenotypes. Indeed, patients infected with *Onchocerca volvulus* and *Brugia malayi* generally have elevated serum levels of IL10 and TGF $\beta$  [713, 714] and a number of studies have shown that *Schistosoma mansoni* and *Heligmosomoides polygyrus* can prevent acute allergic reactions in an IL10-dependent way [715, 716]. Interestingly, when schistosoma eggs are injected, rather than produced *in vivo* during infection, they induce a strong Th2 reaction and cause fatal anaphylaxis following secondary challenge with these eggs [717]. This indicates that schistosoma eggs on their own are strong allergens and that infection with the parasite is crucial for the development of a modified Th2 response.

A possible explanation regarding the mechanism of modified Th2 responses can be found in the theory of "concomitant immunity" [718]. In this theory it is suggested that the mature parasite itself manages parasite numbers by modulating the host's immune system such that only new infective larvae are killed whilst adult worms are unaffected. However as dead larvae would evoke an inflammatory response and lead to pathology which might be detrimental to the host, the parasite actively suppresses inflammation, most likely by secreting

immunomodulatory molecules. A different version of this theory, which would also be plausible with regard to the reduced Th1 and Th2 reactions in parasitic infections, is that live parasites and their offspring can cause severe pathology and hence a location-dependent selective suppression of these immune responses is crucial for the parasite survival. However not all parasitic helminths are able to suppress both Th1 and Th2 responses. For instance *Ascaris lumbricoides* and *Ascaris suum* have been shown to exacerbate allergic reactions in humans and in mice respectively [719-721] and similarly, infection with *Trichinella spiralis* can cause anaphylaxis in mice [722]. This indicates that helminths do not simply use a universal mechanism to suppress immune responses to infection which can be exploited therapeutically in the suppression of allergic and autoimmune processes.

During recent years a number of immunomodulatory molecules, such as ES-62 from *A. vitae*, schistosome lyso-PS and thioredoxin peroxidase from *Fasciola hepatica*, have been identified and their anti-inflammatory properties have been studied in detail [324, 416, 417, 616, 723]. It is currently believed that these molecules may exhibit at least some of their immunomodulatory effects on T cell responses by altering DCs to induce regulatory and/or anti-inflammatory T cell responses. As DCs have been shown to influence development of naturally occurring (*develop in the thymus during normal T cell maturation*) as well as induced (*develop in the periphery under sub-optimal Ag presentation or co-stimulation*) Tregs, modulation of DC phenotype by parasite products could lead to the production of distinct Treg phenotype under different inflammatory conditions [724]. It has been suggested over recent years that defined subtypes of DCs might have particular functions such as inducing tolerance or immunity or even Th1/Th2/Th17 differentiation [59, 61, 725] and consistent with this, it has been shown that different subtypes of DCs can be found in certain areas of the lung. Moreover, certain DC subtypes have been associated with disease eg pDCs in RA [726, 727]. However, presently it is unclear whether a certain DC subtype or maturation state is involved in initiating or indeed, suppressing an allergic response as there is increasing evidence that DC subsets are capable of extraordinary functional plasticity [728, 729]. Nevertheless, parasite products have been shown to have an influence on DC development [402, 413, 414, 418] and this could reflect

that they have the capability to shift production of DCs towards a specific subtype that could affect T cell development in the periphery and possibly even in the thymus. Indeed it has been shown that DCs isolated from the joints and the peripheral blood of RA patients showed phenotypic and functional differences when compared to those from healthy individuals [730, 731].

#### **5.1.4 ES-62 – a parasite product with immunomodulatory effects in murine models of asthma and rheumatoid arthritis**

A few years ago studies in mouse models of experimental colitis showed that infection with a number of helminths can reduce the severity of disease [732]. Furthermore, at the same time, the effects of helminth infection on IBD patients were tested [710-712] by deliberately infecting volunteers with the parasites and in this case an improvement of the disease could also be seen. However, as infection with parasites exposes patients to the risk of side effects, it was considered that a better therapeutic approach would be to exploit the ability of helminths to modulate the immune system. Thus, as helminths produce immunomodulatory molecules to dampen down inflammation and hence sustain their infection of the host, it was suggested that such molecules could be used on their own to mimic the parasite-induced regulatory effects without inducing all the side effects seen during infection. In recent years several of these molecules have been identified and purified [324, 723] and one of the best characterised of these molecules is ES-62, an excretory secretory protein from the filarial nematode *A. vitae*.

The immunomodulatory effects of ES-62 have been studied in depth over the last decade and during the last few years its therapeutic potential in models of inflammatory disease has also been tested [426]. Initially, as ES-62 had been shown to drive Th2 cell development [146] its effects were investigated in the CIA model which was then believed to be a mainly Th1-driven disease. Thus, treatment with ES-62 was expected to reduce the symptoms by promoting a Th2 and/or suppressing the Th1 response. Indeed the results of several studies clearly showed that ES-62 was able to significantly reduce arthritis progression, both prophylactically and therapeutically, suggesting that the molecule could be of

therapeutic use in patients who (i) have already developed the disease; (ii) are in early stages of the disease or (iii) have a strong family history of RA in whom RA is suspected but no destructive features of the disease, can be seen at the time of their first presentation. Interestingly, whilst ES-62 reduced Th1 responses such as IFN $\gamma$ , it did not induce a compensating Th2 (IL4/5) response but rather elevated IL10 [426] suggesting that rather than being simply a TH2-polarising agent, it was also capable of driving Treg or anti-inflammatory responses.

Consistent with this, and as discussed previously, some parasitic helminths, though generally driving a Th2 response, have been shown to exhibit anti-inflammatory effects against inhaled allergens/pathogens. According to the theory of concomitant immunity, an explanation for this is provided by the potential for differential, locally-driven regulatory mechanisms which help the pathogen to prolong survival in its host. Therefore, although in the periphery Th2/Treg cell development may be promoted to overcome Th1-like inflammatory immune reactions against the parasite, this effect can be reversed in an environment where an inflammatory process would be Th2-driven. Thus, in an inversely analogous situation to that described for ES-62-mediated suppression of Th1-like inflammation in the CIA model, this mechanism suggests that the potential ES-62 suppression of allergic airway inflammation would not facilitate an up-regulation of Th1 cells but rather inhibit the Th2 effect, possibly by promoting Treg development. To investigate whether ES-62, which had previously been shown to drive Th2-like responses [146], is a molecule capable of inducing such differential local responses, it was administered in a mouse model of allergic airway inflammation where the parasite product was indeed found to have anti-inflammatory potential, again both prophylactically and therapeutically [733, 734].

Taken together the obtained data so far suggests that ES-62 does not suppress Th1 responses by up-regulating a Th2 response or *vice versa* but rather might promote regulatory mechanisms that prevent excessive inflammation. DCs are mainly responsible for T cell polarisation and therefore perhaps present the best candidate target cells for immunomodulatory molecules such as ES-62. In the previous chapter, ES-62 has been shown to elicit differential functional DC



phenotypes depending on the DC subtypes targeted. Thus as lung DCs have a different phenotype than DCs found in other locations, a possible mechanism by which ES-62 might be capable of mediating differential immune responses in the lungs and systemically is by having a different effect on lung and systemic DC subsets.

## 5.2 Aims

It has previously been established that ES-62 exhibits immunomodulatory, anti-inflammatory potential in the collagen induced arthritis model of rheumatoid arthritis by suppressing pro-inflammatory (eg TNF $\alpha$ ) and Th1 (IFN $\gamma$ ) cytokine production without a compensatory increase in Th2-type (IL4/5) cytokines [426]. Furthermore, preliminary experiments suggested that ES-62 is also capable of reducing the severity of asthma-like disease in an OVA-induced airway inflammation model. This latter finding was initially quite surprising as asthma and allergies are Th2-driven diseases and infection with filarial nematodes usually is associated with an increase in Th2 cytokines. Indeed, ES-62 has previously been shown to promote, via modulation of DC maturation, Th2 cell development *in vitro* [146]. Therefore it was decided to further investigate whether ES-62 could indeed modulate both Th1- and Th2-mediated inflammation depending on the inflammatory process.

In particular, it was planned to confirm and further characterise the effects of ES-62 in the OVA-induced airway inflammation model. Specifically, it was decided to investigate whether

1. the anti-allergic/anti-Th2 effects of ES-62 were restricted to the local site of inflammation in the asthma mouse model
2. exposure to ES-62 *in vivo* altered the phenotype and the cytokine expression profile of bmDCs derived from mice in which airway inflammation had been induced
3. bmDCs derived from such mice exposed to ES-62 *in vivo* failed to drive Th2 responses *ex vivo*
4. ES-62 modulation of DC effector phenotype was facilitating the development of T regulatory cells and if so whether these cells were FoxP3 positive

Similarly, in order to further dissect the immunomodulatory mechanisms employed by ES-62 in the collagen-induced arthritis model it was decided to determine whether

5. ES-62 can differentially modulate the cytokine responses of DC subsets purified from spleens derived from CIA mice,
6. Such DCs induced differential effector T cell responses following exposure to ES-62

## 5.3 Results

### 5.3.1 The OVA-induced airway inflammation model

The OVA-induced airway inflammation model was conducted as described in the Materials and Methods section (Figure 2.2). To confirm that this protocol induced the hallmark features of asthma, mice were sacrificed on d 28 and broncho-alveolar lavage (BAL) was performed to determine cellular infiltration and the cytokine profile. In these investigations it was shown that macrophages were the most dominant cell type in the lavage fluid of all treatment groups. Nevertheless, significantly enhanced levels of eosinophils, which have been proposed to contribute to airway hyper-responsiveness in this model, were always identified in the BAL fluid of “asthma” group mice compared to control group mice. In addition, histologic analyses of the lung tissue from “asthma” group mice revealed profuse peribronchial inflammation, mucosal hyperplasia and eosinophil infiltration (Figure 5.1). Moreover, and consistent with a Th2-inflammatory reaction, cytokine analyses of the BAL fluid demonstrated enhanced levels of Th2 cytokines, IL4 and IL5, whereas no significant levels of the Th1 cytokine IFN $\gamma$  were detected (Figure 5.2).

### 5.3.2 ES-62 decreases OVA-induced Th2 responses in the draining lymph nodes

Th2 cells found in asthma-affected lungs are proposed to have become activated in the draining LNs (DLN) before they migrate to the site of inflammation. Therefore to determine whether ES-62 was mediating its effects *in vivo* by modulating Ag-specific responses by DLN cells, thoracic, pulmonary and cervical LNs were obtained from each treatment group and cultured *in vitro* and stimulated with Ag (OVA). These studies showed an elevated spontaneous proliferation of cells from mice in which airway inflammation had been induced (“asthma” groups) regardless of whether they had been exposed to ES-62 or not, relative to control or ES-62-treated control mice. Furthermore, re-stimulation with OVA induced Ag-specific proliferation of cells from mice in the “asthma” groups and this was not modulated by prior exposure *in vivo* to ES-62. However exposure to ES-62 *in vivo* was shown to inhibit Ag-specific IL4, IL5 and perhaps surprisingly, also IL10 production by lung

DLN cells *in vitro* whereas IFN $\gamma$  and TNF $\alpha$  production were unaltered or elevated respectively. Thus the results of these preliminary studies carried out in this lab suggested that ES-62 was inhibiting Ag-specific Th2 cytokine production and thereby reducing eosinophilia and inflammation in the lungs (Figure 5.3)

### **5.3.3 Differential effects of exposure to ES-62 *in vivo* on Ag-specific systemic and local responses**

In this model of OVA-induced airway inflammation, although the initial sensitisation to OVA was elicited by intraperitoneal injection, OVA challenges were administered intranasally with aerosolised OVA leading to inflammation mediated by the local DLNs. By contrast, each dose of ES-62 was administered subcutaneously to the scruff of the neck and was therefore expected to have a systemic effect. Thus, the effects of asthma were expected to be restricted to the lung DLNs whereas it was thought that ES-62 might induce local (DLN), and systemic (spleen and PLN) responses. To determine whether there were any differences in lymphocyte responses from those sites, OVA-splenic recall responses for splenocytes and peripheral lymph node cells were also characterised.

Similarly to the strong IL4/5 recall responses elicited by stimulation of the “asthma” group DLN cells with OVA, high levels of IL5 and IL4, could be detected in splenocyte cultures, including those of cells derived from mice with airway inflammation. In addition, low levels of IL25 (IL17E), which has been implicated in the pathogenesis of asthma, could be detected in the splenic cultures derived from mice with airway inflammation. Furthermore, although relatively high levels of IL13 were secreted spontaneously by cells from all groups, exposure to ES-62 *in vivo* abolished IL13 and reduced IL4 production in the OVA-stimulated cultures (Figure 5.4). In addition, upon re-stimulation with Ag, IL10 levels showed a marked increase in splenic cultures from “asthma” group mice which had been pre-treated with ES-62 *in vivo*. These results indicated that ES-62 strongly suppressed splenic Ag-specific Th2 responses, possibly via an increase in IL10 levels (Figure 5.4).

To determine whether this increase in Th2 cytokines was accompanied by a compensating increase of Th1 or Th17 cytokines, IFN $\gamma$  and IL17A levels were analysed in each group. Although only low levels were detected, Ag-specific release of IFN $\gamma$  from splenocytes were found to be up-regulated in both the “ES-62 control” and the “asthma + ES-62” groups compared to the “asthma” group in OVA-stimulated but not in control cultures. However, the very low levels of spontaneous production of IL17A were abolished by prior exposure to ES-62 (“asthma + ES-62” group) in healthy control cultures and could not be detected in any group upon OVA-stimulation (Figure 5.4). By contrast, analyses of the pro-inflammatory cytokines IL12p40 and TNF $\alpha$  revealed an Ag-dependent rise in spleen cell cultures from both the “asthma” and “ES-62-control” group mice although the levels of TNF $\alpha$  detected were very low. Interestingly, the highest levels of these cytokines were produced by splenocytes from the “asthma” group, both spontaneously and following OVA re-stimulation. Pre-treatment of “asthma” group mice with ES-62 *in vivo* slightly reduced the Ag induced increase in TNF $\alpha$  and completely inhibited production of IL12p40. Thus, collectively, these data may suggest that ES-62 mediates its anti-inflammatory effects by decreasing an inflammatory Th2 driven response, without inducing a compensatory local or systemic pro-inflammatory/Th1 response but rather, perhaps by promoting Treg induction via up-regulation of IL10 levels.

To further characterise potential differences between local and systemic inflammatory responses, and their potential modulation by ES-62, the Ag-specific responses of the PLN which were likely to function as DLN for ES-62-administration were also assessed from each treatment group. This revealed a quite different recall cytokine expression profile compared to the lung DLN and spleen cell cultures (Figure 5.5). Thus, whilst decreases in Ag-specific IL25 (IL17E) and IL13 levels were detected in splenic cultures derived from mice with airway inflammation pre-treated with ES-62 (“ES-62 + asthma” group), relative to those detected in cultures from mice with disease (“asthma” group), increases in these cytokines were seen in the analogous PLN cultures, although the levels of IL25 (IL17E) detected were very low. Similarly, rather than exhibiting the higher levels of Ag-specific IL10 production, PLN cells from the “ES-62 + asthma” group (as with

those from lung DLN) showed, if anything, reduced IL10 although the cells from this group produced the highest levels of this cytokine spontaneously. Consistent with the *in vivo* challenge with OVA being delivered by aerosol, Ag-specific levels of most other cytokines released by PLN cells were very low or undetectable in all treatment groups, although *in vivo* exposure to ES-62 alone results in very low level IL-5 secretion, which is consistent with its previously published ability to drive Th2 responses. The low level spontaneous production of Th1 (IFN $\gamma$ ) and Th17 (IL17A) cytokines by all groups was suppressed on challenge with Ag and hence displayed a similar pattern to that observed with spleen cultures.

Collectively, the Ag-recall cytokine release profiles from local, systemic and peripheral sites display some very interesting differences. In the “asthma” group, although the initial OVA sensitisation is administered systemically, local challenge by aerosol leads to recall responses in DLNs characterised by a strong Th2 cytokine profile. Furthermore, splenocytes from the “asthma” group respond with a similarly strong Th2 response and some IL25 (IL17E) can be detected. Likewise, ES-62-modulation of the “asthma” group responses is different depending on the immunological site examined. Thus, whilst such *in vivo* exposure to ES-62 significantly decreases the lung DLN Th2 recall response (IL4 and IL5) without a compensatory increase in Th1 cytokine (IFN $\gamma$ ) levels, in splenocyte cultures such ES-62 treatment exhibits an anti-Th2 effect but also strongly reduces the production of pro-inflammatory cytokines and enhances the production of IFN $\gamma$  and IL10. By contrast, although Ag-recall responses were generally very low in PLN cultures, ES-62 treatment of the “asthma” group drives a Th2 response (IL25 and IL13) and induces similar levels of pro-inflammatory cytokines to the ES-62 control group. Thus in conclusion, in mice in which asthma has been induced *in vivo*, treatment with ES-62 appears to exhibit an anti-Th2 effect at the local (lung) site of inflammation and in addition, suppresses pro-inflammatory cytokine production by splenocytes. By contrast, at peripheral sites where no OVA-specific Th2 recall responses can be detected in the “asthma” group, exposure to ES-62 tended to push Th2 responses, consistent with the proposal that ES-62 would drain to these sites.

#### 5.3.4 The effects of ES-62 treatment on bone marrow dendritic cells derived from “asthma” model mice

Following the above investigation of local and peripheral immune responses in asthma model mice and the modulatory effects of ES-62 on such inflammation, it was of interest to investigate the mechanisms employed by ES-62 to drive differential responses at distinct immunological sites and intervene with the inflammatory processes. Previous work conducted by this laboratory demonstrated that ES-62 can modulate the type of Th responses induced by altering the maturation phenotype and cytokine production capabilities of APCs such as DCs and macrophages (Chapter 3 and 4) [146, 418, 425]. Thus, to determine whether the modulation of DC function could also be responsible for the anti-inflammatory effects of ES-62 in the OVA-induced airway inflammation model, the maturation status and functional responses of bmDCs derived from each treatment group was assessed in terms of cell surface markers and cytokine profiles.

It has previously been established that bmDCs from naïve mice treated with ES-62 *in vitro* display a slightly more mature phenotype, characterised by the weak up-regulation of MHC Class II and co-stimulatory molecules, CD40, CD80 and CD86, than bmDCs derived from PBS control mice. However, their phenotype is relatively immature when compared to LPS-matured bmDCs. In contrast, *in vivo* treatment of naïve mice with ES-62 resulted in a less mature phenotype than seen in PBS control mice [403]. Furthermore in this case, *in vitro* stimulation with LPS only partially resulted in up-regulation of co-stimulatory molecules indicating that *in vivo* ES-62 exposure had a stronger suppressive effect on DC progenitors [403]. After establishing that the % of GM-CSF-elicited CD11c<sup>+</sup> cells was the same in all culture groups, it was decided to measure the levels of CD86 and MHCII expressed by such CD11c<sup>+</sup> DCs. Analysis of bmDCs from “asthma” group mice treated with ES-62 (“ES-62 + asthma” group) revealed that a higher % of these CD11c<sup>+</sup> cells expressed CD86 when compared to bmDCs from the “asthma” or “ES-62 alone” groups. By contrast, comparable % of “asthma” and “asthma + ES-62” bmDCS expressed CD86 following culture with LPS. MHCII expression was comparable and very low in all treatment groups relative to the control cells and



was not appreciably up-regulated following LPS-maturation (Figure 5.6). Indeed, the observed levels are much lower than those in normal mice and may reflect a less mature DC phenotype. Semimature DCs have been implicated in the development of regulatory cells and thus, this less mature DC might explain the increased amounts of IL10 found in DLN cultures from “asthma” group and splenocyte cultures from “ES-62 + asthma” group cultures reflecting a potential homeostatic mechanism to counter the observed inflammation.

To obtain a more detailed picture of the phenotype of these bmDCs derived from the asthma model mice, and to further characterise the effect of *in vivo* ES-62 treatment on these cells, culture supernatant was analysed for cytokine content (Figure 5.7). As expected, DCs that had not been further matured *in vitro* with LPS produced very low levels of IL12p40, TNF $\alpha$  and IL6 and only moderate levels of IL10, regardless of the treatment group, indicating that these DCs were still of an immature phenotype. Upon maturation with LPS, however, cytokine levels significantly and substantially increased and major differences amongst the three groups could be observed. More specifically, bmDCs from the “asthma” group of mice, perhaps surprisingly, displayed the highest levels of IL12p40, TNF $\alpha$  and IL6, whereas IL10 levels were similar to those found in ES-62 control cultures. This indicates that although the induction of asthma induces Th2 responses it also promotes the capability to prime general pro-inflammatory responses which may contribute to pathology. Consistent with this, high IL12p40 levels were also detected in splenocyte cultures from “asthma” groups.

By contrast, cells derived from mice treated with ES-62 prior to induction of airway hyper-responsiveness, were clearly inhibited from producing such enhanced levels of IL12p40 and TNF $\alpha$ , but not IL6, upon LPS maturation. In contrast IL10 levels produced by this group were increased in asthma group mice treated with ES-62, perhaps indicating that exposure to ES-62 *in vivo* promoted the consequent induction of a regulatory or anti-inflammatory phenotype of bmDCs presumably by modulating the development of DC precursors as suggested previously [403].

### **5.3.5 *In vivo* treatment of “asthma” model mice with ES-62 modulates the ability of bmDCs to consequently prime T cell responses *in vitro***

DCs are important cells in the immune system as they prime naïve T cells and thereby direct the T cell response. Thus it was investigated whether the effects of *in vivo* exposure to ES-62 modulated the ability of DCs, generated from mice in which airway inflammation was induced, to mediate T cell priming and effector phenotype polarisation. To do this, DCs from each treatment group were generated and then further matured in the presence or absence of LPS before being pulsed with Ag (OVA) and co-cultured with naïve OVA-specific transgenic TCR T cells (Figure 5.8).

T cells co-cultured with “immature” (no LPS maturation) bmDCs from the “asthma” group displayed a cytokine profile that was consistent with a strongly polarised Th2 response. In detail IL4, IL5 and IL-25 (IL17E) levels showed an Ag-dependent increase while the Th1 cytokine IFN $\gamma$  could not be detected (Figure 5.9). Indeed, although T cell priming, as indicated by Ag-dependent induction of IL-2, was comparable in all treatment groups, polarisation to a Th2 phenotype was more pronounced in the Asthma group than in either the “ES-62 control” group or the ES-62-treated “asthma” group. Indeed, although an Ag-dependent increase in IL4 and IL5 production could be detected in cultures containing bmDCs derived from the ES-62-treated “asthma” group, the levels of these cytokines were considerably lower than those obtained from “asthma” group and very similar to those of the “ES-62 control” group (no disease) cultures (Figure 5.9). Furthermore an Ag-dependent decrease of IL-25 (IL17E), a cytokine which is produced by Th2 cells and mast cells and plays an important role in asthma pathology, could be seen (Figure 5.9). By contrast, and perhaps consistent with their overall “inflammatory” phenotype, bmDCs derived from the “asthma” group induced comparable levels of the pro-inflammatory cytokines TNF $\alpha$ , IL12p40, IL6 and IL17A, also in an Ag-dependent manner, although the ES-62-treated “asthma” group induced the highest levels of IL17A (Figure 5.9).

Analysis of the cytokines produced by cultures containing bmDCs matured with LPS revealed a similar profile to that described above for “immature” bmDC-T cell cultures, albeit the levels of cytokines (except IL4 and IL25) were generally increased. The major difference between these two culture conditions was that the ability of bmDCs derived from the “asthma” group to induce IL25 (IL17E) production was abolished if such bmDCs were subsequently matured/immunomodulated by LPS. By contrast, cultures containing LPS-matured bmDCs from the “ES-62-control” group displayed increased levels of IL25 (IL17E) relative to the “asthma” group co-cultures perhaps suggesting that under such conditions ES-62 and LPS could synergise to promote disease (Figure 5.10). Moreover, the increased levels of IL17A induced by the ES-62-treated “asthma” group were maintained and even increased when such DCs were further matured by LPS. However, as it has been suggested in the literature that IL17A might play a role as a counter-regulatory or balancing mechanism in asthma by reducing the effect of IL25 (IL17E) [640], an increase in IL17A production could be one mechanism used by ES-62 to decrease the effects of the disease.

It has also been suggested that induction of Treg effector cells can ameliorate the outcome of asthma and allergies. Therefore, the levels of IL10 and TGF $\beta$  production, two cytokines that have been associated with Treg effector function, were investigated (Figure 5.9). Interestingly, no increase in the levels of IL10 was observed in cultures containing bmDCs derived from ES-62-treated “asthma” group mice whereas TGF $\beta$  levels appeared to be elevated at the highest concentration of OVA. Furthermore, a clear induction of IL10 and TGF $\beta$  was found if such bmDCs were further matured with LPS (Figure 5.10). This could indicate that bmDCs from ES-62-treated “asthma” group mice might only be able to induce the production of Ag-specific Tregs upon full maturation and as the Th2 response elicited by bmDCs from the “asthma” group was reduced by prior exposure to ES-62 *in vivo*, these results perhaps suggest that it is unlikely that increased Treg formation is the (only) mechanism employed by ES-62 to reduce the effects of asthma. Interestingly, the IL10 levels elicited by “immature” bmDCs are highest in the “asthma” group perhaps indicating that such semimature DCs (reduced MHC II) can also induce Ag-specific IL10 production that might act to suppress Th1 responses, and thus

promote a Th2 response or which might alternatively have a regulatory effect on systemic inflammatory (Th1 driving) reactions. As IL10 production in the lung has been shown to correlate with a decrease in the severity of asthma, it is possible that IL10 has site specific effects.

#### **5.3.6 Exposure to ES-62 *in vivo* results in an altered profile of T cell transcription factors expressed by T cells primed by bmDCs derived from “asthma” model mice**

The initiation of polarisation of Ag-specific immune responses by T cells is directed by key T cell transcription factors. Particularly, induction of Th1, Th2 and Treg development appears to require the expression of Tbet, GATA3 and FoxP3, respectively. Thus to further investigate the mechanisms by which ES-62 mediates immune responses, and to determine whether such modulation was initiated at the transcriptional level, it was decided to measure the expression of these transcription factors in the Ag-specific T cells from the above co-cultures by flow cytometry (Figure 5.11).

Consistent with the increased IL4 and IL5 production observed in “asthma” group co-cultures, a slight increase in GATA3 and corresponding decrease in T-bet could be seen in such T cells relative to the “ES-62 + asthma” group. Furthermore, the T-regulatory transcription factor FoxP3 was slightly increased corresponding to the elevated levels of IL10 and TGF $\beta$  in these cultures and perhaps accounting for the decrease in Tbet. Although the responses are low, LPS maturation generally appeared to reverse that GATA3/Tbet and FoxP3/Tbet relationships described above (Figure 5.11).

#### **5.3.7 *In vivo* pre-exposure of asthma model mice to ES-62 affects the expression of transcription factors in T cells from draining and peripheral lymph nodes**

To verify the data from bmDC-T cell cultures and to determine whether ES-62 treatment of mice, in which airway inflammation had subsequently been induced, resulted in an increase in the number of FoxP3 or GATA3 positive CD3<sup>+</sup> cells *in*

*vivo*, lung DLNs and PLNs sections were stained for expression of CD3, the B cell marker B220 and either FoxP3, GATA3 or Tbet and quantitatively analysed by LSC [434]. Analyses of the DLN group slides confirmed the results obtained in bmDC-T cell cultures. In detail, consistent with the induction of an inflammatory immune response, analysis showed that OVA-specific induction of airway inflammation correlated with an increase in GATA3 and also Foxp3 expression to that seen in DLN sections from naïve and ES-62 control groups. No Tbet expression could be detected in any of these groups. Interestingly, *in vivo* exposure to ES-62 prior to sensitisation and challenge with OVA slightly inhibited Foxp3 expression suggesting that ES-62 does not mediate its anti-inflammatory effects via induction of naturally occurring Tregs, although a role for FoxP3<sup>+</sup> Tregs cannot be ruled out. Furthermore the number of GATA3 positive cells was drastically reduced whereas the number of Tbet positive cells was increased indicating that at the affected site ES-62 actively decreases Th2 development and that possibly as a result Tbet positive cells can develop. (Figure 5.12). To investigate whether these effects were also seen in the periphery PLN (which would be the DLN for ES-62 administration) were stained in the same manner. Indeed, analysis of these sections revealed that whilst the number of GATA3 positive cells was still increased in “asthma” group sections, this was also accompanied by a corresponding increase in the number of Tbet positive cells, suggesting an unpolarised phenotype here where it is difficult to detect OVA-specific responses (Figure 5.12). Furthermore FoxP3 positive cells were still moderately high in number, albeit slightly reduced compared to numbers found in DLN sections. Collectively, and consistent with the finding that OVA-specific recall responses cannot be detected in these cells *ex vivo*, these data could indicate that these high GATA3 levels are not resulting in a Th2 response but rather, in conjunction with FoxP3 positive cells, acting to inhibit Tbet-induced Th1 reactions. Supporting the hypothesis that asthma induces some inflammatory responses in the periphery, *in vivo* exposure to ES-62 appeared able to reduce these effects of sensitisation and challenge with OVA, and this may be reflected by the reduced numbers of GATA3 and Tbet positive but increased numbers of FoxP3 positive cells observed (Figure 5.12). In addition to these quantitative data obtained from the LSC studies, it was decided to visualise FoxP3-staining of such sections by using fluorescent microscopy (Figure 5.13 and 5.14).

### 5.3.8 The collagen induced arthritis model

The model of collagen induced arthritis (CIA) is believed to exhibit the greatest similarity to human rheumatoid arthritis as it mimics many features of the human disease, such as joint erosion, histopathological changes and immunological changes including cell infiltrates and cytokine production [735]. Thus this model was chosen for all studies. CIA was induced as previously described in Materials and Methods (Figure 2.1) and the mice were monitored daily for signs of arthritis.

Previous work in this laboratory has demonstrated that prophylactic or therapeutic administration of ES-62 inhibits inflammation in these model mice. In the prophylactic model, this was characterised by significantly reduced arthritic swelling and delayed onset of arthritis which was demonstrated by an inhibition of arthritic score and a reduction in footpad inflammation [426]. When ES-62 was administered in a therapeutic fashion it significantly inhibited progression of joint inflammation and erosion [426]. Further studies indicated that serum samples from the “PBS” group mice (d 33) contained measurable quantities of the pro-inflammatory cytokines, IL1 $\alpha$ , IL12, and IL2. Similarly, KC, MIP1 $\alpha$ , IP10 and MIG, inflammatory chemokines that recruit neutrophils [736, 737] and are important markers of TNF $\alpha$  [738] and IFN $\gamma$ -mediated inflammation [739, 740] were also detected, along with basic fibroblast growth factor (FGF-2,) a potent stimulator of cellular proliferation and angiogenesis [741]. Prophylactic or therapeutic treatment of mice with ES-62, however, did not induce any significant effects on the serum levels of these inflammatory mediators [742].

It has previously been demonstrated that the anti-inflammatory actions of ES-62 in CIA correlate with suppression of Th1 responses in draining lymph node cells (DLN) challenged with Ag (CII), but not mitogen (ConA), *ex vivo* [426]. Thus to further assess the effects of ES-62 on the immune system, cell number and subset distribution following induction of CIA *in vivo* were analysed. These studies showed that ES-62 did not significantly alter the mean number of total lymph node or splenic cell populations after induction of CIA. Moreover, relative proportions of B220<sup>+</sup> B cells, CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic T cells were not modulated

by any of these treatments [742]. Consistent with this, in subsequent *ex vivo* cultures, such DLN and splenic cells were as responsive to stimulation with the mitogen, ConA as those from PBS-treated mice [742]. In contrast, ES-62-treated splenic cells were hypo-responsive to stimulation via the BCR (anti-Ig). Moreover, ES-62-treated cells were refractory to Ag (CII)-stimulation of T cells, results consistent with previous findings on the *in vivo* action of ES-62 in normal and CIA mice [422, 426, 743] (Figure 5.15).

Reflecting these findings, the *ex vivo* responses of DLN and splenic cultures from CIA mice to ConA in producing a wide range (TNF $\alpha$ , IL12, IL6, IL2, IFN $\beta$ , IL17, IL5, IL13 and GM-CSF) of cytokines were not modulated by prior *in vivo* exposure to ES-62 [742]. However, ES-62 significantly inhibited collagen-specific TNF $\alpha$  and IL12 responses. Interestingly, pre-exposure to ES-62 *in vivo* leads to enhanced spontaneous production of the regulatory cytokines IL10 and TGF $\beta$  by splenocytes from CIA mice (Figure 5.16).

### **5.3.9 Investigating the effects of ES-62 on the *in vitro* function of DCs derived from CIA model mice**

A preliminary study carried out in this laboratory investigated the effects of exposing bmDCs derived from CIA model mice to ES-62 *in vitro*. In this study it was shown that DCs from the CIA model exhibited a more mature phenotype compared to naïve mice of the same strain, which was characterised by an up-regulation of co-stimulatory molecules MHC II, CD40, CD80, CD86 and CD54. In addition to surface marker analyses, the cytokine profile of these cells was analysed and shown to display increased production of IL12, TNF $\alpha$ . *In vitro* stimulation of DCs from both the CIA model and naïve control mice with LPS resulted in a further maturation characterised by a higher expression of surface markers and increased pro-inflammatory cytokine production. To determine whether treatment of such mice with ES-62 *in vivo* induced a modulation of the bmDC phenotype and cytokine production profile, bmDCs from ES-62-treated mice were analysed. Interestingly, these studies showed that, despite ES-62 treatment, these DCs displayed an activated mature phenotype similar to that observed with

CIA model bmDCs. However, upon further stimulation with LPS, ES-62-treated CIA bmDCs exhibited a reduced CD40, CD54 and CD80 expression compared to LPS stimulated CIA bmDCs indicating that bmDCs from ES-62-treated CIA model mice might result in a reduced ability of these cells to respond to inflammatory stimuli. Consistent with this, bmDCs derived from CIA model mice pre-exposed to ES-62 *in vivo* also produced similarly lower amounts of TNF $\alpha$  although subsequent LPS-stimulation of these DCs reversed this trend (D. E. Kean and M. M. Harnett, unpublished results).

Therefore, to dissect the mechanism by which ES-62 mediates its actions in CIA, it was decided to further investigate the effects of the parasite product on DC function. However, as many different DC subtypes have been discovered over the last decade, which have different abilities to induce specific immune reactions (see chapter 3), it was decided to focus on the effects of ES-62 on the most well characterised DC subsets from the spleen as their *in vitro* responses to an inflammatory pathogen product (LPS or CpG) versus ES-62 have already been characterised from normal BALB/c mice (see chapter 3).

To determine the effects of ES-62-treatment *in vitro* on the interaction between splenic DC subtypes and T cells, spleens and LNs were harvested from CIA model mice. The induction of arthritis in these mice was confirmed by the clinical score and by front and rear paw measurements (Figure 5.17). Mice were monitored daily for signs of arthritis for which severity scores were derived as follows: 0 = normal, 1 = swollen digit, 2 = erythema plus swelling, 3 = swollen paw 4 = loss of function, and total score = median of the sum of four limbs. One group was treated with dexamethasone and used as a control group but not examined further in the *ex vivo* experiments.

To set up CIA-derived DC subtype cultures, spleens were harvested from the disease model mice and the different cDC subtypes and pDCs purified using FACS sorting. Initially it was planned to characterise the CD4<sup>+</sup>, CD8<sup>+</sup> and DN subsets of the cDC population present in CIA mice, however the proportion of DN DCs was found to be below 1% (normally around 20% of CD11c<sup>+</sup> cells) of all cDCs and



therefore, only the CD4<sup>+</sup> and CD8<sup>+</sup> cDCS and pDC populations were further analysed. This discovery was rather surprising and appears to be a result of the CIA induction as there are no reports in literature stating that DBA/1 mice have a different ratio of DC subtypes in the spleen. However as DCs are involved in the pathogenesis of CIA, and effects on their maturation status have been found in bmDC cultures, it is possible that either CD4<sup>+</sup>, CD8<sup>+</sup> and pDCs are preferentially produced in this disease model or that DN DCs change their phenotype in such a way that they did not match the sorting settings and hence were not detected.

DCs were then cultured in the presence of ES-62, LPS or CpG (pDCs only) for 24 h before their cytokine profile was analysed. Consistent with a mature phenotype, all DC groups secreted pro-inflammatory cytokines including TNF $\alpha$ , IL12p40, IL12p70 and IL6 (Figure 5.18). Furthermore, low IL10 secretion was detected in response to either ES-62 or LPS in cDC subtypes but only in the LPS-exposed group of pDCs (Figure 5.18). More specifically, comparison of cytokine production under ES-62- and LPS-treatment did not generally reveal any significant differences between the two cDC subtypes although ES-62-, but not LPS-maturation induced substantial IL12p70 release. However, and rather surprisingly, LPS-maturation of pDCs generally induced higher levels of these cytokines than maturation with either CpG or ES-62. Indeed in case of IL12p40 and IL6 these differences were highly significant, once again supporting the observation that pDCs can respond to LPS. Furthermore, rather surprisingly only LPS was able to induce an IL10 response (Figure 5.18).

In summary the obtained data showed that LPS- and ES-62-maturation generally caused similar levels of pro-inflammatory cytokine production in cDCs, albeit a slight trend towards higher levels (apart from IL12p70) by LPS-matured DCs could be observed. By contrast, pDCs responded with low cytokine production to ES-62- and CpG-induced maturation whereas LPS caused significantly higher levels of cytokine production in some cases. Thus, as slight differences in the cytokine profiles of the three DC subtypes could be detected it is possible that these cells play a different role in driving T cell responses in the CIA model.

To investigate such effects, spleen DC subtypes from CIA-model mice were matured with ES-62, LPS or CpG (pDCs only) in the presence or absence of Ag (collagen 10 or 50 µg/ml) and then co-cultured with activated CD62L<sup>-</sup>CD4<sup>+</sup> T cells from CIA model mice for 72 h before cytokine production was measured in the culture supernatant. Detectable levels of the pro-inflammatory cytokines TNFα, IL12p40, IL12p70, IL6 and of the regulatory cytokine IL10 could be found (Figure 5.19) however there was no evidence of either Th1 or Th2 cytokine production in these culture supernatants (data not shown).

In more detail, analyses of ES-62-CD4<sup>+</sup> DC- T cell cultures revealed a slight Ag-dependent decrease of TNFα and IL12p70 indicating that the parasite product could act to reduce priming of inflammation. Furthermore and by contrast, a slight Ag-dependent increase in IL6 and IL12p40 could be detected which could reflect Th17 polarisation but, as IL17 levels were barely detectable, the ES-62-driven polarisation towards Th17 cells is unlikely. As perhaps might be expected, LPS-exposed cultures displayed slightly elevated levels of IL12p40 at high levels of Ag restimulation and significantly higher production of IL12p70 compared to ES-62-cultures. Interestingly, a substantial increase in IL10 could also be observed which could either be DC-derived or a result of an induction of adaptive Tregs.

Collagen-specific T cells primed by CD8<sup>+</sup> DCs generally responded in a very similar manner to these stimulated by CD4<sup>+</sup> DCs. Although, in general, higher cytokine levels could be detected upon ES-62-treatment there was hardly any significant differences between the responses elicited by ES-62- and LPS-treated DCs. Furthermore, an Ag-dependent decrease in the production of all cytokines (TNFα, IL12p40, IL12p70, IL6) could be seen indicating that not even the maturation of the DCs with LPS could facilitate the production of pro-inflammatory cytokines upon rechallange. Interestingly, and in contrast to that observed with CD4<sup>+</sup> DCs, LPS stimulation did not lead to increased production of IL10. However ES-62-treated CD8<sup>+</sup> DCs were able to prime an IL10 response at moderate Ag concentrations which might suggest that ES-62 induces some kind of anti-inflammatory phenotype in these cells.

Co-cultures containing pDCs treated with either ES-62 or LPS generally displayed a similar TNF $\alpha$ , IL6 and IL17A secretion profile to that seen with cDCs with hardly any difference in cytokine levels between these treatment groups. However, although no Ag-dependent changes could be detected, levels of IL12p40 were significantly enhanced in cultures matured with LPS or CpG compared to cultures matured with ES-62 whilst IL12p70 levels were only found to be elevated upon CpG-treatment. Furthermore, IL10 levels were almost abolished in co-cultures containing pDCs treated with CpG whereas ES-62-treatment induced moderate production and LPS-exposure caused an Ag-dependent rise in the production of this cytokine. Taken together, these data suggest that while both CpG- and LPS-matured DCs both induce production of the IL12p40 subunit, only CpG-maturation facilitates the production of active IL12 by this DC subtype, although this induction appears to be Ag-independent. Furthermore when IL10 production was analysed, it was determined that the cytokine was Ag-dependently increased in cultures containing LPS-matured pDCs.

Collectively as the cytokine profile is very similar to that of DCs cultured on their own, and as hardly any Ag-dependent changes can be seen, it appears that restimulation of activated (CD62L<sup>+</sup>) T cells with collagen II has had very little effect on T cell cytokine production. However bidirectional signalling between macrophages and other immune cells is thought to contribute greatly to the pathologic process and hence low cytokine responses in DC-T cell cultures might be due to a lack of this interaction. To be more precise, McInnes et al formulated a theory [744] in which macrophages and fibroblast-like synoviocytes and activated T cells communicate in a cell-contact and cytokine-secretion-dependent manner which facilitates inflammation and arthritis pathology. In this model T cells reciprocally activate macrophages and fibroblast-like synoviocytes to produce pro-inflammatory cytokines. These in turn re-stimulate T cells to further activate macrophages and thus a positive feedback loop is created which keeps up inflammation in an inflammatory environment that is lacking in these *in vitro* studies.

## 5.4 Discussion

### 5.4.1 *In vivo* exposure to ES-62 inhibits OVA-specific Th2-mediated immune responses associated with airway inflammation in the local DLN but induces Th2-like responses systemically in the PLN

Preliminary work in this laboratory has shown that ES-62 is able to reduce asthma-like pulmonary inflammation in OVA-sensitised and challenged mice by inhibiting local IL4 production and influx of eosinophils into the lungs [733]. The current studies, characterising the effects of *in vivo* exposure to ES-62 on responses to OVA-sensitisation and challenge at different immunological sites, have confirmed that the *ex vivo* recall cytokine profile of asthma model mice reflects ES-62-mediated inhibition of the Ag-dependent Th2 cytokine production by DLN cells. By contrast, analysis of the Th2 cytokine recall profile of PLN cell cultures did not reveal any Ag-specific production of the classic Th2 cytokines, IL4 and IL5 indicating that in asthma model mice such cytokine production is restricted to the local site of inflammation. However IL17E and IL13, two Th2-related cytokines that have been implicated in the pathogenesis of asthma, as well as pro-inflammatory cytokines (IL12p40 and TNF $\alpha$ ) were found to be elevated in spleen but not in PLN cell cultures (Figure 5.4 and 5.5). In addition, in spleen cell cultures, Ag re-stimulation further enhanced the production of all of these cytokines indicating that induction of asthma did not only have local effects, but also to some degree altered the systemic immune response while leaving peripheral immune responses unaffected.

With the aim of addressing whether ES-62 was specifically targeting local asthma-induced immune responses, recall cytokine analyses were conducted on DLN, spleen and PLN cultures derived from asthma model mice that were pre-exposed to ES-62 *in vivo*. Interestingly, whilst Ag-specific IL25 (IL17E) and IL13 production was decreased in DLN and spleen cell cultures, the opposite was true for PLN cell cultures. Furthermore, ES-62 induced an enhanced production of the regulatory cytokine IL10 in spleen cell cultures upon Ag re-challenge whereas a decrease was found in PLN cultures under the same conditions (Figure 5.4 and 5.4). Thus these data indicated that in those parts of the immune system which played a role

in mediating the response to airway inflammation (DLN and spleen), ES-62 was inhibiting Ag-specific Th2 responses whereas in the sites not involved in this allergic immune response but presumably responsible for responding to ES-62 administration (PLN), the parasite product was facilitating a more Th2-like response commensurate with that associated with the immune response to ES-62 itself [146]. Furthermore, *in vivo* exposure to ES-62 also reduced the pro-inflammatory cytokine production capabilities of splenic cells from asthma model mice whilst slightly stimulating their production in the periphery, a finding in keeping with the known adjuvant properties of ES-62 [146, 418]. Thus, it appears that, rather than simply driving Th2 effects to modulate the host immune responses away from damaging Th1 pathology during filarial infection, ES-62 can act to dampen local aberrant inflammation irregardless of the phenotype involved.

A clue to the mechanism by which ES-62 could be mediating its anti-Th2/anti-inflammatory effects in this model might be found in the elevated levels of IL10 observed in splenic, and to some extent DLN cultures (Figure 5.3 and 5.4). IL10, a regulatory cytokine, has been shown to be produced by natural occurring Tregs, adaptive Tregs [745] B1 cells [746] and also by some DCs, including lung DCs [653]. Several studies have indicated that increased levels of IL10 in the lung correlate with a reduced severity of asthma [747-749] and indeed a recent study has suggested that allergic disease may reflect a result of an imbalance of IL10-secreting regulatory T cells and Th2 cells [750]. Therefore one possible mechanism employed by ES-62 for facilitating an anti-allergic immune reaction, that is localised to the affected areas, would be to re-establish this regulatory/effector cell balance. This could be established by promoting enhanced secretion of IL10 from lung DCs or DCs in the spleen which in turn could lead to the enhanced production of adaptive T regulatory cells. Another possibility would be the induction of natural T regulatory cells in the thymus and/or their increased migration into the affected LNs.

#### **5.4.2 *In vivo* treatment with ES-62 suppresses the inflammatory potential of bone marrow-derived DCs from asthma model mice**

The above described experiments have clearly shown that ES-62 is able to inhibit Th2-mediated immune responses associated with local airway inflammation whilst inducing Th2-like responses in the periphery, indicating that ES-62 must employ a regulatory mechanism which enables it to respond to, and counteract inflammatory responses, independently of whether they are Th1 or Th2 based.

Naturally occurring Tregs, which originate in the thymus and express the transcription factor FoxP3, are thought to have a specialised role in controlling immune responses [332, 395, 396]. One of the mechanisms by which natural Tregs exhibit their functions is, as stated above, through the induction of regulatory cytokines such as IL10 and TGF $\beta$  [348, 751-754]. To dissect the mechanism by which ES-62 mediates its anti-inflammatory action in this model, the consequences of pre-exposure of asthma model mice to ES-62 *in vivo* on the subsequent maturation of bone marrow-derived DCs, and their ability to induce Treg cell effector function was analysed. DCs are crucial in the initiation of adaptive immune responses as they efficiently present Ag to naïve T cells. To properly polarise these T cells, DCs express cell surface molecules and secrete specific cytokines. Therefore as several studies in this laboratory have shown that ES-62 can exert at least some of its immunomodulatory actions by altering the surface marker expression and cytokine profile of bmDCs [146, 418, 425], it was proposed that DCs in the asthma model might be modulated by similar mechanisms. Examination of the DC phenotype revealed that bmDCs from ES-62-treated asthma model mice, bmDCs from “asthma” model mice as well as bmDCs from mice treated with ES-62 alone (no disease ES-62 control) displayed a less mature phenotype than bmDCs from PBS control mice (Figure 5.6). However, no significant difference in phenotype could be seen between the bmDCs derived from “ES-62”, “asthma” and ES-62-treated “asthma” model mice. However, when the cytokine expression profile was analysed, clear differences between these three groups were obtained. More specifically, *in vitro* LPS-matured bmDCs from “asthma” model mice displayed a pro-inflammatory cytokine profile. By contrast, bmDCs from ES-62-

matured “asthma” model mice behaved fairly similarly to bmDCs from “ES-62 control” mice in producing significantly less pro-inflammatory cytokines (IL12p40 and TNF $\alpha$ ). Interestingly, a slight increase in IL10 production could be seen by bmDCs from ES-62-treated “asthma” model mice, compared to those from the other groups, indicating that these DC might have been rewired to be able to induce adaptive Tregs (Figure 5.7). Furthermore as these bone marrow-derived cells were not fully differentiated DCs, when the *ex vivo* cultures were initiated, these results indicate that exposure to ES-62 *in vivo* has the capacity to alter DC precursor maturation to potentially promote anti-inflammatory responses in this model.

#### **5.4.3 *In vivo* treatment of “asthma” model mice with ES-62 inhibits the development of Th2 cells in *in vitro* cultures**

To determine whether ES-62-modulated bmDCs could indeed drive regulatory responses, these cells were subsequently cultured with OVA-specific T cells and the resulting Ag-specific cytokine profile was analysed. Consistent with the hypothesis that these bmDCs might be able to induce regulatory cells, Ag-specific production of TGF $\beta$  was detected in these cultures (“ES-62 + asthma” group). However, pro-inflammatory cytokine levels were also increased in these co-cultures suggesting that ES-62 treatment *in vivo* was possibly not sufficient enough to fully inhibit the inflammatory process suggesting that further doses of ES-62 throughout the model protocol or at the *in vitro* stage may have induced more dramatic effects. Nevertheless, even though only these progenitors were exposed to ES-62 *in vivo*, such modulated bmDCs (“ES-62 + asthma” group) produced significantly reduced Th2 responses relative to DCs generated from the “asthma” group. To corroborate this ES-62-mediated reduction of a Th2 response at the transcriptional level GATA3, the key transcription factor for Th2 responses, was analysed. Consistent with the cytokine findings, GATA3 was reduced compared to control T cells whilst Tbet, the key transcription factor for Th1 responses was slightly increased. However, perhaps surprisingly, given the elevation of TGF $\beta$  levels in the context of a reduced Th2 response, and absence of a compensatory increase in Th1 phenotype, the decrease in GATA3 was not accompanied by an

increase in FoxP3. These results therefore suggested that ES-62 was not in fact modulating DC maturation to drive Treg induction, although a role for Foxp3 negative Tregs could not be ruled out.

#### **5.4.4 *In vivo* treatment of asthma model mice with ES-62 reduces the number of natural regulatory cells in DLNs and PLNs**

To further investigate whether ES-62 facilitated its anti-allergic effects in the “asthma” model by increasing the number of naturally-occurring T regulatory cells in DLNs, sections were stained for FoxP3 expression and visualised by fluorescence microscopy (Figure 5.13). Consistent with this, whilst sections obtained from naïve mice, ES-62-treated control mice and indeed even the “asthma” model mice all revealed the presence of FoxP3 positive cells, analyses of the stained sections did not show any evidence of natural occurring Tregs in ES-62-treated “asthma” model mice. To confirm this data, and to determine the number of FoxP3 positive cells in the T cell areas of each LNs section, sections were analysed by LSC (Figure 5.12). Similar numbers of FoxP3 positive cells were indeed found in ES-62 and PBS control mice. Interestingly, although rather surprisingly quite a large increase in the number of FoxP3 positive cells was found in “asthma” model mice, this was completely suppressed in the ES-62-treated “asthma” model mice. Consistent with these findings, FACS analyses of FOXP3 expression in T cells co-cultured with bmDCs from the various groups showed an increase in the asthma model group which was reversed by ES-62 treatment (Figure 5.11).

A very similar picture was seen in sections from PLNs with the only difference being that, in contrast to DLN sections, PLN sections from ES-62-treated “asthma” model mice displayed similar numbers of naturally-occurring Tregs compared to “asthma” model mice (Figure 5.12 and 5.14). Interestingly, analyses of the FACS data, from LPS-exposed bmDC-T cell cultures, displayed a similar FoxP3 expression profile to the one found in PLNs indicating that LPS-maturation induces these bmDCs to induce responses similar to those found in PLN. Thus, these



results supported the mRNA data in that ES-62 treatment indeed did appear to induce a loss of natural occurring Tregs in the local DLN but not in peripheral LNs. A possible explanation for this could be that ES-62 rather directly works on reducing Th2 response or maybe even enhances up-regulation of Th1 promoting factors. Consistent with this proposal, GATA3 levels were strongly reduced and Tbet expression was up-regulated in both DLN sections and co-cultures from the ES-62-treated “asthma” model mice (Figure 5.11 and 5.12). By contrast no such switch in transcription factor expression could be detected in T cells from PLNs indicating that this effect was indeed restricted to cells at the affected site.

Furthermore, these data indicated that the IL10 detected in the ES-62-treated “asthma” group spleen cultures, is unlikely to be produced by FoxP3 positive cells but rather might be a product of either B1 cells, DCs or adaptive FoxP3 negative Tregs. As ES-62 is known to alter the DC phenotype it is possible that, under the conditions found in the “asthma” model, the parasite product facilitates the production of IL10-producing FoxP3 negative Tregs by modulating DC effector function. Furthermore, as relatively high IL10 levels were spontaneously produced by PLN cultures before Ag re-challenge *in vitro*, it can be speculated that the production of such adaptive FoxP3 negative Tregs is not restricted to DLNs and the spleen but occurs throughout the body. Interestingly, a recent study showed that infection with the gastrointestinal nematode *Heligossomoides polygyrus* can suppress allergic airway inflammation by inducing adaptive Tregs [755]. This adaptive Treg population was found in the MLNs and was transferred into uninfected sensitised recipients where they exhibited a protective effect. Interestingly however, these MLN-derived adaptive Tregs were FoxP3<sup>+</sup> and associated with a high TGFβ production. Indeed, a recent study confirmed that gut DCs can induce differentiation of FoxP3<sup>+</sup> Tregs from FoxP3 negative precursors using endogenous TGFβ [756]. It was also shown that these adaptive Tregs, although able to produce IL10, did not depend on this cytokine to develop. Thus, as ES-62 treatment, which mimics infection with *A. viteae* does not induce differentiation of FoxP3<sup>+</sup> cells despite inducing high levels of IL10 in “ES-62-asthma” group samples from the affected sites (DLN and spleen), it is likely that

this parasite product facilitates induction of a different type of Treg cell or has found a different mechanism of suppressing local allergic responses.

In summary the data indicate that ES-62 is capable of driving differential immune responses at distinct sites within an animal, including down-regulating aberrant inflammatory immune responses locally whilst inducing a similar phenotype of responses, but at a low level, in the periphery. In detail, it was shown that ES-62-treatment in “asthma” model mice reduced the local lung-associated Th2 response, at both the transcriptional and also cytokine secretion level, whilst in PLNs, the opposite response was found. Furthermore this immunomodulation of the DLN response did not induce a compensatory increase in Th1 cytokines, but rather, reflected an elevation in the levels of IL10 that could be detected. However, in spleen cell cultures, a slight increase in IFN $\gamma$  could be seen and consistent with this, Tbet expression was increased in ES-62-treated “asthma” group T cells. However, and perhaps unexpectedly, ES-62-treatment was shown to result in a complete loss of natural T regulatory cells in “asthma” model LNs. Whether this effect is a result of an increase in adaptive Tregs, or due to some other process, has still to be investigated.

#### **5.4.5 The effects of ES-62 exposure on individual DC subtypes derived from spleens of CIA model mice**

ES-62 has been shown to exert anti-inflammatory effects, both prophylactically and therapeutically, in CIA models of arthritis as manifested by prevention of the onset of disease or by reduction in the severity of existing inflammation [426]. Furthermore it is believed that DC play a role in the pathogenesis of CIA and preliminary studies from this lab have shown that bmDCs from CIA model mice exhibit a more active and mature phenotype compared to bmDCs from naïve mice. However, as many different DC subtypes have been discovered over the last decade, that have been reported to differentially induce specific immune reactions (see chapter 3), it was decided to study the potential immunomodulatory effects of ES-62 on a number of individual DC subtypes typically found in the spleen from CIA model mice.

Interestingly, the first main difference between CIA model mice and naïve mice became obvious when purifying the individual DC subtypes of interest. Specifically, in naïve mice, three major cDC subsets, namely CD4<sup>+</sup>, CD8<sup>+</sup> and DN DCs can be found: CD4<sup>+</sup> DCs make up about 65% of all cDCs whereas the other subtypes account for 15-20% each. However, whilst a slight increase in the % of the CD4<sup>+</sup>DC and the CD8<sup>+</sup> DC subpopulations could be detected, compared to the % found in spleens of naïve mice, the DN DC population was hardly detectable and accounted for less than 1% of all splenic cDCs in CIA model mice. The proportion of pDCs among all splenic DCs remained the same as in naïve mice. This suggested that either CIA-induction selectively inhibits DN DC development or that DN DCs undergo substantial phenotypic changes. As DCs are very plastic cells and can alter their surface phenotype quite easily in response to various stimuli it is possible that these cells either down-regulated or up-regulated certain surface markers, like CD11b, that are widely used to distinguish them and hence render them “undetectable “ by the FACS sorting protocol optimised for naïve cells.

When analysing the cytokine data obtained from the remaining 3 subtypes (CD4<sup>+</sup>, CD8<sup>+</sup>, pDC), very few differences amongst these subtype cultures could be detected (Figure 5.18). Specifically, a very similar pattern was obtained regarding the production of TNF $\alpha$ , IL12p40, and IL10 by the CD4<sup>+</sup> and CD8<sup>+</sup> cDC subtypes in response to maturation with ES-62 or LPS. Interestingly, this was also seen with pDCs where levels of these cytokines did not vary greatly between the ES-62 and CpG group. However LPS stimulation of pDCs increased all cytokines but IL12p70. This was a quite surprising finding as pDCs were predicted to be fairly unresponsive to LPS as they lack the expression of TLR4. However as discussed in chapter 3, experiments in this laboratory have shown that pDCs can indeed respond to treatment with this pathogen product. Furthermore, to investigate whether the IL12p40 subunit was used to form active IL12p70 or generate IL23, a cytokine crucial for a Th17 response, IL12p70 and IL6 levels were analysed. With regard to the IL6 production no differences between the DC subtypes or the treatment groups could be obtained, indicating that if Th17 cell formation was promoted at least IL6, an important cytokine for the initial development of these

cells, had to be produced by a different cell type, possibly by macrophages. However evaluation of the IL12p40 versus p70 production of the three DC types indicated that ES-62 exposure generally reduced production of IL12p40, but not IL12p70, compared to LPS- and CpG-treated cultures indicating that the parasite product did not contribute to IL23 production.

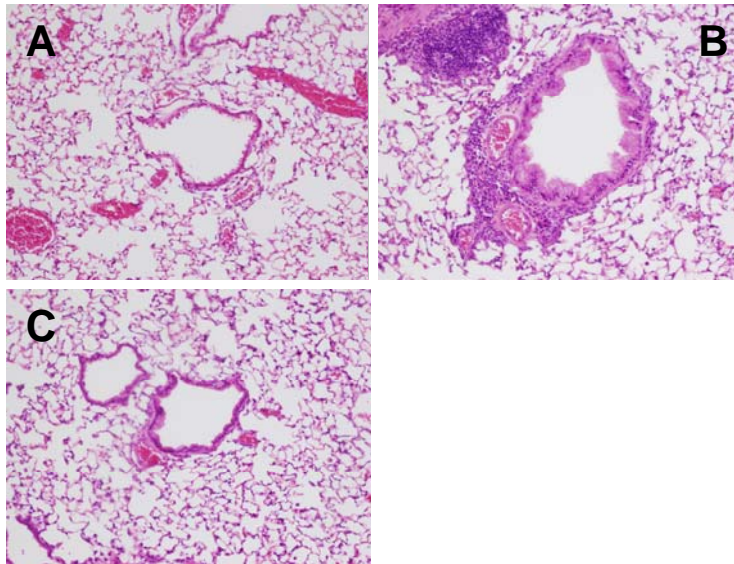
#### **5.4.6 The effects of *in vitro* exposure to ES-62 on the interaction of splenic DC subtypes and T cells from CIA model mice**

To further investigate the potential immunomodulatory effects of ES-62 on DC subtype function in arthritis, the ability of ES-62 to modulate T cell priming and effector function development driven by the individual DC subsets from CIA model mice was investigated.

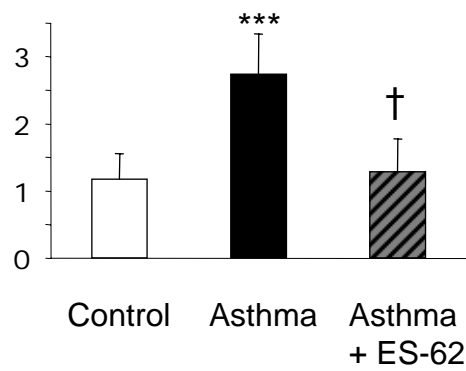
No trace of Th1 or Th2 cytokines could be found in any culture indicating that, although some pro-inflammatory cytokines (TNF, IL12) were produced, these did not lead to a Th1 response. As it has already been indicated in literature that the pathogenesis of RA is not due to a Th1 immune response but rather might be Th17 driven, the lack of Th1 or Th2 cytokines was perhaps not surprising. However, only traces of IL17A were detected in these culture supernatants and hence an increased production of Th17 cells could not be confirmed. Furthermore ES-62 was unable to enhance IL10 or TGF $\beta$  production indicating that under these conditions the parasite product was unlikely to be driving regulatory responses.

**Figure 5.1 ES-62 treatment of asthma model mice reduces severity of airway pathology**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d -2, 12, 25 and 27 and control mice received PBS. Intact lungs were carefully dissected from mice in each treatment group and fixed in formalin. Sections of lung tissue from Control (A), Asthma (B) and Asthma + ES-62 (C) mice were stained using H & E staining and viewed using a x10 objective. D, 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. Histological analysis was performed by C. McSharry.



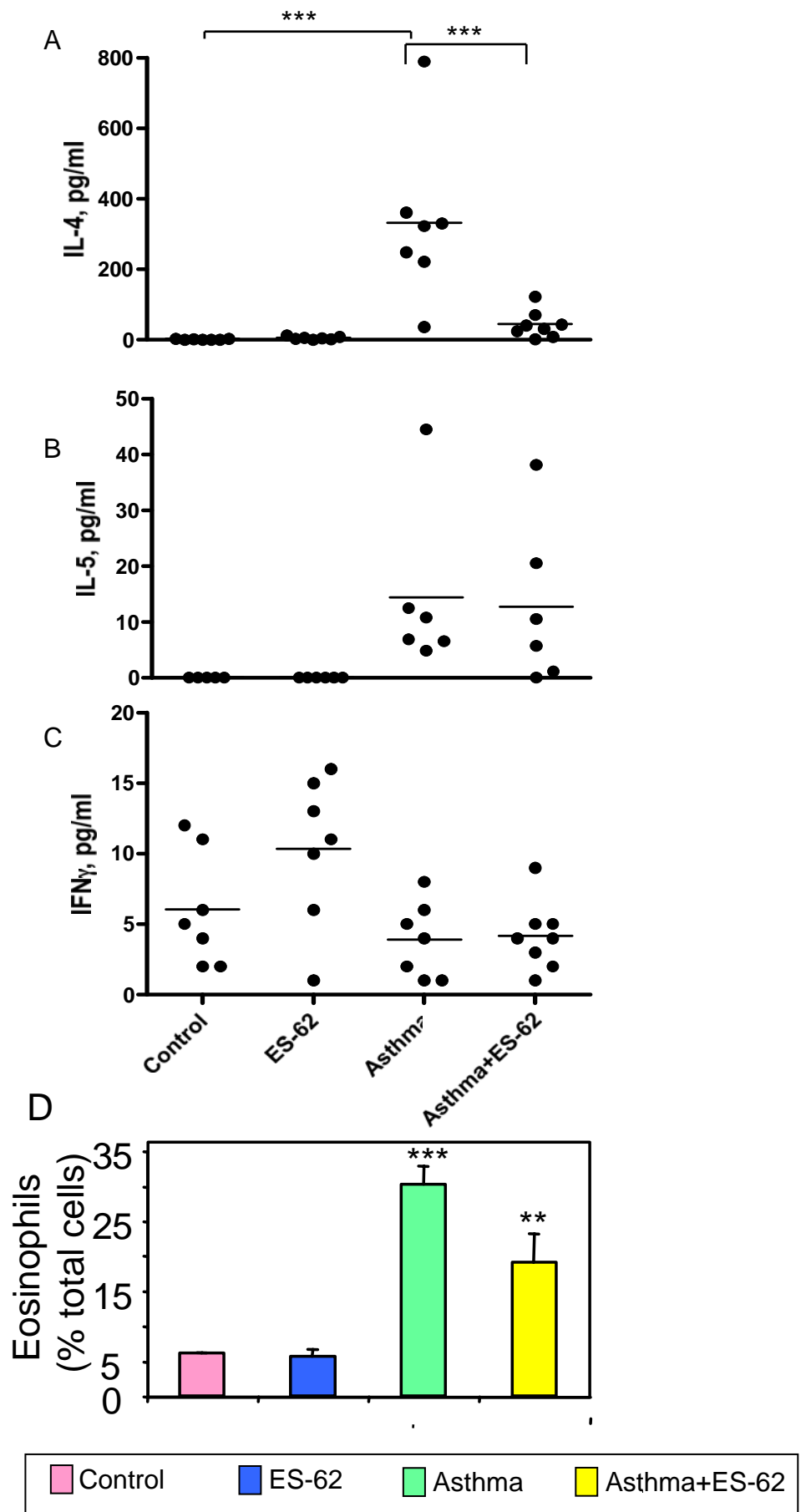
**D**



## **Figure 5.2 ES-62 inhibits ovalbumin-induced airway Th2 cytokine production**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d -2, 12, 25 and 27 and control mice received PBS. Mice were sacrificed on d 28 and broncho-alveolar lavage (BAL) performed as described in Chapter 2. The concentration of IL-4 (A), IL-5 (B) and IFN $\gamma$  (C) in the BAL fluid was analysed for each mouse by ELISA. (D) Eosinophil count data are expressed as mean  $\pm$  SEM ( $n=6$  mice/group). 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.

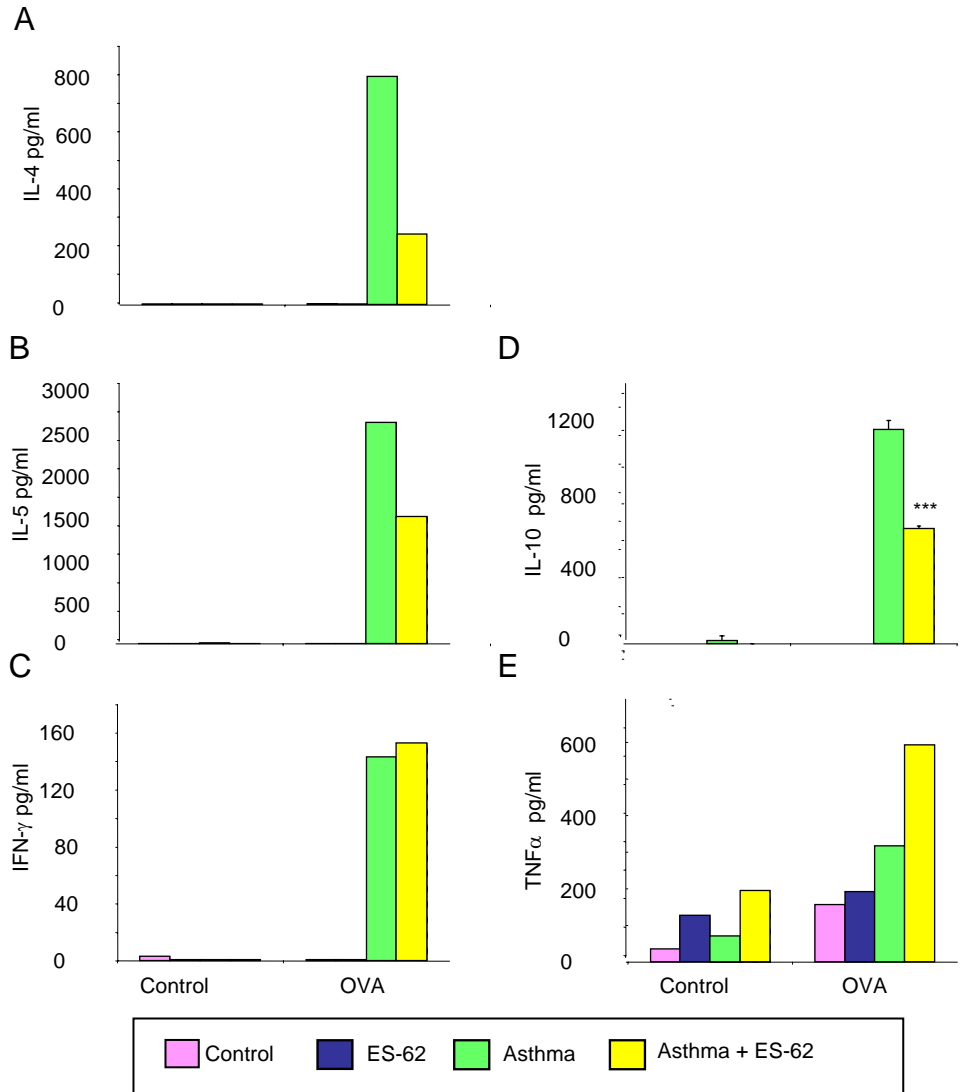
\*\*\*,  $p<0.001$ (by ANOVA).





**Figure 5.3 ES-62 treatment *in vivo* inhibits draining lymph node cell Ag-specific TH2 cytokine production during recall responses**

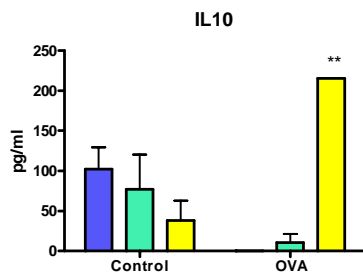
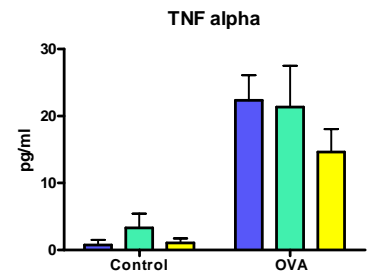
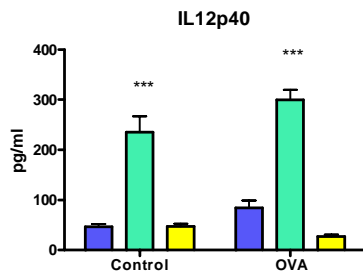
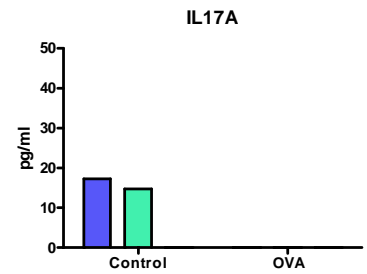
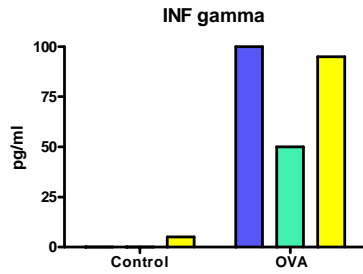
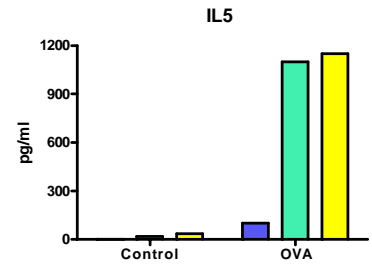
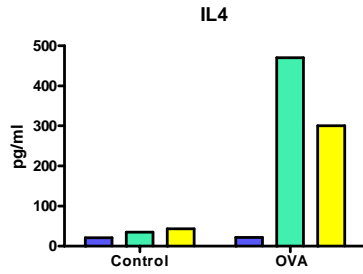
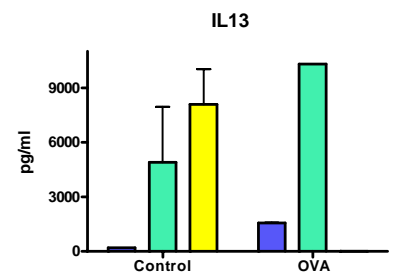
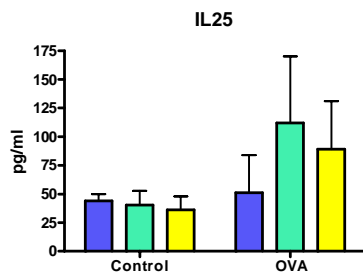
Mice were treated and sacrificed as described in the legend 4.2 and thoracic, pulmonary and cervical lymph nodes from each mouse removed. Lung draining lymph node cells from mice in each group were pooled and cultured with medium alone (Control) or OVA (Ova; 500 µg/ml) for 72h. Culture supernatant concentrations of IL-4 (B), IL-5 (C), IFN $\gamma$  (E) and TNF $\alpha$  (F) were measured by Luminex. Luminex data is expressed as mean of duplicate samples ( $n=2$ ) and are representative of 3 experiments. Culture supernatant concentrations of IL-10 (D) were measured by ELISA and data are expressed as mean  $\pm$  SD ( $n=3$ ) and are representative of 3 experiments. \*\*\*,  $p<0.001$  vs Asthma treatment group.



**Figure 5.4 ES-62 treatment *in vivo* inhibits spleen cell Ag-specific TH2 cytokine production during recall responses**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Spleens were harvested and cells from each group were pooled and cultured in the presence or absence of OVA (500 µg/ml) for 72 hrs. Cytokine production in the culture supernatant was analysed using ELISA and data are expressed as mean ± SD. Data representative of at least 3 experiments

\*\*\* p<0.005 AS vs ES-62 +AS and ES-62, \*\*p<0.01ES-62 +AS vs ES-62 and AS

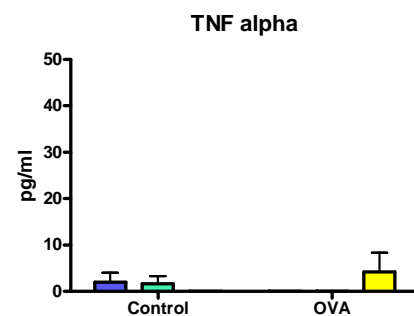
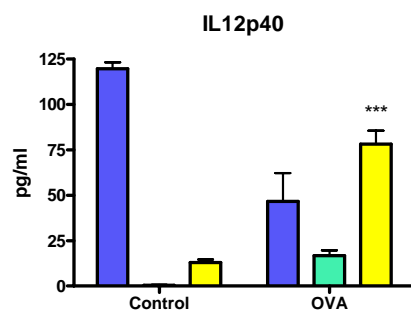
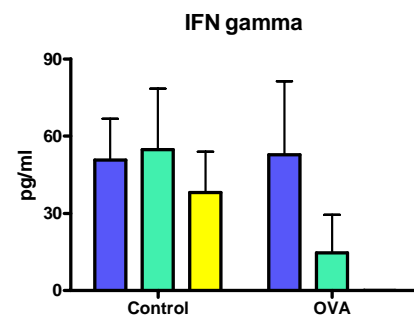
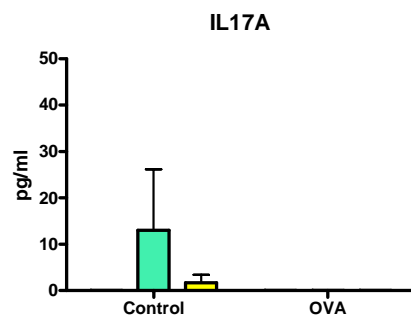
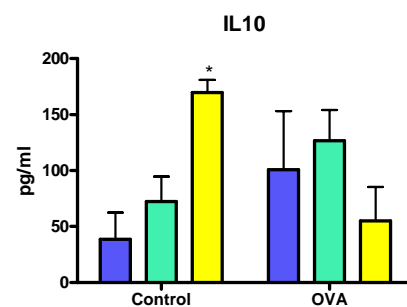
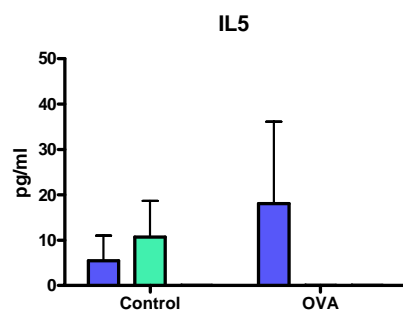
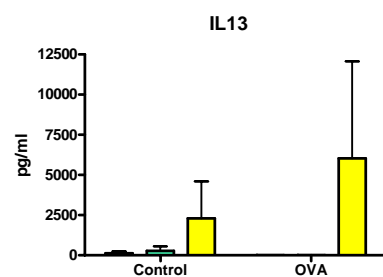
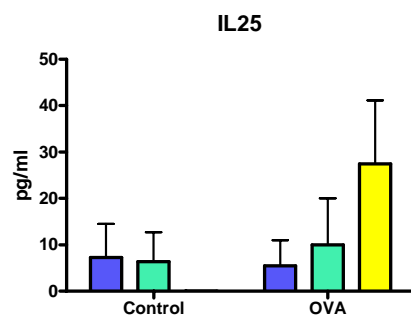


ES-62 AS ES-62 AS

**Figure 5.5 ES-62 treatment *in vivo* inhibits peripheral lymph node cell Ag-specific TH2 cytokine production during recall responses**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Peripheral lymph nodes were harvested and cells from each group were pooled and cultured in the presence or absence of OVA (500 µg/ml) for 72 hrs. Cytokine production in the culture supernatant was analysed using ELISA and data are expressed as mean ± SD. Data are representative of at least 3 experiments.

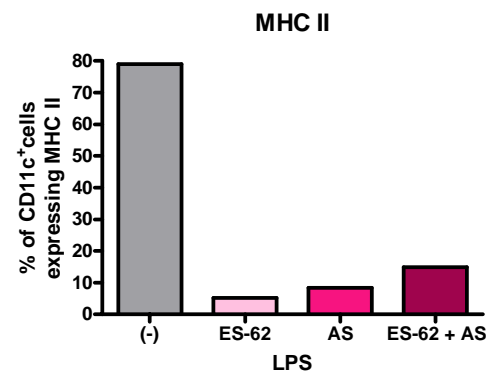
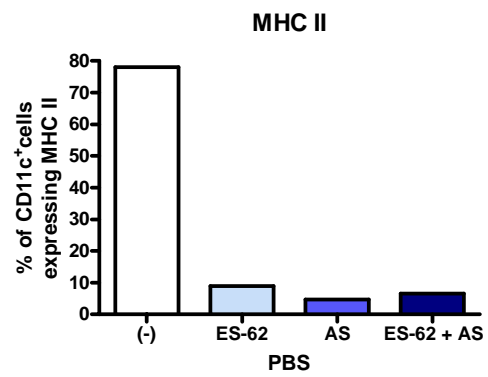
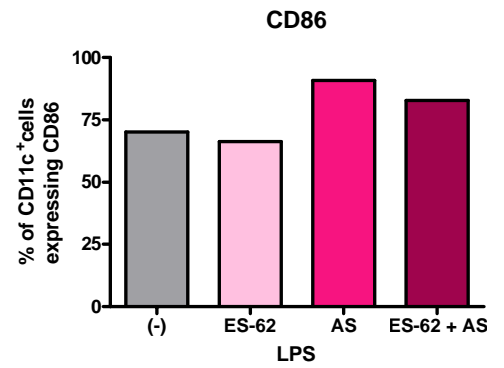
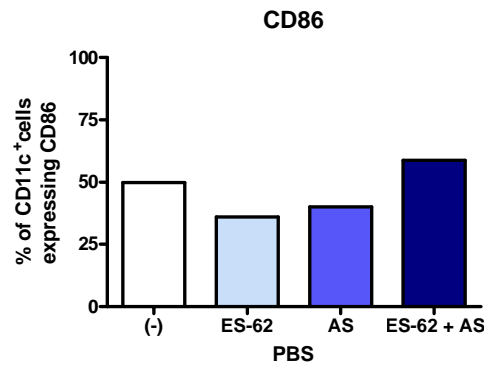
\*\*\*  $p < 0.001$  ES-62 + AS vs AS, \*  $p < 0.05$  ES-62 + AS vs ES-62 and AS



ES-62 AS ES-62 AS

**Figure 5.6 ES-62 treatment of the ovalbumin-induced asthma model *in vivo* modulates bone marrow-derived dendritic cell surface marker expression**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Bone marrow was harvested pooled and cultured *in vitro* in the presence of GM-CSF for 7 d. On d 7 cells were stimulated with PBS or LPS (1 µg/ml) for 24 h. Expression of CD86 and MHC class II was analysed by flow cytometry. Data were gated on CD11c<sup>+</sup> population and expressed as percentage of surface marker positive cells.

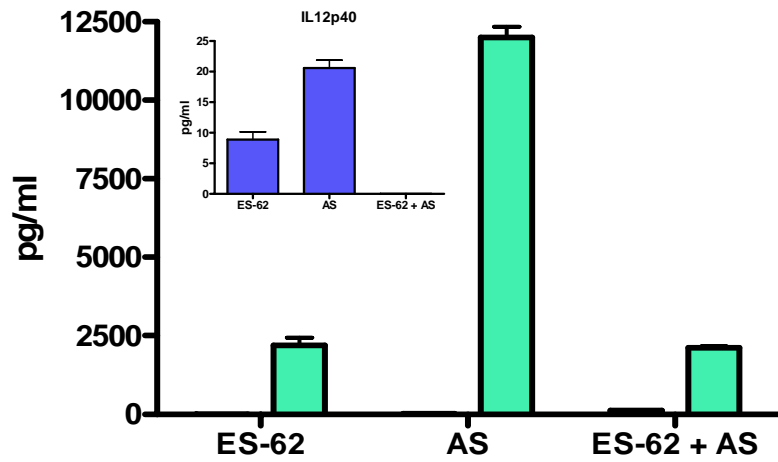
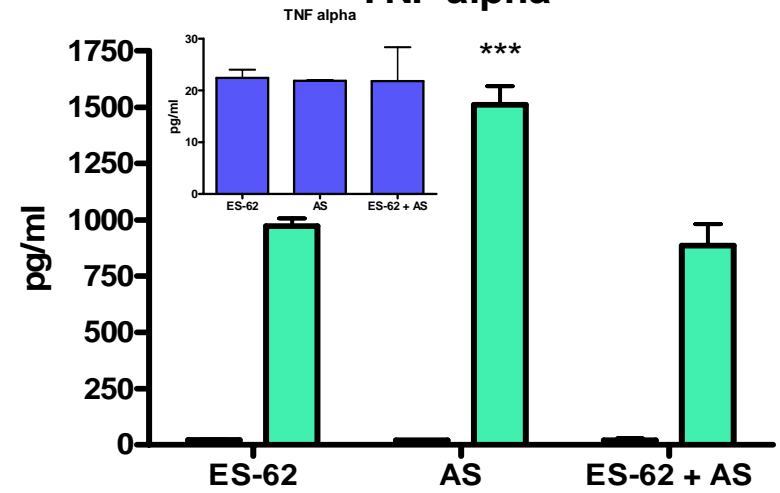




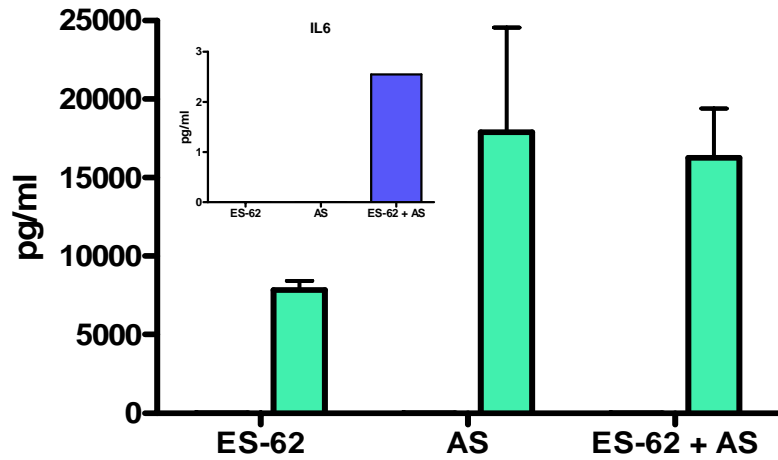
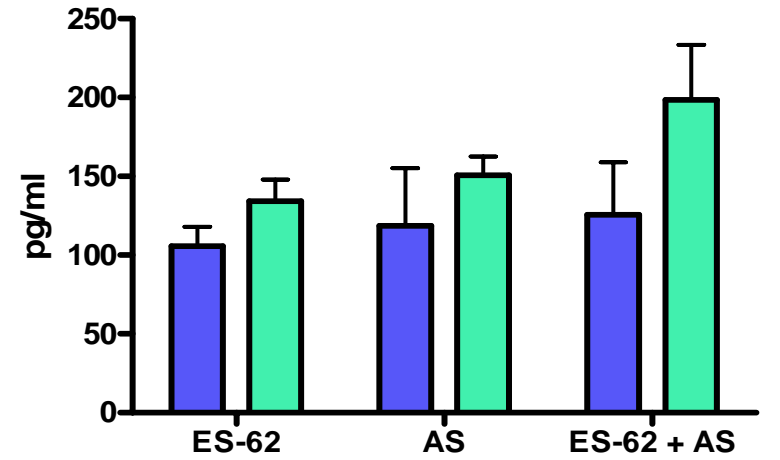
**Figure 5.7 ES-62 treatment of the ovalbumin-induced asthma model *in vivo* modulates bone marrow-derived dendritic cell production of cytokines**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Bone marrow was harvested pooled and cultured *in vitro* in the presence of GM-CSF for 7 d. On d 7 cells were stimulated with PBS or LPS (1 µg/ml) for 24 h. Cytokine production in the culture supernatant was analysed by ELISA and data are expressed as mean ± SD (n=3)

\*\*\* p<0.001

**IL12p40****TNF alpha**

(-) LPS

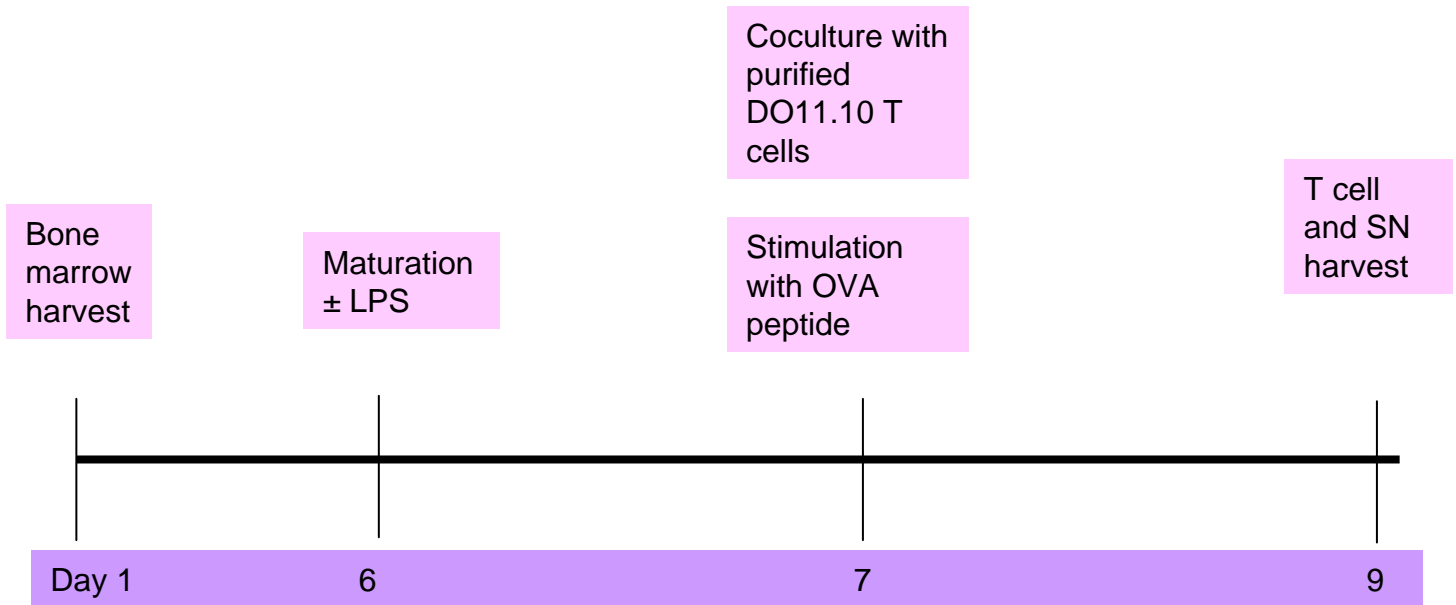
**IL6****IL10**

**Figure 5.8 Protocol for co-cultures containing asthma model derived bmDCs and DO11.10 OVA-TCR transgenic T cells**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Bone marrow was harvested pooled and cultured (culture d 1) *in vitro* in the presence of GM-CSF for 6 d. On d 6 cells were stimulated with PBS or LPS (1 µg/ml) for 24 h. On d 7 LNs and spleens from DO.11.10 mice were harvested and T cells purified and then cultured in the presence or absence of whole bmDCs loaded with different OVA peptide concentrations with the bmDCs for 72 h. At that time point culture supernatant was harvested for further analyses

bmDCs are from 3 groups:

1. asthma
2. ES-62+asthma
3. ES-62



**Figure 5.9 The effects of exposure to ES-62 *in vivo* on the ability of “immature” bone marrow DCs derived from OVA induced airway inflammation model mice to prime cytokine production by Ag-specific T cells**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Bone marrow was harvested and cells were cultured *in vitro* in the presence of GM-CSF for 6 d. On d 7 cells were pulsed with OVA peptide and co-cultured with DO11.10Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells for 72 h before cytokine production was measured from culture supernatants by ELISA. The data is presented as means ± SD.

Significances: \*p<0.05, \*\*p<0.01, \*\*\*p<0.005

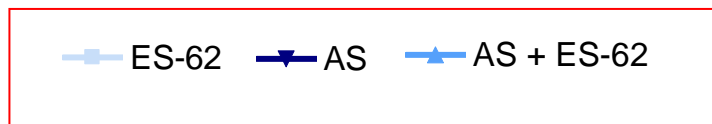
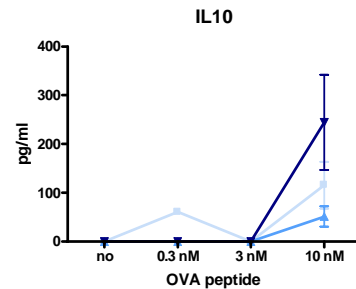
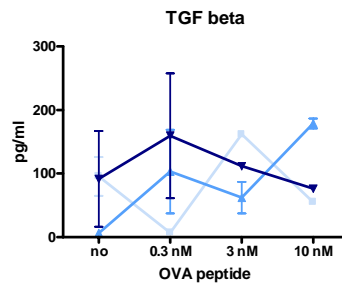
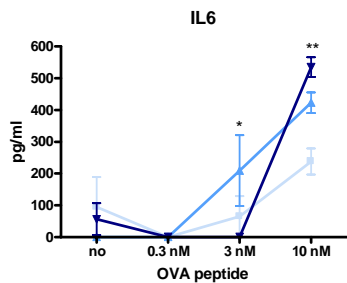
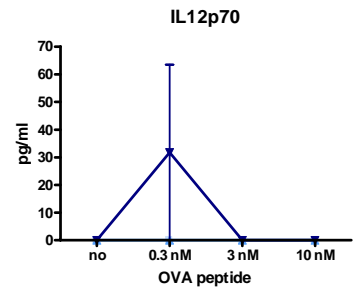
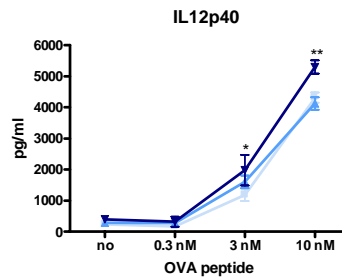
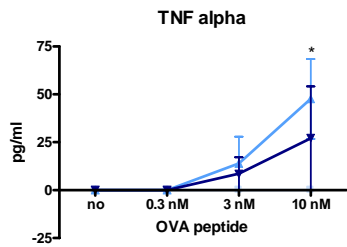
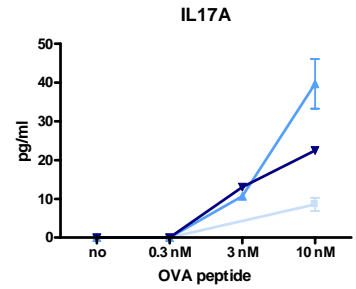
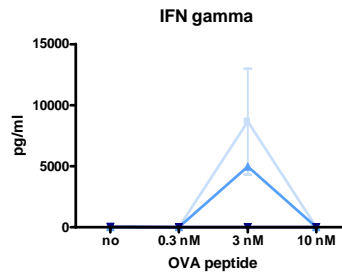
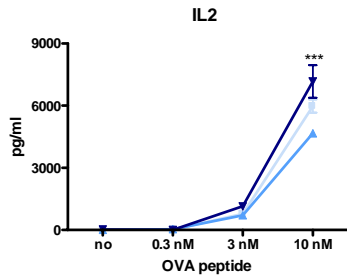
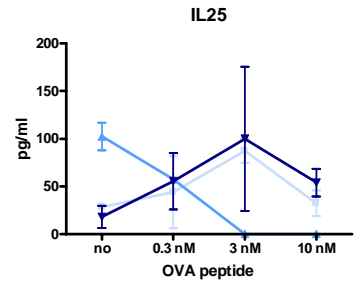
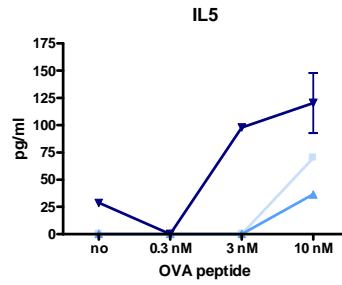
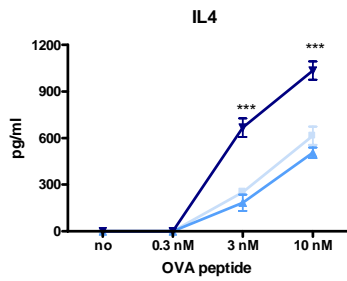
IL4: \*\*\* AS vs ES-62 and ES-62+AS

IL2: \* ES-62 vs AS and ES-62+AS, \*\*\* AS vs ES-62+AS

TNFα: \*ES-62 vs ES-62+AS

IL12p40: \*\* AS vs ES-62 and ES-62+AS, \* ES-62 vs AS

IL6: \*\* ES-62 vs AS, \* AS vs ES-62+AS



**Figure 5.10 The effects of exposure to ES-62 *in vivo* on the ability of LPS-matured bone marrow DCs derived from OVA induced airway inflammation model mice to prime cytokine production by Ag-specific T cells**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Bone marrow was harvested and cells were cultured *in vitro* in the presence of GM-CSF for 6 d. On d 6 cells were stimulated with PBS or LPS (1 µg/ml) for 24 h. On day 7 cells were pulsed with OVA peptide and co-cultured with DO11.10Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells for 72 h before cytokine production was measured from culture supernatants by ELISA. The data is presented as means ± SD.

Significances: \*p<0.05, \*\*p<0.01, \*\*\*p<0.005

IL4: \* ES-62 vs ES-62+AS

IL5: \* ES-62 vs AS

IL2: \*\* AS vs ES-62 and ES-62+AS

IFN $\gamma$ : \* ES-62 vs AS

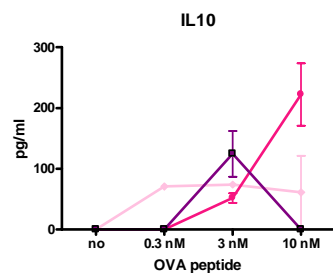
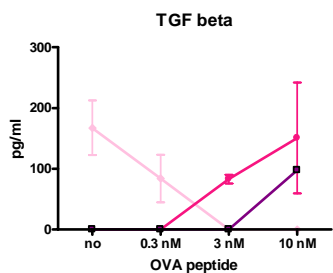
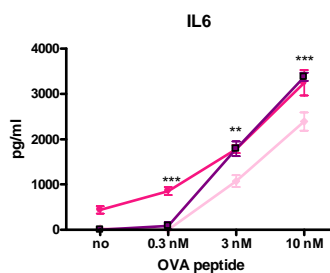
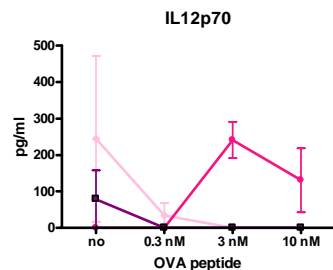
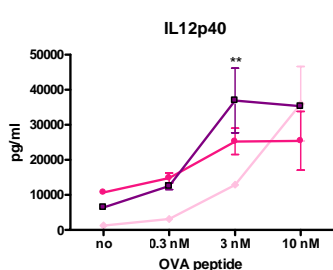
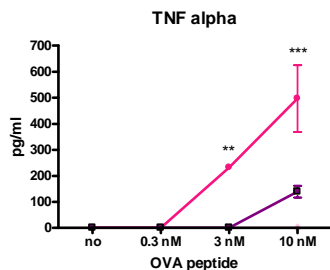
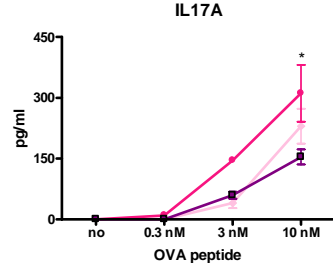
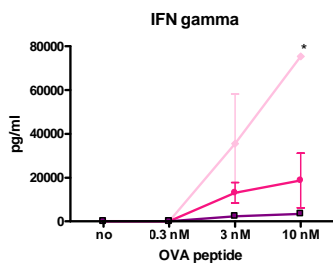
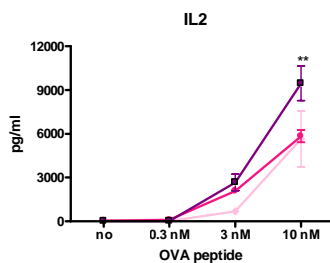
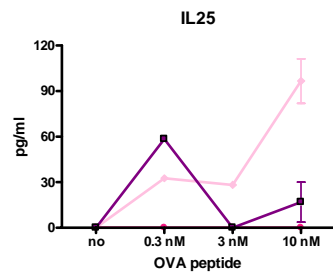
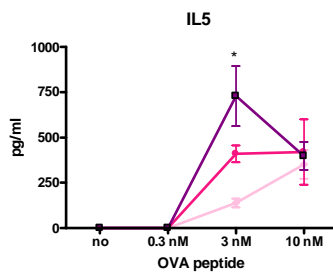
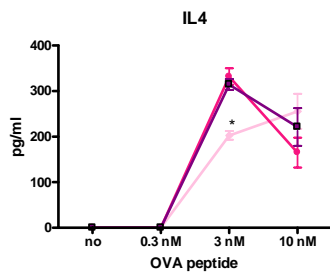
IL17A: \*AS vs ES-62+AS

TNF $\alpha$ : \*\*, \*\*\* ES-62+AS vs ES-62 and AS

IL12p40: \*\* AS vs ES-62

IL6: \*\* ES-62 vs AS and ES-62+AS, AS vs ES-62+AS,

\*\*\* ES vs ES-62+AS and AS

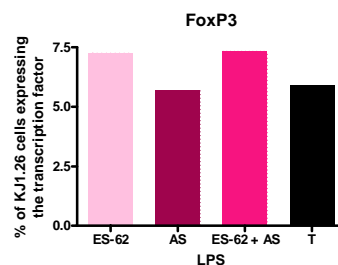
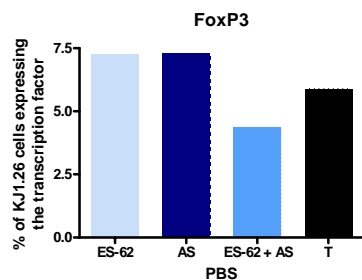
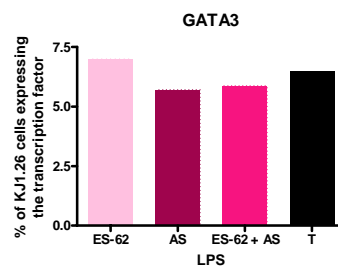
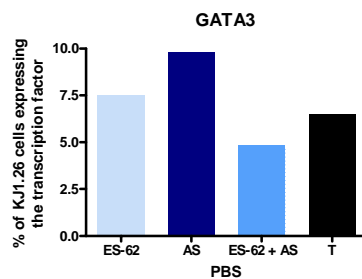
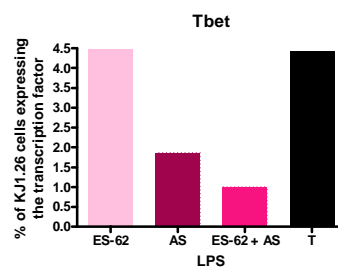
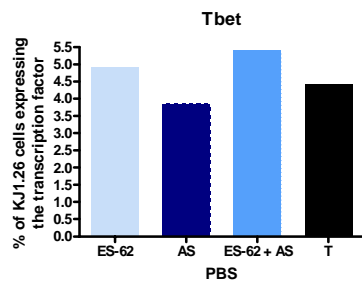
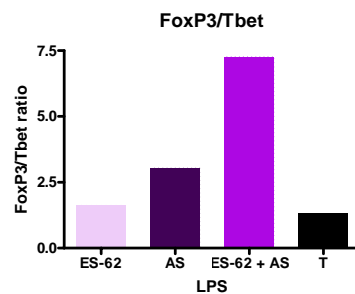
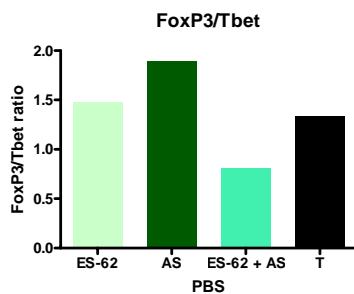
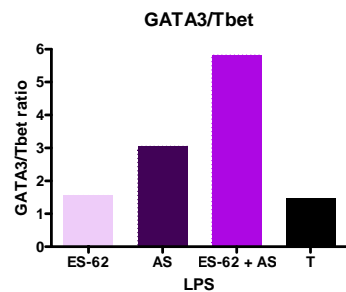
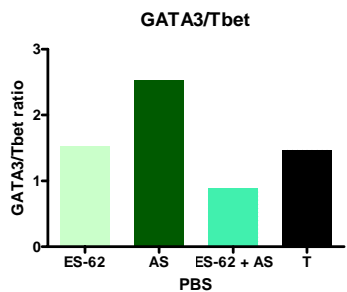


—◆— LPS ES-62    —■— LPS AS    —●— LPS AS + ES-62



**Figure 5.11 The effects of exposure to ES-62 *in vivo* on the ability of “immature” bone marrow DCs derived from OVA induced airway inflammation model mice to induce transcription factor expression by Ag-specific T cells**

BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Bone marrow was harvested and cells were cultured *in vitro* in the presence of GM-CSF for 6 d. On d 6 cells were stimulated with PBS or LPS (1 µg/ml) for 24 h. On d 7 cells were pulsed with OVA peptide and co-cultured with DO11.10Tg CD4<sup>+</sup>CD62L<sup>+</sup> T cells for 72 h. At the end of the culture period T cells were harvested and stained for the transgenic marker KJ1.26, CD4 and one transcription factor (FoxP3, Tbet, GATA3) before analysing them by flow cytometry. (a) Data were gated on the CD4, KJ1.26 population and expressed as percentage of transcription factor positive cells. (b) Data are presented as the ratio of expression of GATA3/Tbet and FoxP3/Tbet.

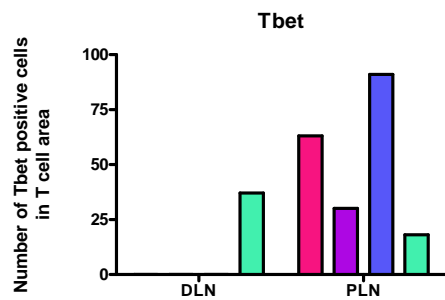
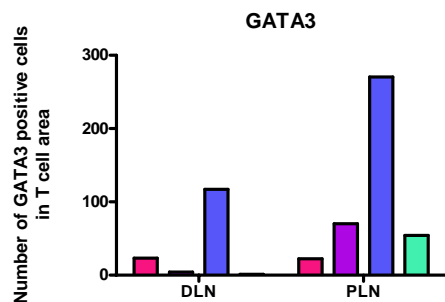
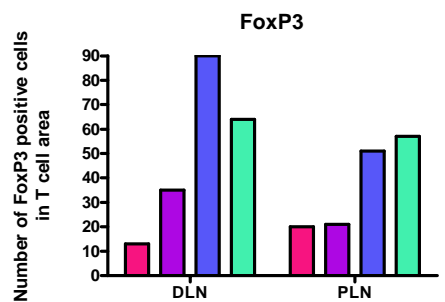
**a****b**

### **Figure 5.12 Localisation of transcription factor positive cells within DLNs and PLNs**

DLN and PLN from naïve BALB/c, (PBS), ES-62-treated BALB/c, “asthma” model, and ES-62-treated “asthma” model mice (see Material and Method section for description of the model) were harvested, snap frozen and 6 µm sections were cut. These sections were subsequently stained for B220<sup>+</sup> B cells and for the indicated transcription factor (either FoxP3, GATA3, or Tbet). T cell area was then located within the B cell follicular regions and the transcription factor positive cells were quantified within the T cell regions.

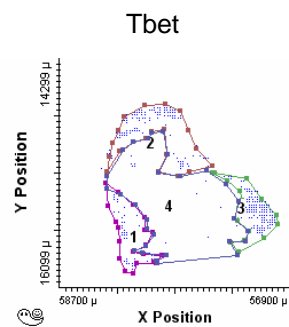
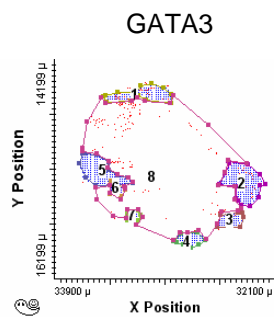
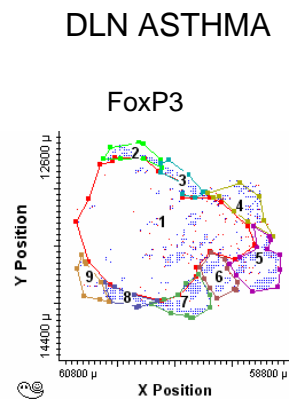
- (A) Number of transcription factor positive cells within the T cell area of the respective LN (DLN or PLN)
- (B) Tissue map of DLNs from asthma and ES-62-treated “asthma” model mice displaying the distribution of transcription factor positive cells within the T cell region

A

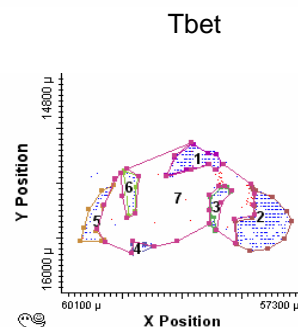
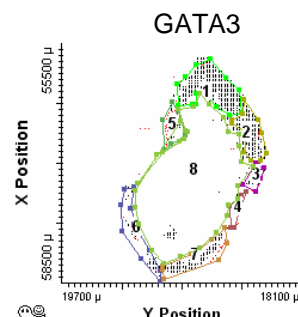
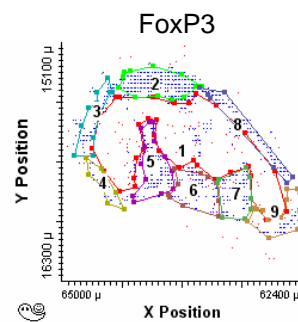


■ PBS ■ ES-62 ■ AS ■ AS + ES-62

B



**DLN ES-62 + ASTHMA**



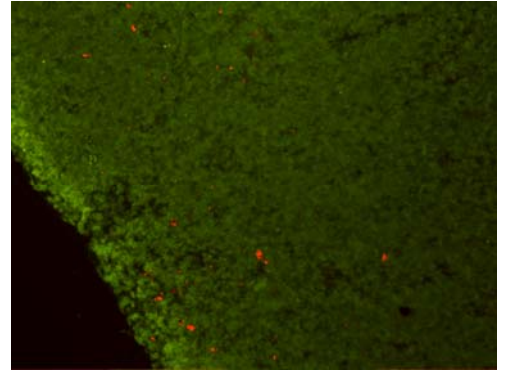
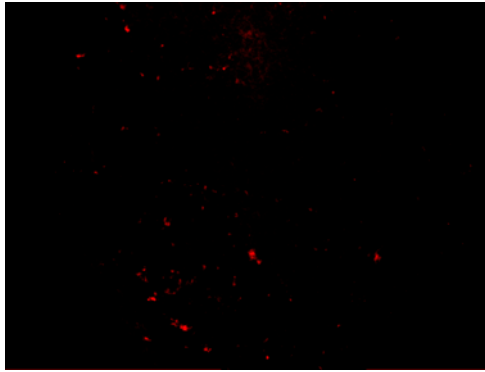
■ Transcription factor ■ B220

**Figure 5.13 *In vivo* treatment with ES-62 leads to the loss of naturally occurring T regulatory cells in draining lymph nodes from OVA induced airway inflammation model mice**

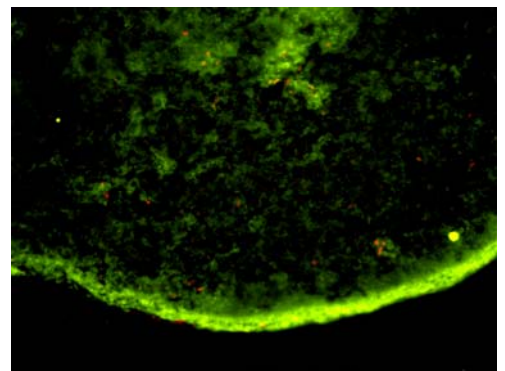
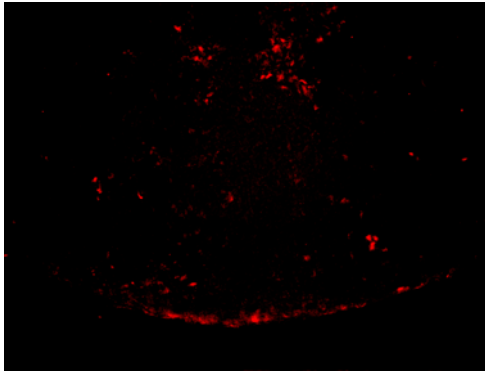
BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Intact draining LNs from each treatment group were snap frozen in liquid nitrogen and cut into 6 µm sections. Sections were stained with anti-B220-FITC (not shown), anti-CD3-biotin + streptavidin Qdot-705 and FoxP3-PE and digital images were obtained using the Openlab 3.0.9 software.

## Draining Lymph Nodes

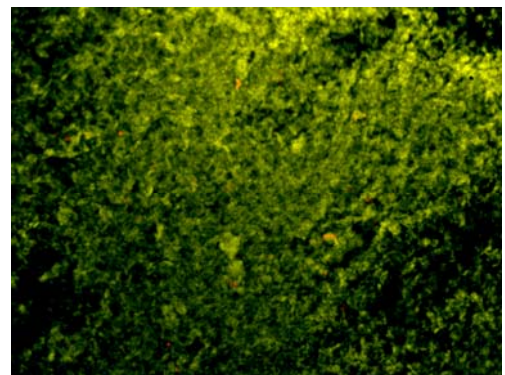
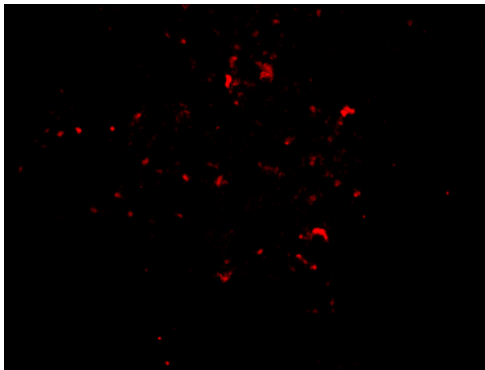
PBS



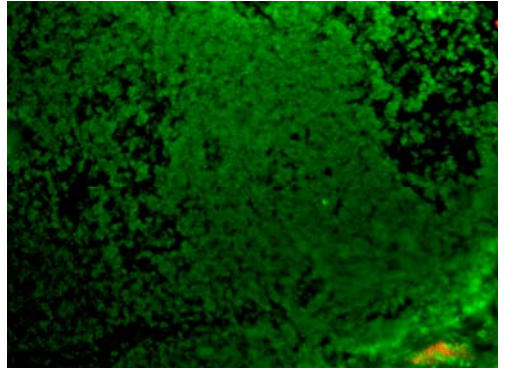
ES-62



ASTHMA



ASTHMA + ES-62



PE: FoxP3

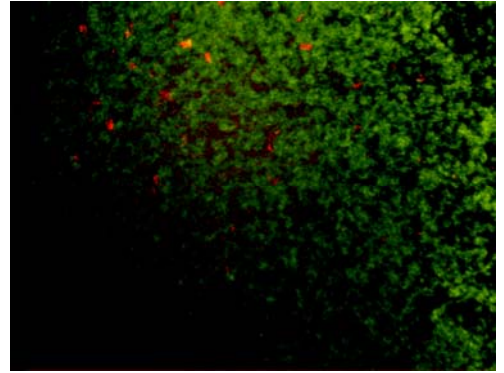
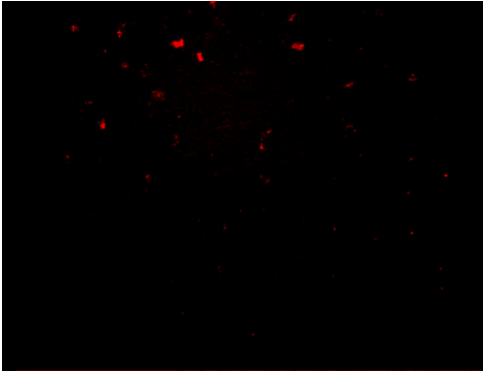
QDot-705:CD3

**Figure 5.14 *In vivo* treatment with ES-62 leads to the loss of naturally occurring T regulatory cells in draining lymph nodes from OVA induced airway inflammation model mice**

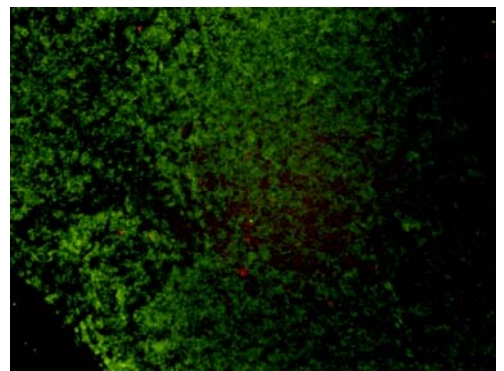
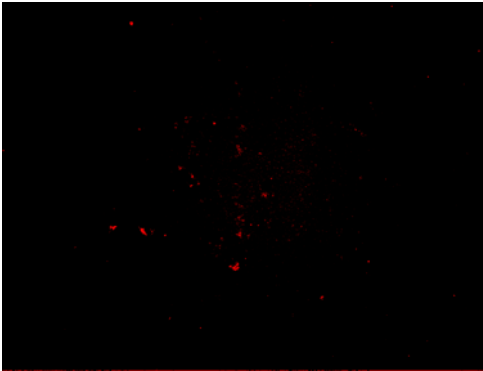
BALB/c mice sensitised with OVA were challenged intranasally on d 14, 25, 26 and 27. ES-62 (2 µg) was administered on d-2, 12, 25, and 27 and mice were sacrificed on d 28. Intact peripheral LNs from each treatment group were snap frozen in liquid nitrogen and cut into 6 µm sections. Sections were stained with anti-B220-FITC (not shown), anti-CD3-biotin + streptavidin Qdot-705 and FoxP3-PE and digital images were obtained using the Openlab 3.0.9 software.

## Peripheral Lymph Nodes

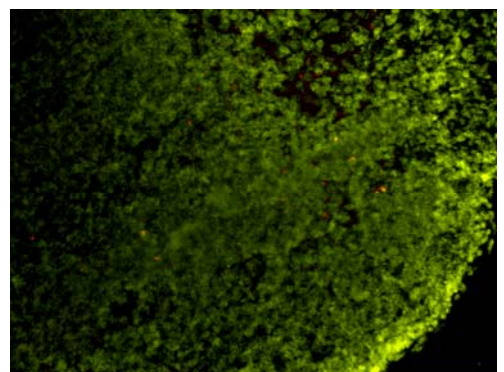
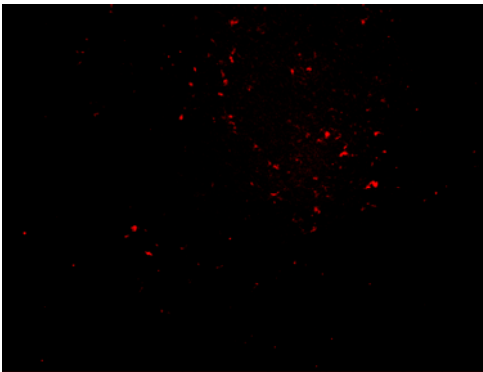
PBS



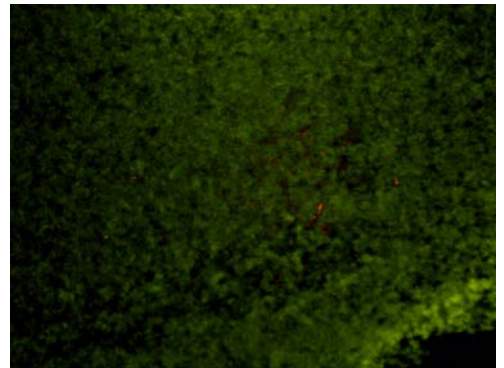
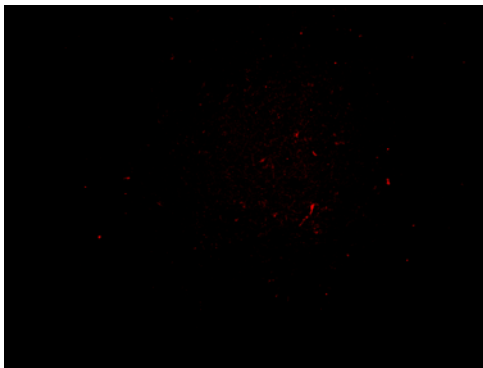
ES-62



ASTHMA



ASTHMA + ES-62



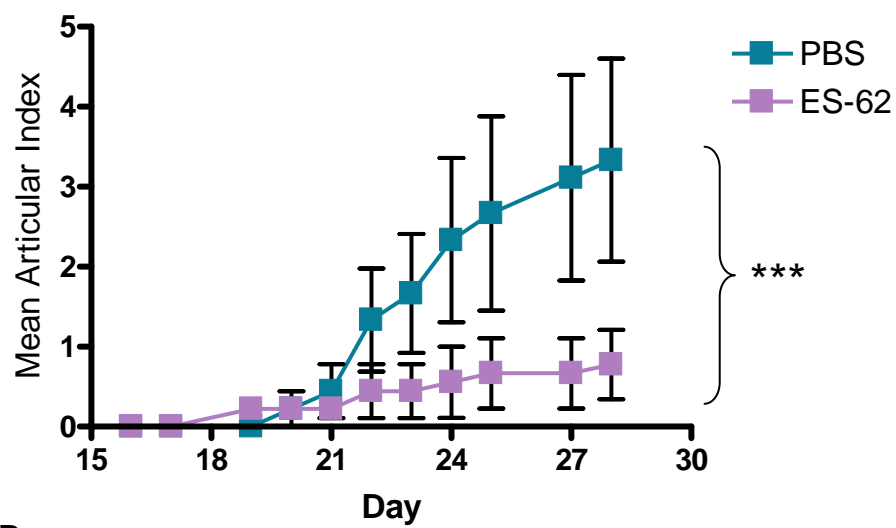
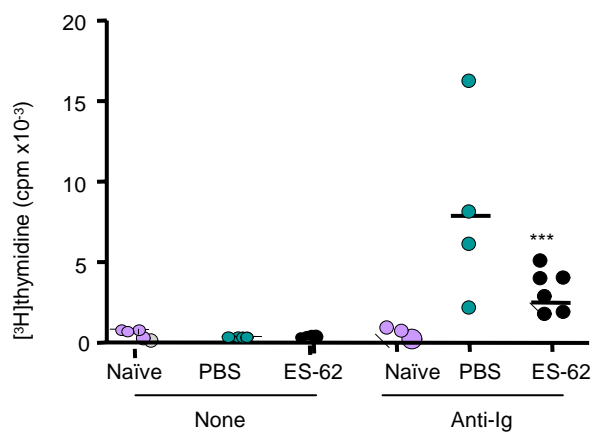
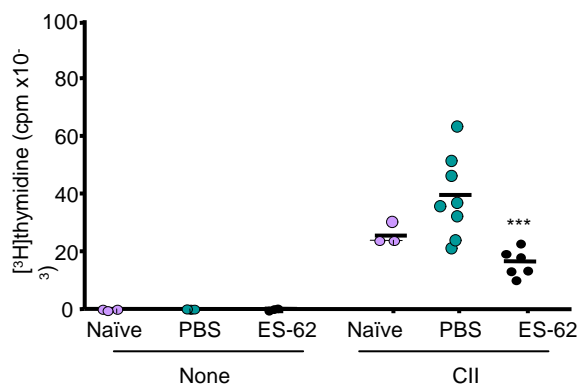
PE: FoxP3

QDot-705:CD3



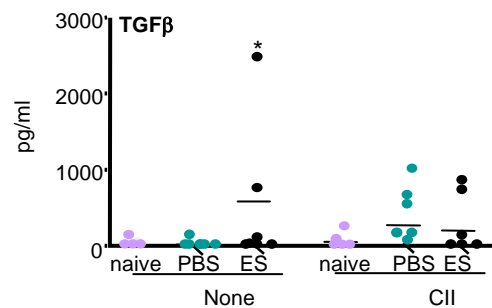
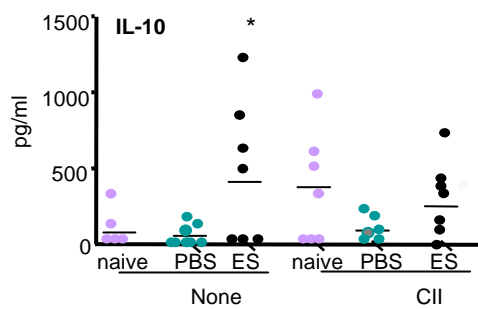
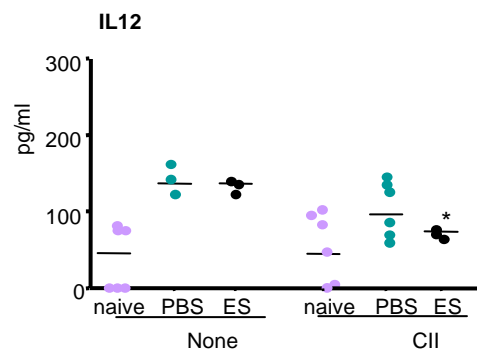
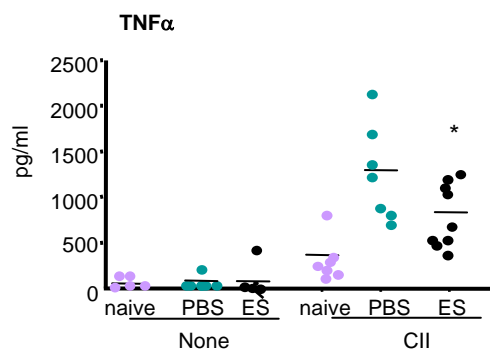
**Figure 5.15 ES-62 mediated inhibition of proliferation in LN and spleen cells from CIA model mice.**

DBA/1 mice were immunised on d 0 and 21 with collagen, and were treated with ES-62 (n = 9), or PBS (n = 9) on d -2, 0 and 21. Clinical score was monitored daily (**A**). Draining lymph nodes and spleens were obtained for each treatment group at sacrifice and cellular proliferative responses assessed by [<sup>3</sup>H]thymidine uptake. Spleen cells (**B**) were stimulated with media or F(ab')<sub>2</sub> fragments of anti-Ig antibodies (anti-Ig 50 µg/ml) for 72 h and DLN cells (**C**) were stimulated with media or collagen (**C**; CII, 50 µg/ml) for 72 h. Data are plotted as the mean values from individual mice (of triplicate determinations) from each treatment group. In A, \*\*\*p<0.001 for ES-62 versus PBS and in B, \*\*\*p<0.001 for ES-62 versus PBS (Students t-test). All data are representative of at least 3 independent experiments.

**A****B****C**

**Figure 5.16 Comparison of *ex vivo* collagen-stimulated cytokine responses from CIA model mice treated with or without ES-62 *in vivo*.**

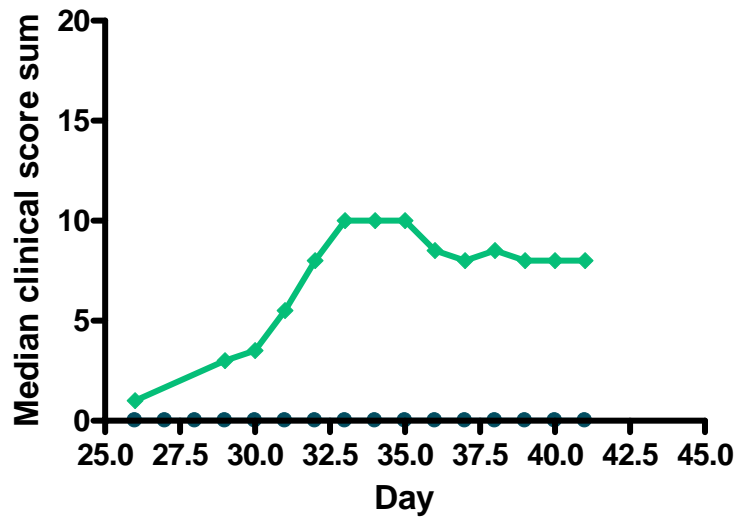
DBA/1 mice were immunised on d 0 and 21 with collagen, and were treated with ES-62 (n = 9), or PBS (n = 9) on d -2, 0 and 21. Clinical score was monitored daily (Figure 5.15). Splenic cells were obtained from naive mice and from each treatment group at d 33 and stimulated with media or collagen (CII, 50 µg/ml) for 96 h and cytokine secretion assessed by ELISA. Data are plotted as the mean of triplicate samples from individual mice in each of the treatment groups; the bar represents the mean of mean values for the group. Data are representative of 3 independent experiments. \*p<0.05 for ES-62 versus PBS (Students t-test).



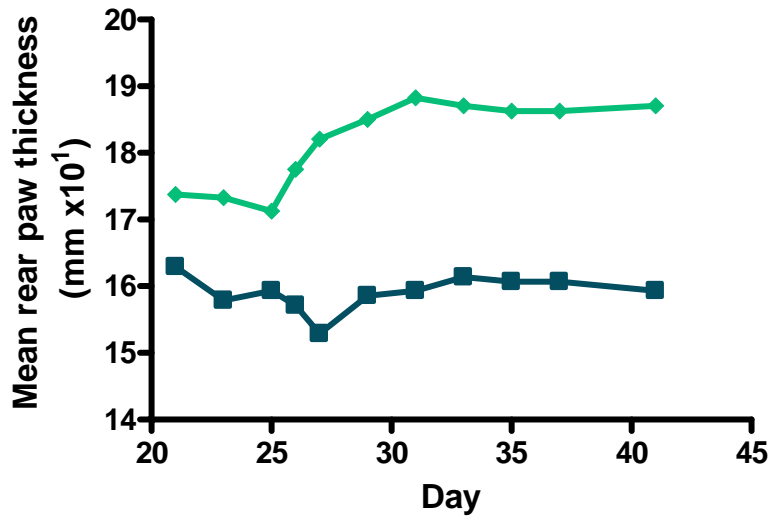
**Figure 5.17 Comparison of the clinical scores and the paw measurements from CIA model mice with and without dexamethasone treatment**

DBA/1 mice were immunised with bovine collagen type II/CFA (100 µg) intradermally on d 0 and again with bovine collagen type II/CFA (200 µg) on d 21 intra peritoneally and sacrificed on d 41. To monitor the induction of arthritis, the mean clinical score and rear and front paw measurements were conducted daily. To compare the severity of arthritis, data are presented as a comparison between the disease positive control mice (CIA model) and dexamethasone treated mice which do not exhibit any symptoms. Data are mean  $\pm$  SD and represent 12 mice per group

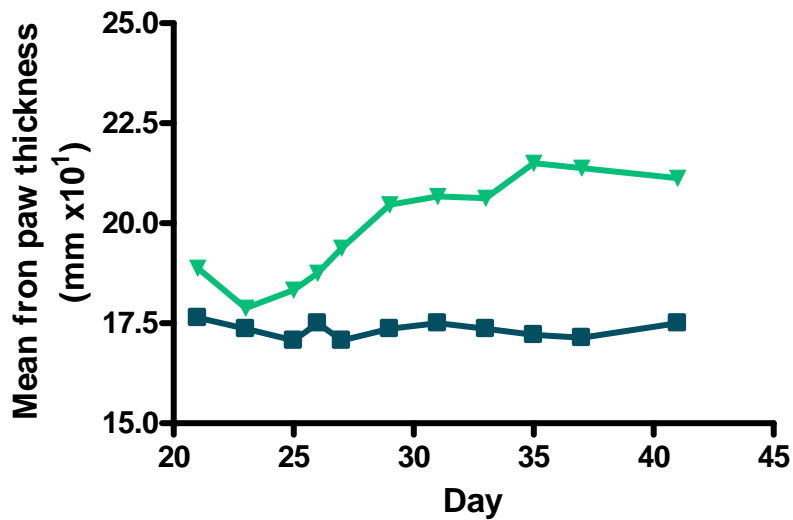
## Median clinical score



## Mean rear paw calliper measurement



## Mean front paw calliper measurements

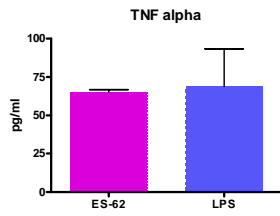


—◆— Disease positive mice    —■— 200mg/kg Dexamethasone

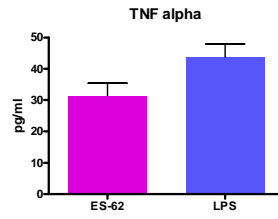
**Figure 5.18 The cytokine profile of *in vitro* ES-62 exposed spleen DC subsets from collagen induced arthritis model mice**

DBA/1 mice were immunised with collagen on d 0 and d 21 as described in figure legend 5.16 and sacrificed on d 41. Spleens were harvested and cells were pooled and stained for DC surface phenotype marker and subsequently sorted into CD4<sup>+</sup> DCs, CD8<sup>+</sup> DCs and pDCs according to the protocol described in Materials and Methods. Cells were then cultured with either ES-62 (2 µg/ml) or LPS (1 µg/ml) for 24 h. Cytokine production in the culture supernatant was analysed using ELISA and data are expressed as mean ± SD. \*\*p< 0.01 LPS vs CPG

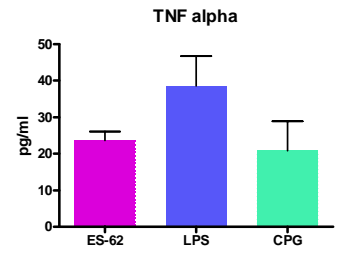
## CD4<sup>+</sup>DC



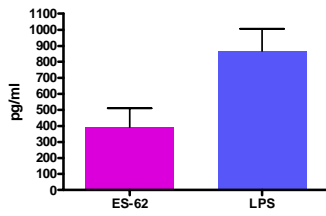
## CD8<sup>+</sup>DC



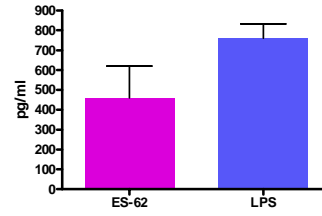
## pDC



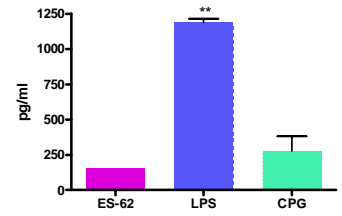
## IL12p40



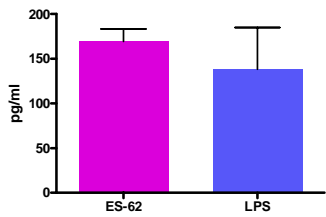
## IL12p40



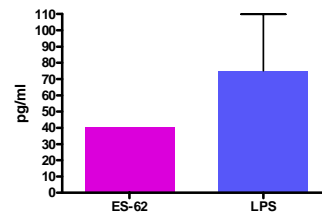
## IL12p40



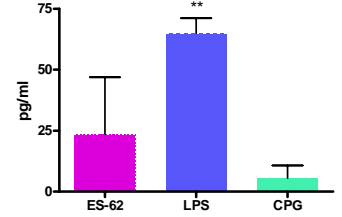
## IL6



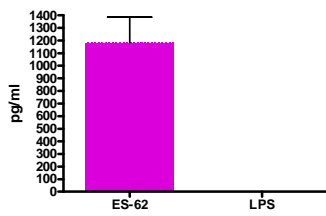
## IL6



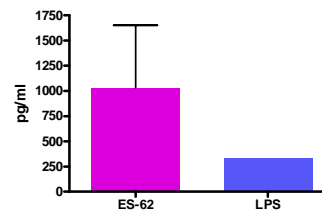
## IL6



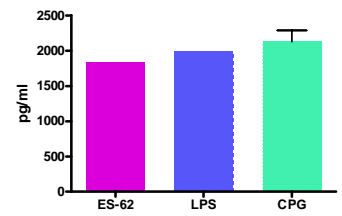
## IL12p70



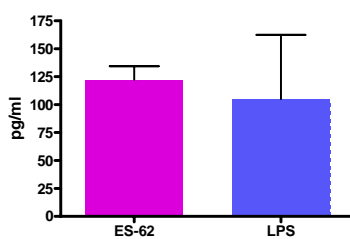
## IL12p70



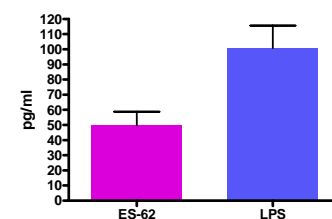
## IL12p70



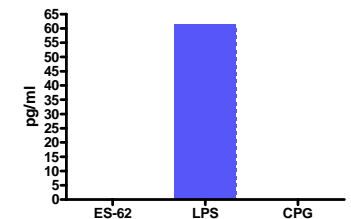
## IL10



## IL10



## IL10

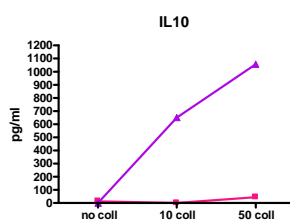
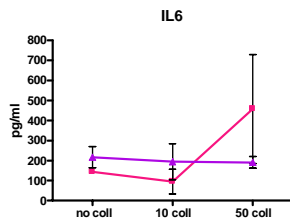
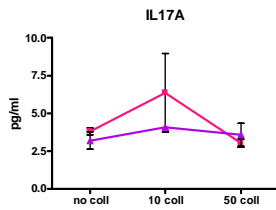
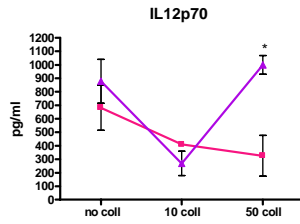
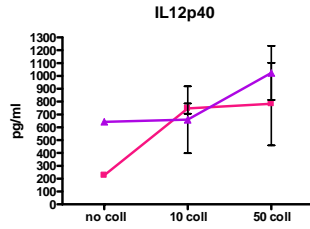
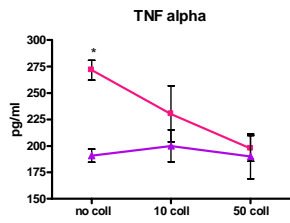




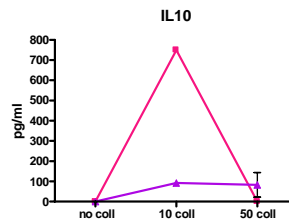
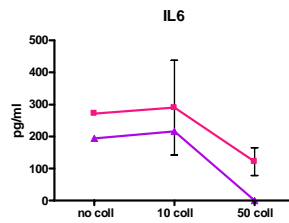
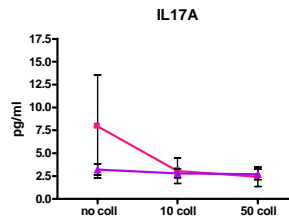
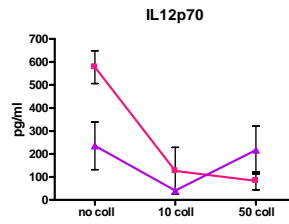
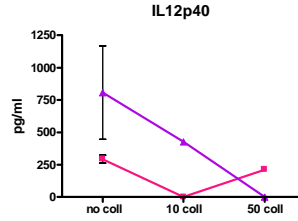
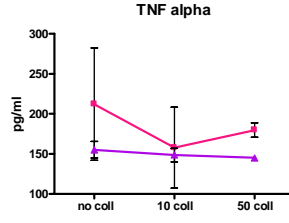
**Figure 5.19 The effects of *in vitro* ES-62 exposed spleen DC subsets from collagen induced arthritis model mice on the cytokine production profile of T cells from this model after Ag recall**

DBA/1 mice were immunised with collagen on d 0 and d 21 as described in Figure legend 5.16 and sacrificed on d 41. Spleens were harvested and cells were pooled and stained for DC surface phenotype marker and subsequently sorted into CD4<sup>+</sup> DCs, CD8<sup>+</sup> DCs and pDCs according to the protocol described in Materials and Methods. DC subtypes were then cultured in the presence or absence of 10 mg/ml and 50 mg/ml collagen for 2 h. After this incubation period either ES-62 (2 µg/ml) or LPS (1 µg/ml) was added to the culture for another 3 h. DCs were then co-cultured with purified CD4<sup>+</sup> T cells for 72 hrs. The T cells were extracted from mice of the same treatment group from the same experiment. Cytokine production in the culture supernatant was analysed using ELISA and data are expressed as mean ± SD. \*p<0.05, \*\*p<0.01, \*\*\*p<0.005

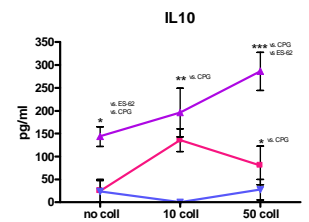
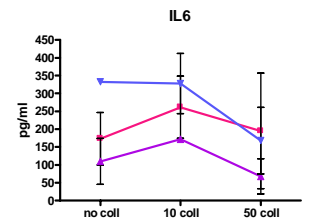
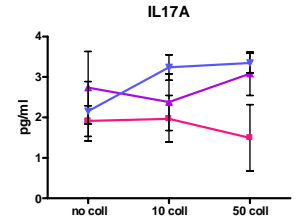
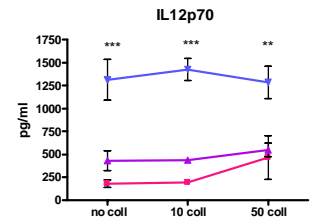
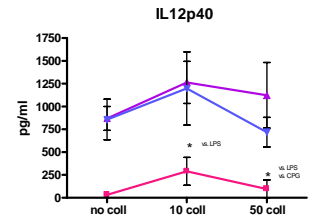
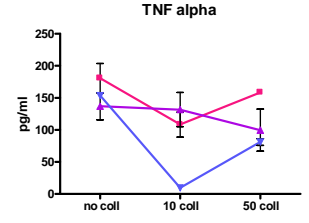
## CD4<sup>+</sup>DC + T



## CD8<sup>+</sup>DC + T



## pDC + T



## **Chapter 6**

### **General Discussion**

## 6.1 Conclusions and future prospects

Parasitic filarial nematodes are often tolerated in the human hosts for decades with little evidence of pathology. Evidence to date suggests that the mechanism by which parasites manage to survive in the host for such a prolonged time is due to the production of excretory-secretory molecules. One such molecule is ES-62, which is secreted by the rodent filarial nematode *Acanthocheilonema viteae*. Previous studies have demonstrated that this parasite product exhibits immunomodulatory activities that are broadly anti-inflammatory in nature. Indeed, recent studies of the effects of ES-62 treatment in disease models of asthma and arthritis have provided evidence that ES-62 can ameliorate inflammatory symptoms regardless of whether the disease is Th1 or Th2-based, in nature [403, 426, 733]. As additional recent studies have also shown that ES-62 does not directly influence T cells but rather interacts with dendritic cells (DCs), a major focus of this thesis was to further characterise the effects of ES-62 on this type of cell and to investigate if DCs are cardinal therapeutic targets of ES-62 in inflammatory disease.

### 6.1.1 How does ES-62 manipulate DC function?

DCs are Ag presenting cells, localised in many different sites of the body. Depending on their location these cells encounter different types of Ag and interact with different micro-environments requiring them to express specific receptors and surface components. Thus over the last decade, several distinct DC subtypes have been discovered in different lymphatic organs displaying varying potential to initiate different types of T cell responses. The mechanisms by which ES-62 modulates DC function to promote particular T effector responses are as yet unknown, however one possibility would be to change the DC phenotype or the ratio of DC subtypes at a particular site. As individual DC subtypes have been shown to mediate different immune functions, even a minor change in their phenotype or function could have a dramatic effect on the overall T effector response. Thus it was not surprising to discover that there was indeed a difference in the modulation of bm CD11c<sup>+</sup> DC and sp CD11c<sup>+</sup> DC phenotype, indicating that due to the

observed increase in CD205 the number of CD8<sup>+</sup> DCs had increased in these cultures. However as DCs are very plastic cells, which can up- and down-regulate surface marker in culture, the change in surface phenotype does not necessarily implicate a change in subtype. Consistent with this, the cytokine profile displayed a rather low secretion of pro-inflammatory cytokines which is reportedly untypical for the CD8<sup>+</sup> DC subtype [59, 465, 466]. Interestingly though the observation that led to the assumption that CD8<sup>+</sup> DCs were either induced or had a longer survival, was based on the up-regulation of the surface marker CD205, a molecule which belongs to the group of mannose like receptors [581]. As ES-62 has been shown to bind the mannose receptor, it is possible that the parasite molecule can also bind CD205 and thus promote up-regulation of the expression of this surface receptor. As analyses of cytokine profiles and surface marker expression profiles of mixed CD11c<sup>+</sup> DC populations only illustrated the overall pattern of how such DCs react, it was decided to investigate the effects of ES-62 on individual spleen cDC subtypes and to study their ability to induce T cell priming.

Although a late DC precursor has been found in the spleen [449], the majority of splenic DC subtypes are considered to be fully developed but not activated. Thus in contrast to bmDCs which have to be differentiated *in vitro*, splenic DC subtypes can be used without any delay. The benefit in using spleen-derived DC subtypes therefore lies in the fact that no culture is required prior to use, and as culturing DCs has been shown to cause phenotypical changes, avoiding any culture period before the experiments was thought more likely to provide results more closely related to the *in vivo* situation. However, the downside of this experimental set up is that it was impossible to study the effects of ES-62 on DC precursors. In general, and consistent with previous data [146] obtained from mixed DC-T cell co-cultures, ES-62 promoted a Th2 effector cell polarisation. Nevertheless, some differences in the cytokine production profile could also be detected in these co-cultures, concerning the production of both regulatory cytokines, TGFβ and IL10 and the Th17 cytokine IL17A. Whilst both the CD4<sup>+</sup> DC and the DN DC subtype induced relatively high production of IL10, moderate production of TGFβ and no IL17A, CD8<sup>+</sup> DCs only secreted high amounts of IL10 and moderate amounts of IL17A. Initially it was unclear as to why ES-62 would promote the production of IL17A, a

cytokine associated with an inflammatory response, especially as no IL17A production was observed in these co-cultures upon LPS exposure, a Th1-driving reagent. Thus it was speculated that ES-62 might promote the development of Th17 cells to balance or counteract a possible Th1 development which is the response that CD8<sup>+</sup> DCs are reported to preferentially prime. However, during the writing of this thesis, a new hypothesis regarding Th17 cells was published indicating that regulatory Th17 cells might exist which have no inflammatory function [536]. Furthermore these cells have been reported to produce IL10 and thus, they fit the cell type found in the co-cultures containing ES-62-modulated CD8<sup>+</sup> DC. Therefore it appears that ES-62 not only can promote Th2 development, but it might also be able to promote secretion of regulatory cytokines and possibly induce the development of adaptive FoxP3 negative Tregs and regulatory Th17 cells.

Following the investigations of ES-62-mediated immunomodulation in cDCs it was decided to investigate whether this parasite product could also have an effect on pDC function. Not only are pDCs very plastic cells exhibiting many different functions, but they do not express TLR4, a receptor thought to be of critical importance for ES-62 signalling. Interestingly though, ES-62 was able to interact with pDCs and indeed induced the production of several cytokines. Surprisingly, LPS also proved to have effects on pDCs as LPS-pDCs were able to induce some Th1 (IFN $\gamma$ ) polarisation. Furthermore LPS and CpG induced different effects in pDC-T cell co-cultures, indicating that the observed LPS responses were not just weak or baseline responses but indeed displayed a specific response to LPS-matured pDCs. Thus while the LPS-mediated T cell response in these cultures could be explained by LPS-contaminants [618] signalling through TLR2, the receptor and the signalling pathway resulting in the ES-62-mediated modulated pDC responses is, as yet, unclear.

### **6.1.2 Which receptors are involved in ES-62 mediated immunomodulation**

Previous work has shown that expression of TLR4 but not necessarily its ability to function, is required for induction of bmDC maturation upon ES-62-exposure [403].

Furthermore, in this thesis, it has been shown that such ES-62-treated bmDCs from TLR4 KO mice are also unable to polarise T cells towards a Th2-like effector phenotype. Thus, these data indicate that ES-62 might require certain structures to bind and/or signal via TLR4 to induce a bmDC phenotype that can prime Th2 responses. Hence it was decided to investigate whether ES-62 also requires the presence of MyD88, an adaptor molecule in most TLR-mediated signalling pathways. Previous results have determined that ES-62 can induce partial maturation of bmDCs from MyD88 KO mice [403]. Interestingly, it was determined that ES-62-modulation of the functional phenotype of bmDCs was critically dependent on MyD88-signalling to exhibit its Th2 polarising effects and indeed, T cells primed by MyD88 KO bmDCs exposed to ES-62 showed a Th1 phenotype (increased levels of IFN $\gamma$ ). Thus it was concluded that at least in cells expressing TLR4, ES-62 is likely to signal in a TLR4-MyD88 dependent manner. However, as ES-62 was able to induce T cell responses in pDCs which do not express TLR4, the question was raised as to whether ES-62 is able to utilise additional receptors in cells that do not express TLR4. Furthermore, as previous studies had shown that TLR4 did not need to be functional for ES-62 to exhibit its anti-inflammatory functions it was suggested that ES-62 might use some structures of TLR4 for binding and/or entry to cells but that a co-receptor may be required to transducer ES-62 signals.

As it had been shown that ES-62 was able to bind the mannose and the PAF receptor it was decided to investigate whether ES-62 was dependent on these receptors for signalling. However, data obtained from these studies did not reveal any striking differences between MR KO, PAF receptor KO and WT bmDCs with regard to their response to ES-62, indicating that at least in bmDCs these receptors were not crucial for ES-62-mediated maturation. Interestingly though, when LPS-mediated bmDC maturation was analysed in cells derived from PAF receptor KO mice, a slight inhibition of IL12p40 and IL12p70 production was detected indicating that PAF receptor might play a role in LPS-mediated pro-inflammatory signalling and thus, might be a receptor targeted by ES-62 to suppress pro-inflammatory signals. To further elucidate the potential role of the PAF receptor as an ES-62 target, PAF receptor KO bmDCs matured with ES-62

and LPS were used to prime T cells. Whilst T cells from ES-62-treated cultures showed a rather stronger Th2/ anti-inflammatory profile, T cells from LPS-matured cultures were unable to mount the expected Th1/pro-inflammatory response, but instead induced production of Th2 cytokines. A possible explanation for this is that LPS-mediated DC maturation leads to the production of PAF and hence signalling through the PAF receptor that is required for a Th1-promoting phenotype. Furthermore, LPS itself has been shown to bind PAF and thus could activate signalling pathways downstream of the receptor leading to inflammation. Thus it is possible that for a full Th1 cell response the expression of PAF receptor is required and that ES-62 is not using the receptor to signal but rather acts as a strong antagonist competing for the receptor with PAF, or other agonists and therefore inhibiting most signalling through the receptor. Such a strong antagonist could have similar effects to those seen in PAF receptor KO mice and might explain why ES-62-exposure prior LPS-maturation can suppress LPS-mediated Th1 polarisation. In summary, these data indicated that although the PAF receptor might not be of crucial importance for ES-62-mediated Th2 polarisation, it could be targeted by ES-62 to suppress pro-inflammatory/Th1-signalling.

### **6.1.3 How does ES-62 manipulate the immune system in inflammatory and allergic disease**

ES-62 has been shown to exert anti-inflammatory effects in murine models of allergic airway inflammation and arthritis (CIA). In the CIA model *in vivo* application, both prophylactically and therapeutically, has been shown to result in delayed onset of disease, or a reduction in severity of existing inflammation, respectively [426]. To study the underlying mechanisms for this immunomodulation, the effects of ES-62 on splenic DC subtypes from CIA model mice were investigated *in vitro*. One of the most interesting findings of these experiments was the apparent lack of DN DCs recovered from spleens from CIA model mice. In WT mice, this DC subtype accounts for about 20% of all cDCs in the spleen. However as DCs are very plastic cells known to undergo phenotypic changes upon stimulation, it is possible that this cell type was not missing, but rather not detected due to a switch in surface marker expression. Interestingly,



investigations of the effects of ES-62 on the maturation of the individual subtypes did not reveal any significant differences between the subtypes or, with LPS-matured DCs. Furthermore, when these cells were cultured with T cells from CIA model mice, no significant Th1 or Th2 cytokine production could be found and collectively the cytokine profile from co-cultures looked very similar to that of DCs cultured on their own. Thus with respect to the theory formulated by McInnes [744] which states that bidirectional signalling between macrophages and other immune cells such as synoviocytes and activated T cells contributes greatly to the disease process, it is possible that the low cytokine responses are a result of the lack of this interaction.

ES-62 has been shown to drive Th2 responses, thus it was decided to test its immunomodulatory effects in a murine model of allergic airway inflammation (“asthma” model) which is a Th2 associated disease. Rather surprisingly, it was shown that ES-62 was capable of driving two different responses in these mice. For example, in the lung, DLN and the spleen, ES-62 inhibited inflammation and Th2 development by reducing Th2 cytokines whereas the reverse was true for PLN responses. Furthermore, ES-62-exposure resulted in a switch in the expression of key transcription factors for Th2 (GATA3) and Th1 (Tbet) development at local sites resulting in an increase of Tbet. Interestingly, although clinical improvement is associated with an increase in the expression of the Treg cytokine IL10, and high levels were found under ES-62-treatment of asthma disease mice, no increase in FoxP3 could be seen. By contrast, a decrease of such FoxP3 positive cells was found in DLN indicating that the IL10 production is not due to naturally occurring Tregs but rather to induced Tregs or other cells such as B1 cells. Thus collectively the data obtained from the “asthma” model showed that ES-62 is capable of driving differential immune responses at distinct sites within an animal.

#### **6.1.4 Future work**

The effects of ES-62 on individual splenic DC subtypes and their ability to prime T cells have been described in Chapter 3. However as splenic DCs are mostly fully differentiated, and ES-62 was administered *in vitro* to these cells, it was not

possible to study the effects of this parasite product on DC precursor cells. During infection with the parasite, such precursor cells would be exposed to the parasite product and thus further investigations concerning the ability of splenic DC subtypes to prime T cell responses after *in vivo* exposure to ES-62 would be helpful in determining whether the parasite product could alter DC phenotype or promote development of a specific subtype.

Another question to be answered is whether ES-62 can utilise the PAF receptor if TLR4 is not expressed. It has been shown that the parasite protein can enter cells via this receptor and thus it might function as a signalling receptor for ES-62 in the absence of TLR4. Hence it would be interesting to test this theory by using PAF receptor KO pDCs, a cell type that naturally does not express TLR4 but has been shown in chapter 3 to be able to respond to ES-62.

Finally, ES-62 has been shown to bind the mannose receptor. However DCs in lymphoid organs do not express this receptor although the CD8<sup>+</sup> DC population expresses CD205, another member of the mannose receptor family. Furthermore surface markers like CD205 have been shown to become up-regulated during infection, inflammatory processes or even cell culture. Thus CD205 could be a potential ES-62 receptor and might even become up-regulated on other DC subtypes upon exposure to the parasite product. Therefore future work would include investigating whether CD205 could be a receptor or co-receptor for ES-62 mediated immunomodulation.

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