Modulation of Th1 and Th2 type immune responses

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Abstract

The Th1/Th2 cell balance is crucial for the outcome of many infections and autoimmune diseases. The identification of novel Th1 and Th2 cell specific markers can expand our knowledge about Th1 and Th2 cell function, and these markers can be used to manipulate Th1/Th2 cell responses. Recently, ST2L (an orphan receptor of the IL-1R family) was identified to be selectively expressed on Th2, but not on Th1 cells. Factors that determine this differential expression pattern are still unknown. Antibodies against ST2L as well as a ST2-Fc fusion protein, mimicking the soluble form (ST2), were able to decrease Th2 effector functions. ST2 is thought to compete with ST2L for the ligand and therefore prevent ST2L signalling. These studies demonstrated that ST2L functions are closely linked to Th2 responses *in vitro* and *in vivo*. However, with the ligand of ST2L still unidentified, the function of ST2L and especially of ST2 are controversial.

The aim of my thesis was to expand the knowledge about the role and regulation of ST2 and ST2L in murine T helper cells. In my initial experiments (Chapter 3) I established a method to express and purify ST2 from baculovirus-infected insect cells and from stably transfected mammalian CHO cells. The protein was tested for its biological activity on LPS-stimulated macrophages, where it was able to decrease the production of inflammatory cytokines (IL-6 and IL-12).

Next, I examined the effects of ST2 on CD4(+) T cell activation (Chapter 4). ST2 could bind to naïve CD4(+) T cells and suppressed Th1 and Th2 type cytokine production. It worked directly on Th cells without the need for APC. CD4(+) T cells,

whose TCR had been engaged before, did not bind ST2 and therefore did not show any decrease in cytokine expression.

I then assessed the regulatory effect of Th1 and Th2 cytokines on the gene expression of ST2 and ST2L at protein, mRNA and promoter level to clarify whether these cytokines are responsible for the differential expression of ST2 and ST2L (Chapter 5). IL-4, a major cytokine for Th2 development and proliferation, increased ST2L protein expression, while IFN-γ, the main Th1 cytokine, decreased ST2L protein expression. Similar results were obtained at the mRNA level, where IL-4 upregulated and IFN-γ downregulated the ST2L mRNA expression. Interestingly, IL-4 induced an early increase in ST2 mRNA expression and a delayed increase in ST2L mRNA, suggesting differential regulation of the two splice variants. The upstream promoter showed increased activity when treated with IL-4, but decreased activity when IFN-γ was added. Hence, IL-4 was able to upregulate ST2L and drive Th2 cell development simultaneously. IFN-γ had opposite effects to IL-4.

In order to stably transfect Th cells with a ST2L antisense construct, toxicity studies for G418, the selection marker, were carried out on Th1 and Th2 cells (Chapter 6). G418 and its structural analogue gentamicin were found to modulate the Th1/Th2 balance *in vitro* by selectively suppressing Th2 cells to a greater extent than Th1 cells. To investigate the ability of gentamicin to regulate the Th1/Th2 cell balance *in vivo*, I treated *Leishmania major*-infected BALB/c mice with high doses of gentamicin for 12 days. Complete suppression of the disease was achieved, providing gentamicin treatment was started early after infection.

Analysis of antigen-specific proliferation, cytokine release and antibody production revealed a complete suppression of antigen-responding Th lymphocyte activity. However, a shorter period of gentamicin treatment (7 days), started immediately after infection, selectively suppressed Th2 but not Th1 cytokine expression, confirming the ability of gentamicin to modulate Th1/Th2 type responses. Gentamicin also caused a partial suppression of macrophage viability. The appearance and activity of parasites changed upon short term gentamicin treatment, but this did not affect their infectivity *in vivo*. *In vitro* experiments suggested an increased uptake of the aminoglycoside by macrophages in the presence of parasites, promoting increased clearance of the organism.

In this thesis I undertook a series of defined studies which together further advance our knowledge on the regulation of Th1 and Th2 cells. This is based on the function of ST2L/ST2 and gentamicin, both of which have significant effects on the balance between Th1 and Th2 cells. These results can contribute to our ability to modulate some of the most important infectious and inflammatory diseases.

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Xu D, Chan WL, Leung BP, Hunter D, Schulz K, Carter RW, McInnes IB, Robinson JH, Liew FY (1998) Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. *J Exp Med.* **188,** 1485-1492

Xu D, Trajkovic V, Hunter D, Leung BP, Schulz K, Gracie JA, McInnes IB, Liew FY IL-18 induces the differentiation of Th1 or Th2 cells depending upon cytokine milieu and genetic background. *Eur J Immunol.* **30,** 3147-3156

Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, Campbell CC, Xu D, Liew FY A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol.* **166,** 6633-6639

Abbreviations

Ag	antigen
Ab	antibody
APS	ammonium-persulphate
APC	antigen presenting cell
BSA	bovine serum albumin
BMM(s)	bone marrow macrophage(s)
CFTR	cystic fibrosis transmembrane receptor
DC	dendritic cells
DEPC	diethyl-pyrocarbonate
DNA	Deoxy-ribonucleic acid
dNTP	mix of deoxy-nucleotides (dATP, dCTP, dGTP, dTTP) at 10 mM
DC	dendritic cell
DTH	delayed type hypersensitivity
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylene diamine tetra-acetic acid
Fc	Fc part of IgG1
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	Fluorescein
h	hour
HRO	horse radish peroxidase
Ig	Immunoglobulin
IL	interleukin
i.p.	intra peritoneal
iNOS	inducible nitric oxide synthase
kb	kilobase
kDa	kiloDalton
LPS	Lipopolysaccharide

mAb	monoclonal antibody
mdr	multiple drug resistance
MHC	major histocompatibility complex
min	minute
mRNA	messenger ribonucleic acid
MRP	multiple drug resistance protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Thiazolyl blue
NK	natural killer
NO	nitric oxide
OD	optical density
OVA	ovalbumin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	Phycoerythrin
pfu	plaque-forming units
PI	propidium iodide
R	receptor
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
s	second
SCID	severe combined immuno-deficiency
SD	standard deviation
SDS	sodium dodecyl sulfate
STAT	signal transducer and activators of transcription
SOCS1	suppressor of cytokine signaling 1
TEMED	N,N,N',N'-tetramethylenediamine
TGF	Transforming growth factor
Th	T helper
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor

TMB	tetramethylbenzidine
TNF	Tumor necrosis factor
U	unit
wt	wild type

L	litre
M	molar
mM	millimolar
mg	milligramm
ml	millilitre
µM	micromolar
µg	microgramm
µl	microlitre
nM	nanomolar
pg	picogramm

Chapter 1 General Introduction

The human body is constantly under siege. It must defend itself from a whole host of bacterial parasitic and viral invaders. The immune system is the primary line of defense avoiding "self" antigens, while vigorously attacking foreign ones. T lymphocytes, the strategists in this game, both direct the responses of the rest of the immune system and play an active role themselves. T cells orchestrate the immune response via the production of cytokines - secreted proteins that instruct other cells to behave in specific ways. T cells may be broadly classified as either helper T cells (Th cells), distinguished by surface expression of the molecule CD4, or cytotoxic cells (Tc cells, expressing CD8). Th cells consisted of two major subsets - Th1 and Th2 cells. The balance between Th1 and Th2 cells determines the outcome of a wide range of infections with extra- and intracellular pathogens, and an inappropriate Th1- and Th2-dominated response can lead to autoimmune diseases. In recent years, several molecules have been identified which are differentially expressed on Th1 and Th2 cells. These findings gave more insight into the molecular basis of the different functions of Th1 and Th2 cells. Additionally, some of these molecules can be used to modulate the Th1/Th2 cell balance *in vitro* and *in vivo*. The purpose of the work described in this thesis was to explore the ability of two molecules, ST2 and gentamicin, to modulate the balance of Th1 and Th2 type immune responses.

1. T helper cells in the immune response

1.1 Subsets of CD4(+) T cells

CD4(+) T cells leave the thymus as naïve Th0 cells, destined to recognise major histocompatibility complex II (MHCII)- peptide complexes on antigen presenting cells (APC). At this stage the Th cell will produce IL-2 and clonally expand. Following activation, naïve Th0 cells are capable of "polarising" or differentiating into several Th cell subsets (Table 1.1, Fig. 1.1).

Previously, two major subsets of effector T cells have been defined on the basis of the cytokines that they secrete and the immunomodulatory effects provided by these cytokines (reviewed in Mosmann and Coffman, 1986 and 1989; Paul and Seder, 1994). The two most discernible populations were termed Th1 and Th2, with effector Th1 cells producing IL-2 and proinflammatory cytokines such as interferon (IFN)-γ and lymphotoxin-α, and Th2 cells producing IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13.

The two helper cell classes also differ in the type of immune response they produce. Th1 cells tend to generate responses against intracellular pathogens such as bacteria, parasites, yeast and viruses, mainly by expression of the Th1 cytokines which can activate microbicidal activity, as well as cytokine production by macrophages. A Th1 response is often accompanied by the production of complement-fixing antibodies of the IgG2a isotype, as well as the activation of natural killer (NK) cells and cytotoxic CD8(+) T cells expressing IFN-γ and perforin (reviewed in O'Garra and Arai, 2000). Th2 cells produce immune responses

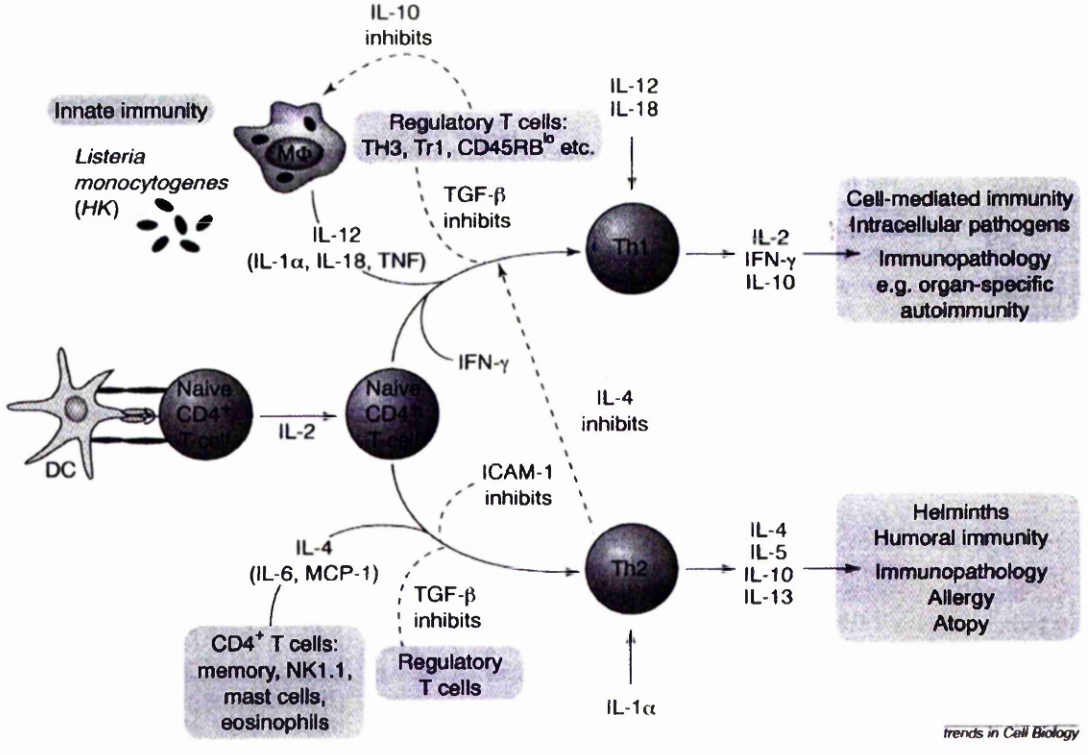


Fig. 1.1 Responses of innate and adaptive immunity

Overview of different T helper cell subsets involved in innate and adaptive immune responses (from O'Garra and Arai, 2000).

Subtype	Defining cytokines	Effect(s)
Naïve Th0	IL-2	Proliferate and differentiate to effector cells
Th1	IL-2, IFN-γ, lymphotoxin-α	Cell-mediated immunity, opsonizing antibody
Mature Th0	IFN-γ, IL-4, others	Unclear
Th2	IL-4, IL-5, IL-9, IL-10, IL-13	Humoral immunity, inhibits inflammation
Th3	TGF-β	Regulates mucosal immunity and stimulates B cell secretion of IgA
T-regulatory 1	High levels of IL-10, some TGF-β	Suppression of immune response

Table 1.1 Subtypes of CD4(+) T-helper (Th) cells and their characteristic cytokines and effects (from Spellberg and Edwards, 2001)

against helminth and other extracellular parasites (reviewed in Perkel, 2001) by activating mast cells and eosinophils. However, these cells also mediate allergic and atopic manifestations, which is in keeping with findings that Th2-derived cytokines can induce hyperresponsiveness to an allergen (Tournoy *et al.,* 2001) as well as the production of IgE (reviewed in O'Garra and Arai, 2000).

Mature Th0 cells are characterised by the secretion of both IL-4 and IFN-γ as well as IL-2 (Miner and Croft, 1998). It is yet to be clarified whether the Th0 phenotype represents a transitory state of priming cells during their development into a Th1/Th2 phenotype, or whether the Th0 phenotype indeed represents a fully differentiated subpopulation of cells (Firestein *et al.*, 1989, Murphy *et al.*, 1996, Bucy *et al.,* 1995). Th0 cells can be generated *in vitro* by antigenic stimulation of CD4(+) T cells in the presence of IL-2 (Rogers *et al.*, 1998) or by priming antigen-specific naïve CD4(+) T cells with a moderate dose of exogenous IL-4 in combination with endogenous IFN-γ (Miner and Croft, 1998). In the presence of IL-12, differentiated Th2 cells have been shown to revert into a population predominantly containing Th0 and Th1 cells (Sornasse *et al.*, 1996). *In vivo* Th0 cells have been found to be involved for example in the killing of melanoma cells (Imro *et al.*, 1995), in resistance to *Listeria monocytogenes* infections (Hiromatsu *et al.*, 1996) or in autoimmune diseases such as Sjogren's syndrome (Matsumoto *et al.,* 1999). It has also been suggested that Th0 cells are involved in eliminating many pathogens, where a balance of both regulated cell-mediated immunity and an appropriate humoral response will eradicate an invading pathogen with minimum immunopathology (reviewed in O'Garra, 1998).

Its now well established that immune responses driven by Th1 and Th2 cells are sometimes also influenced by a third T cell type whose main function is counter-regulation or suppression of immune responses mediated by Th1 and Th2 cells (Chen *et al.*, 1994; Fukaura *et al.*, 1996). It has been shown, for instance, that the induction of oral tolerance by the feeding of relatively low amounts of myelin basic protein leads to the induction of immunoregulatory T cells, which prevent the development of experimental autoimmune encephalitis (Chen *et al.*, 1994; Fukaura *et al.,* 1996). Such T cells have a unique cytokine production profile in that they produce high levels of TGF-β without necessarily producing either Th1 or Th2 cytokines; therefore, they have been dubbed Th3 T cells (Fukaura *et al.*, 1996). Th3 cells producing TGF-β have also been shown to occur in experimental models of colitis or diabetes or in $HgCl_2$-induced autoimmune diseases, and in these instances it is thought that such T cells play an important role in disease prevention or cure (Neurath *et al.*, 1996; Powrie *et al.*, 1996; Han *et al.*, 1996; Bridoux *et al.*, 1997). The cytokine TGF-β is implicated in oral tolerance (Neurath *et al.*, 1996), in the prevention of colitis in mice (Powrie *et al.*, 1996), in the control of adjuvant-induced encephalitogenic T cells in mice (Chen *et al.*, 1994), in the prevention of the autoimmunity induced by Th2 cells (Bridoux *et al.*, 1997) and in airway hypersensitivity (Haneda *et al.*, 1997).

Recently, another type of regulatory T cell has been identified (in mice and humans) that may be related to the aforementioned Th3 T cells. This cell, termed T regulatory cell 1 (Tr1), is induced *in vitro* by stimulation of T cells in the presence of IL-10 and is a T cell that produces high levels of IL-10 and also low levels of

TGF-β (Groux *et al.*, 1997; Buer *et al.*, 1998). Functionally, these T cells have inhibitory effects on Ag-specific activation of naïve autologous T cells, which is partially mediated by IL-10 and TGF-β (Groux *et al.*, 1997). In addition, in a murine model of inflammatory bowel disease in SCID mice, cotransfer of Tr1 cell clones together with pathogenic CD4(+) $CD45RB^{high}$ T cells prevented the induction of disease (Groux *et al.*, 1997). Donor-derived T cells specific for host alloantigens that possess a Tr1 profile of cyrokine production were also isolated from tolerant SCID patients who had been reconstituted with HLA-mismatched stem cells (Bacchetta *et al.*, 1994; Bacchetta *et al.*, 1993; Bacchetta *et al.*, 1990). Together, these data indicate that Tr1 cells can function as regulatory cells *in vivo*.

Additionally, naturally anergic and suppressive CD4(+) CD25(+) T cells have been identified, that prevent autoimmune diseases *in vivo* and are naturally unresponsive (anergic) to TCR stimulation *in vitro.* Upon TCR stimulation, which upregulates their CD25 expression levels strongly, they are able to suppress the activation/proliferation of other T cells. The function of these cells may be dependent on secretion of IL-10 (Read *et al.*, 2000; Hara *et al.*, 2001; Annacker *et al.*, 2001). There are strong similarities between effector functions and the cytokine profiles of CD25(+) suppressive T cells and Tr1, Th3, which suggest that these subsets may overlap.

1.2 Cytokines

1.2.1 Th1 type cytokines

IFN-γ (reviewed in Farrar and Schreiber, 1993; De Maeyer and De Maeyer-Guignard, 1992), first identified by its anti-viral activity (Wheelock, 1965), is produced by activated CD4(+) and CD8(+) T cells and NK cells, although the CD4(+) Th1 cells are considered the major producer in response to antigen (Mosmann and Coffman, 1989). IFN-γ has potent immunoregulatory effects on a variety of cells including activation of macrophages, induction of FcγRI on macrophages and FcγRII on granulocytes, enhanced production of IgG2a by B lymphocytes and enhanced expression of class I MHC gene products and class II MHC gene products on most cells *in vitro*, thus rendering them able to participate in class II-mediated immune reactions. IFN-γ enhances the differentiation of IgG2a secreting B cells and counteracts the effects of IL-4 on B cells (Mosmann and Coffman, 1989; Hasbold *et al.*, 1999). The proliferation and effector functions of Th2 cells is suppressed by IFN-γ (Gajewski *et al.*, 1988; Fitch *et al.*, 1993). IFN-γ is also responsible for inducing non-specific cell mediated mechanisms of host defence through its macrophage activation ability and is required for the resolution of microbial infections such as *Leishmania*.

IL-2 (reviewed in William *et al.,* 1991; Smith *et al.*, 1988) is produced mainly by CD4(+) T cells following activation by mitogen or antigen. Effector Th1 and Th0 cells produce large amounts of this cytokine upon stimulation. It can cause proliferation of T cells, inducing cell cycle progression in resting cells and thus

allows clonal expansion of activated T lymphocytes. This growth-promoting property is the best characterized and perhaps most important function of this cytokine. IL-2 appears to be a T cell differentiation factor, as it is able to induce production of other cytokines such as IFN-γ and IL-4 (Farrar *et al.*, 1982). IL-2 also promotes the proliferation of activated B-cells, although this also requires the presence of additional factors such as IL-4. NK cells as well as monocytes and macrophage precursors proliferate and increase their cytolytic activities in response to IL-2 (Trinchieri *et al.*, 1984; Baccarini *et al.*, 1989, Malkovsky *et al.*, 1987, Ralph *et al.*, 1988).

Lymphotoxin-α (TNF-β) is expressed by naïve CD4(+) T cells, differentiated Th1, but not Th2 cells, due to an IL-4 induced downregulation of LT-α expression (Gramaglia *et al.*, 1999). Apart from activated CD4(+) T cells, CD8(+) T cells, B cells and NK cells express this cytokine (Murphy *et al.*, 1998). LT-α and β (membrane bound form) and their close relative TNF are involved in lymphoid organogenesis. LT-α-deficient mice lack all peripheral lymph nodes and Peyer's patches, have disorganized splenic architecture with no mature follicular DCs and are not able to form germinal centres (Banks *et al.*, 1995; De Togni *et al.*, 1994, Matsumoto *et al.*, 1996). Other activities ascribed to either the membrane or secreted forms of LT include a direct cytotoxic effect (Makay *et al.*, 1997; Browning *et al.*, 1996), promoting inflammatory reactions and homing of lymphocytes (Kratz *et al.*, 1996; Sacca *et al.*, 1998), and controlling effector functions by stimulating responses of B cells, T cells and macrophages (Estrov *et al.*, 1993).

1.2.2 Th2 type cytokines

IL-4 (reviewed in Boulay and Paul, 1992; Paul, 1987) is mainly produced by Th2 cells, which are the biologically most active helper cells for B-cells. Non-T/Non-B cells of the mast cell lineage also produce IL-4. This cytokine promotes the proliferation and differentiation of activated B cells, T cells and mast cells. It induces the expression of class II MHC antigens and of low-affinity IgE receptors in resting B-cells. In activated B-cells, IL-4 stimulates the synthesis of IgG1 and IgE and inhibits the synthesis of other antibody isotypes. Another property of IL-4 is the ability to drive precursor T cells to Th2 cells (Le Gros *et al.*, 1990), which is supported by IL-13 (see below). Pretreatment of macrophages with the cytokine prevents the production of inflammatory mediators, such as IL-1 and TNF-α, in response to activation by LPS or IFN-γ and it therefore counteracts the Th1-mediated effects on macrophages.

IL-5 production is restricted to a few cell types, which include T cells (Robinson *et al.,* 1992), mast cells (Plaut *et al.,* 1989) and eosinophils (Broide *et al.,* 1992), the predominant source being Th2 cells (Robinson *et al.,* 1992). IL-5 gene expression in Th2 cells has been shown to be stimulated by antigen and cytokines (Bohjanen *et al.,* 1990; Lee *et al.*, 1993). IL-5 plays a central role in orchestrating the inflammatory response in asthma, for example by influencing the development of eosinophilia (Sanderson, 1992; Walker *et al.*, 1991). It promotes the proliferation, activation, differentiation and recruitment of eosinophilic granulocytes. It primes basophils and is also involved in the response to allergens for increased histamine production (Bischoff *et al.*, 1990). IL-5 is a late-acting factor in the differentiation of

primary B cells (O'Garra *et al.*, 1986) and has been suggested to be involved in the production of IgA (Sonoda *et al.*, 1992).

IL-6 is produced by many different cell types including macrophages, fibroblasts, T and B lymphocytes, synovial cells, endothelial cells, glial cells and keratinocytes (reviewed in Ray *et al.*, 1989 and Hirano, 1992). LPS enhances IL-6 production by monocytes and fibroblasts. IL-6 is a pleiotropic, proinflammatory cytokine with several roles in the immune response (reviewed by Hirano, 1992). For example, it plays a role in the terminal differentiation of B cells (Hirano *et al.*,1985) through induction of IL-2 receptor expression on B cells (La Flamme *et al.*, 1999). It induces immunoglobulin synthesis by activated B cells (Yoshizaki *et al*, 1982; Hilbert *et al.*, 1989; Splawski *et al.*, 1990), promotes proliferation of endothelial cells (Giraudo *et al.*, 1996) and cloned T cells by inducing expression of IL-2 receptor (Akira *et al.*, 1993; Lee *et al.*, 1988). IL-6 can mediate Th2 cell differentiation via enhanced IL-4 production and Th1 cell inhibition via the suppressor of cytokine signalling 1 (SOCS1) (Diehl *et al.*, 2000). It is a differentiation factor for cytotoxic T cells (Takai *et al.,* 1988). During a septic reaction, IL-6 stimulates the production of acute phase proteins by hepatocytes (Steel *et al.*, 1994).

The IL-9 gene is expressed in freshly isolated T cells upon stimulation by lectins or anti-CD3 antibodies and is enhanced by PMA. Its expression has been detected in Th2 but not on Th1 cells (Schmitt *et al.*, 1990). IL-9 promotes growth of mast cells (Renauld *et al.*, 1995), previously activated T cells but not freshly isolated T cells (Houssiau *et al.*, 1993). IL-9 has been suggested as a candidate gene for asthma

(Doull *et al.*, 1996; Noguchi *et al.*, 1997), which was supported by the findings in IL-9 transgenic mice challenged with allergen *in vivo* (McLane *et al.*, 1998; Temann *et al.*, 1998). Transgenic mice with lung-specific expression of IL-9 develop airway inflammation, eosinophilia, mast cell hyperplasia and increased airway hyper-responsiveness (Temann *et al.*, 1998).

IL-10 (reviewed in de Waal-Malefyt *et al.*, 1992; Howard and O'Gara, 1992) is produced mainly by murine Th2 cells following stimulation. It is also expressed late in the activation process of B cells, monocytes/macrophages, mast cells and keratinocytes (Moore *et al.*, 1993; Rennick *et al.*, 1992; de Waal Malefyt *et al.*, 1992). IL-10 inhibits the synthesis of a number of Th1 cytokines such as IFN-γ, IL-2 and TNF-α. The inhibitory effect on IFN-γ production is indirect and appears to be the result of suppression of IL-12 synthesis by accessory cells (Fiorentino *et al.*, 1991). In LPS or IFN-γ stimulated macrophages, IL-10 inhibits the synthesis of IL-1, IL-6 and TNF-α by promoting for example the degradation of cytokine mRNA. It inhibits IFN-γ-induced production of reactive oxygen intermediates and nitric oxide by macrophages. Therefore, IL-10 has anti-inflammatory activities (Moore *et al.*, 1993; Bogdan *et al.*, 1991). In contrast, IL-10 can exhibit pro-inflammatory activities, for example it synergizes with LPS and IFN-γ to increase the expression of the leukocyte chemoattractant calcium binding protein S100A8 (A8, Xu *et al.*, 2001). It also leads to an inhibition of antigen presentation by downregulating the expression of class II MHC molecules on the surface of monocytes and macrophages (de Waal Malefyte *et al.*, 1991). IL-10 acts as a costimulator of the proliferation of mast cells and peripheral lymphocytes (mature and immature

thymocytes). In B cells activated via their antigen receptors or via CD40, IL-10 induces the secretion of IgG, IgA and IgM (reviewed in de Waal-Malefyt *et al.*, 1992).

Murine IL-13 is produced by activated Th2 cells, but not by Th1 cells (Zurawski *et al.*, 1994). It has also been detected in mast cells (Burd *et al.*, 1995). IL-13 down-modulates macrophage activity, reducing the production of pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-12, TNF-α) in response to IFN-γ or LPS (de Waal-Malefyt *et al.*, 1993). IL-13 can decrease the production of nitric oxide by activated macrophages, leading to a decrease in parasiticidal activity (Doherty *et al.*, 1993). It induces human monocyte differentiation and human B cell differentiation, proliferation and isotype switching to IgE (Briere *et al.*, 1993; Zurawski *et al.*, 1994). Mice deficient for IL-4 have been shown to develop residual Th2 responses (Kopf *et al.,* 1993; Pearce *et al.*, 1996; Kopf *et al.*, 1995), suggesting that there is a functional equivalent of IL-4. IL-13 is a cytokine that shares some, but not all biological activities with IL-4, for example isotype switching to IgE (Minty *et al.*, 1993; Zurawski *et al.*, 1994). There is now compelling evidence that IL-4 and IL-13 share receptor components, including IL-4R and IL-13R1 (Callard *et al.,* 1996; Hilton *et al.*, 1996). *Nippostrongylus brasiliensis*-infected IL-4Rα-deficient mice show an increased impairment of Th2 differentiation when compared to infected IL-4 deficient mice, suggesting a regulatory role for IL-13 on Th2 cell development (Barner *et al.*, 1998). Supporting observations were made in IL-13- or IL-4/IL-13-deficient mice (McKenzie *et al.*, 1998; McKenzie *et al.*, 1999), where Th2 responses were completely abolished. In aggreement with these results, IL-13

contributes to the susceptible phenotype in *Leishmania major* infections (section 1.9.3.1), but prevents *Schistosoma* egg-induced pathology and allergic asthma (reviewed in Brombacher, 2000).

1.3 Generation of Th1 and Th2 cells

Although a number of factors (Fig. 1.2) have been proposed to influence the induction of Th1 versus Th2 cells, cytokines present in the local milieu appear to play an overriding role. It is generally recognized that IL-12 produced by APC (macrophages and DC), together with TCR activation, drives the differentiation of precursor T cells to Th1 cells. Among CD4(+) lymphocytes, functional receptors for IL-12 appear to be restricted to recently activated, uncommitted cells and to Th1 cells, and are lost on differentiated Th2 cells (Szabo *et al.*; 1995). In the mouse, IL-12 induces Th1 differentiation, while IFN-α has no effect; in contrast, Th1 differentiation of human T cells can be induced by either IL-12 or IFN-α (reviewed by Murphy *et al.*, 2000; O'Garra *et al.*, 1997). The cytokine IL-18 (IFN-γ-inducing factor), related to IL-1, synergizes powerfully with IL-12 to promote Th1 differentiation, in part by increasing the efficiency of Th1 differentiation and in part by acting on differentiated Th1 cells to enhance cytokine expression (reviewed by Akira, 2000; Kohno *et al.,* 1997). Mice deficient in both IL-12 and IL-18 have a more severe defect in IFN-γ production than mice lacking IL-12 or IL-18 alone (Akira, 2000). IL-23, assembled from a novel protein (p19) related to IL-12 p35 and IL-12 p40, also stimulates IFN-γ production and proliferation in PHA blast T

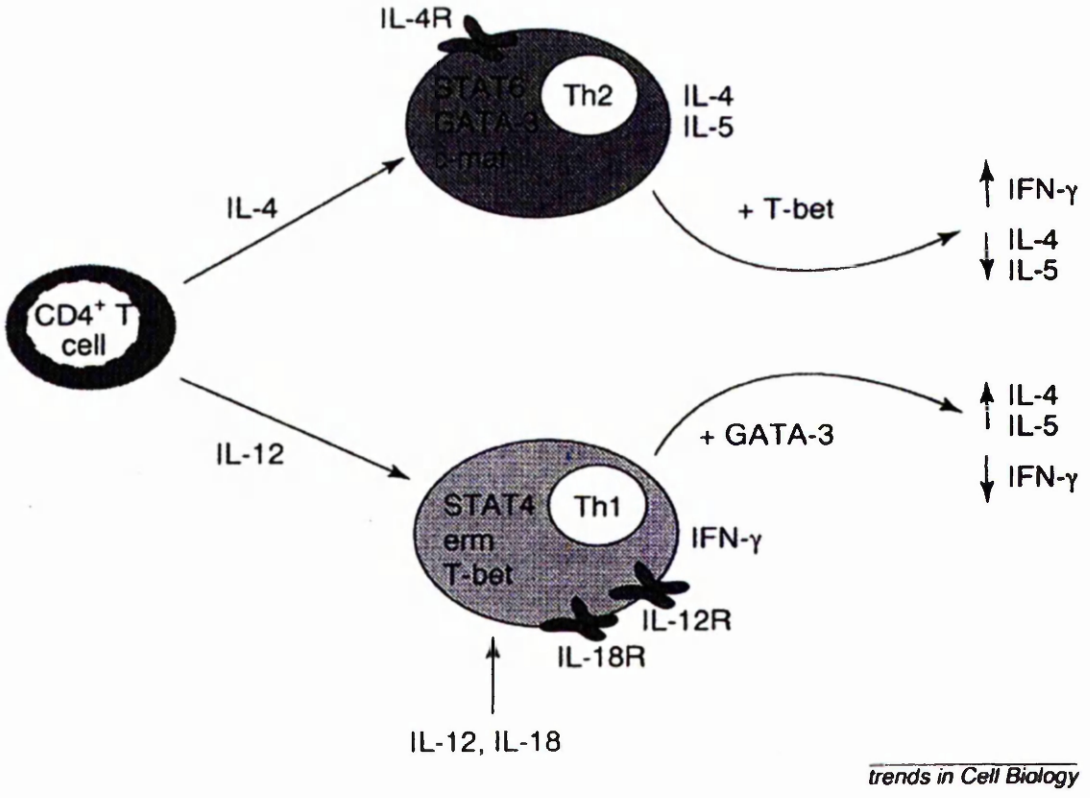


Fig.1.2. Generation of Th1 and Th2 effector cells

The local cytokine environment determines if a naïve CD4(+) T cell polarises towards the Th1 or the Th2 phenotype. IL-12 drives the differentiation of precursor T cells to Th1 cells. The cytokine IL-18 synergises with IL-12 to promote Th1 differentiation. Binding of IL-12 to its receptor activates the STAT4 signalling pathway and the Th1-specific transcription factors ERM and T-bet, which controls the expression of IFN-γ. IL-4 induces the differentiation of Th2 cells through activation of the STAT6 signalling pathway and transcription factors c-Maf and GATA-3. The introduction of GATA-3 into Th1 cells leads to an increase in IL-4 and IL-5 production, while IFNγ is downregulated. T-bet transfected into Th2 cells causes the development of a Th2 phenotype (reviewed in O'Garra and Arai, 2000).

cells, as well as in memory T cells (Oppmann *et al.*, 2000). Hence, IL-23 might also promote Th1 cell differentiation.

IL-4 is generally thought to induce the differentiation of Th2 cells (O'Garra, 1998a; Constant and Bottomly, 1997). Sources of the early burst of IL-4 could be the Th cell themselves (Schmitz *et al.*, 1994; Chen *et al.,* 1999) and/or CD4(+) NK1.1(+) T cells which may recognise antigens presented in association with the non-polymorphic beta2-microglobulin-associated molecule CD1 (Yoshimoto and Paul, 1994). However, mice lacking NK1.1(+) T cells develop normal Th2 responses (Daser *et al.*, 1998; Wang *et al.*, 1998; Chen *et al.*, 1997).

Several factors apart from the cytokine microenvironment influence differentiation along the Th1 or Th2 pathways. These include the concentration of antigen, costimulation of the T cells, the type of APC, the route of antigen entry and the presence of immunologically active hormones (reviewed in Abbas *et al.,* 1996; Romagnani, 1997). Low antigen concentrations and low dose infections tend preferentially to induce Th1 responses, whereas high doses induce Th2 development (Bretscher *et al.*, 1992; Hosken *et al.*, 1995). Very high antigen dose ranges and therefore the highest microbial burdens in infected patients suppress cell-mediated immunity (Livingston *et al.*, 1999). This might seem contradictory to recent findings that Th1 responses are associated with T cells expressing higher affinity TCR (Tao *et al.*, 1997; Rees *et al.*, 1999). However, it is probable that the higher affinity TCR clones are preferentially selected when the Ag dose is low and reciprocally, lower affinity TCR clones, necessarily augmented by costimulatory molecules interactions, are more likely to be activated when the concentration of

Ag is high (Jankovic *et al.*, 2001). Costimulatory signals are widely reported to modulate Th1/Th2 type immunity. CD28 ligation can dramatically favour the development of Th2 cells (Burr *et al.*, 2001; Howland *et al.*, 2000), perhaps by enhancing IL-4 production (Rulifson *et al.,* 1997) or by direct activation of STAT-6 (Oki *et al.*, 2000). Mouse inducible costimulatory molecule (ICOS) is expressed by activated T cells and has homology to CD28 and CD152. Its expression is enhanced by CD28 costimulation and regulates differentiation of CD4(+) T cells to Th2 cells (McAdam *et al.*, 2000). It is thought that CD28 primes T cells and ICOS regulates effector responses (Gonzalo *et al.*, 2001). CD40-CD40L interaction selectively induces Th1 cells as a result of the production of IL-12 by APC (Howland *et al.*, 2000). OX40-OX40L interaction favours Th2 responses (Akiba *et al.*, 2000; Ohshima *et al.*, 1998) as does CD30-CD30L (Del Prete *et al.*, 1995) and CD4 signals (Rogers and Croft, 2000). In contrast, LFA-1 signals appear to potently switch cells towards the Th1 phenotype (Salomon *et al.*, 1998).

The type of APC stimulating the T cell may also play a role in the polarisation of a lymphocyte to a Th1 or a Th2 phenotype (Aloisi *et al.*, 1999). Some studies have reported that B cells are particularly prone to inducing type 2 outcomes (Liano and Abbas, 1987), which is consistent with their need to drive a type 2 response to stimulate their own production of antibody. Conversely, macrophages may be more likely to stimulate type 1 outcomes (Schmitz *et al.*, 1993). Dendritic cells, the most potent antigen-presenting cells (Croft *et al.*, 1992; Cassell *et al.*, 1994; Masten *et al.*, 1999), are capable of driving both type 1 and type 2 outcomes, depending on the local cytokine environment (Hilkens *et al.*, 1997). DC

phenotypes that elicit Th1 or Th2 development have been described as DC1 and DC2, respectively. In mice, these distinct subsets selectively induce Th1 or Th2 responses as a result of their differential secretion of IL-12 (Maldonado-Lopez *et al.,* 1999).

Further, the route of antigen entry also influences the type of immune response. For example, intramuscular injections of DNA vaccine induced Th1 type responses , whilst intravenous injections gave a mixed Th1/Th2 type response as assessed by antigen-specific antibody classes (McCluskie *et al.*, 1999).

Finally, hormones such as glucocorticoids, are powerful stimulators of type 2 responses and powerful inhibitors of type 1 responses, directly inducing IL-4 and IL-10 production from lymphocytes and APC (Daynes *et al.*, 1990; van der Poll *et al.*, 1996; Ramirez, 1998). Like glucocorticoids, estrogens, progestins and catecholamines inhibit type 1 cytokine production and stimulate the secretion of type 2 cytokines (Guilbert, 1996; Szekeres-Bartho and Wegmann, 1996; Kanda and Tamaki, 1999; Sanders *et al.*, 1997; Coqueret *et al.*, 1994).

1.4 Transcription factors for Th1 or Th2 development and commitment

IL-12 binds to a receptor that consists of two chains, IL-12Rβ1 and IL-12Rβ2, causing binding and activation of the transcription factor Janus kinase (JAK)1 and tyrosine kinase (TYK)2 which in turn will then activate the signal transducers and activator of transcription (STAT)1, STAT3 and STAT4. STAT4 signalling is especially crucial for Th1 responses (reviewed by O'Garra and Arai, 2000, Fig. 1.2). The ectopic expression of IL-12Rβ2 in developing Th2 cells results in the

ability of IL-12 to activate STAT4 and proliferation in these cells, however, it does not lead to significant production of IFN-γ. These results confirms that IL-12Rβ2 and IL-12-dependent STAT4 activation is required for Th1 responses, but activation of this pathway is not sufficient to restore a Th1 phenotype in developing or committed Th2 (Nishikomori *et al.*, 2000; Heath *et al.*, 2000).

Szabo *et al.* (2000) have recently isolated T-bet, a protein selectively expressed in Th1 but not in Th2 cells. T-bet belongs to the T box family of transcription factors that play crucial roles in diverse developmental processes (Szabo *et al.*, 2000). IL-12 seems to induce expression of T-bet, which is enhanced by IL-18 (Heath *et al.*, 2000). It controls the expression of the hallmark Th1 cytokine, IFN-γ. In contrast, T-bet expression represses the activation of the IL-2 promoter, explaining findings that differentiating Th1 cells downregulate IL-2 expression. Retroviral gene transduction of T-bet into polarised Th2 and Tc2 primary T cells redirected them into Th1 and Tc1 cells, respectively. Remarkably, introduction of T-bet into a stably committed Th2 population also dramatically increased the number of IFN-γ-producing cells and reduced the number of IL-4- and IL-5-producing cells (Szabo *et al.*, 2000). Thus, T-bet can convert Th2 into Th1 cells.

A number of transcription factors have been implicated in Th2 differentiation: STAT6, NF-IL-6, NFATc/2, AP-1/Jun-B, c-Maf, SKAT2 and GATA-3 (O'Garra, 1998; Murphy *et al.*, 1999; Rincon and Flavell, 1997; Szabo *et al.*, 1997a; Zheng and Flavell, 1997; Zhang *et al.*, 1997; Ho *et al.*, 1996; Blanchard *et al.*, 2000).

Ligation of IL-4 with its receptor activates signalling through several pathways. JAK1 and JAK3 activate STAT1, STAT3 and the Th2 specific STAT6 (Hou *et al.*,

1994). STAT6-deficient mice showed defects in Th2 responses (Kaplan *et al.*, 1996; Shimoda *et al.*, 1996; Takeda *et al.*, 1996), similar to the phenotype of IL-4-deficient mice (Kuhn *et al.*, 1991; Kopf *et al.*, 1993), indicating a crucial role for STAT6 in inducing Th2 differentiation. When STAT6 was transduced retrovirally into naïve T cells driven under Th1 conditions, activation of STAT6 induced the production of IL-4, IL-5 and IL-10 and repressed Th1 development and production of IFN-γ at least in part through inhibition of IL-12 Rβ2 (Kurata *et al.*, 1999). Additionally, GATA-3 and c-maf expression was induced, suggesting that these factors are downstream from STAT6 in the IL-4R signalling cascade (Kurata *et al.*, 1999).

Although NFAT and AP-1 proteins are important for inducing IL-4 and IL-5 transcription, they are present in both Th1 and Th2 subsets and do not appear to account for the Th2-restricted expression of IL-4 (Rooney *et al.*,1995). Recent findings show that NFATp and NFAT4/x can excert a negative regulatory role on IL-4 expression *in vivo*, whereas NFATc apparently positively transactivates the IL-4 promoter (reviewed in Szabo *et al.*, 1997a).

c-Maf is another transcription factor selectively expressed in naïve T cell at low level and in Th2, but not Th1 cells (Ho *et al.*, 1996). It directly augments IL-4 promoter activity through cooperative interaction with Nip-45, NF-AT (Ho *et al.*, 1996, 1998) and JunB (Rincon and Flavell, 1997). c-Maf-deficient mice have been shown to be defective in IL-4 production (Kim *et al.*, 1999), but retroviral infection of developing Th1 cells with c-Maf did not induce the expression of IL-4 (H.J. Lee *et al.*, unpublished).

SKAT2 has also been identified in Th2 but not in Th1 cells (Blanchard *et al.*, 2000). Transfection studies suggest that it upregulates the activity of the gene promoter for IL-4 but not IL-5 (Blanchard *et al.*, 2000). Thus, SKAT2 and GATA-3 (see below) might contribute to the selective regulation of Th2 cytokine genes.

The importance of GATA-3 in Th2 cytokine gene expression and/or Th2 differentiation has been demonstrated by several investigators. GATA proteins (1-6) are members of a zinc-finger superfamily of DNA-binding proteins and recognize a common consensus motif through a conserved zinc-finger DNA-binding domain (Orkin, 1992). Zheng *et al.* (1997) reported that the transcription factor GATA-3 is expressed in naive T cells and Th2 but not Th1 cells and is critical for IL-5 gene expression by activating the IL-5 promoter. A GATA-3 binding site was identified in the IL-5 promoter (Zheng and Flavell, 1997; Lee *et al.*, 1998). In a different study, GATA-3 was shown to be important and sufficient for the expression of other Th2 cytokines including IL-4, IL-6 and IL-10; GATA-3-dependent enhancer activity has been found within several regions surrounding the IL-4 gene (Zheng and Flavell, 1997; Ranganath *et al.*, 1998). This suggests that GATA-3 controls also IL-4 expression through distal enhancer elements outside the IL-4 promoter. cAMP enhanced the GATA-3 effects to induce IL-4 and IL-5, although it had no effect on inducing these cytokines in Th1 cells in isolation (Lee *et al.,* 2000). Further, STAT6 and CD28-mediated signalling can also upregulate the expression of GATA-3 (Kurata *et al.*, 1999; Rodriguez-Palmero *et al.*, 1999). Retroviral infection of naïve T cells with GATA-3 when cultured under Th1 conditions induced the production of Th2-specific cytokines and

downregulated IFN-γ production at least in part through repression of IL-12 signalling (Ferber *et al.*, 1999; Ouyang *et al.*, 1998). In addition to modulating cytokine production, ectopic expression of GATA-3 induced the expression of endogenous GATA-3 in developing Th1 but not in committed Th1 cells, suggesting autoregulation of GATA-3 in Th2 cells (Ouyang *et al.*, 2000; Lee *et al.*, 2000). It is thought to be a major factor in the control of the production of all Th2-specific cytokines, thus maintaining the Th2 phenotype (reviewed in O'Garra and Arai, 2000).

1.5 Th1 and Th2 cytokine gene regulation at the chromatin level

Th1 and Th2 differentiation are accompanied by changes in Dnase I hypersensitivity and the methylation state of the *IFN-γ* and linked the *IL-4/IL-13/IL-5* genes (Takemoto *et al.,* 1998; Agarwal *et al.*, 1998). These changes are associated with selective accessibility of gene regulatory regions to DNA-binding proteins. While the cytokine IL-2 is produced by antigen-stimulated naïve T cells even prior to S phase, the production of IFN-γ becomes apparent after one cell cycle, the production of IL-4 after 3-4 cycles (Bird *et al.*, 1998; Gett *et al.*, 1998). In naïve T cells the *IFN-γ* and the linked *IL-4/IL-13/IL-5* genes reside in a chromatin environment that is not accessible to transcription factors (reviewed in Bird *et al.*, 1998; Agarwal *et al.*, 1998). The first exposure of naïve T cells to antigen and cytokine results in specific changes in the chromatin structure and DNA methylation of the relevant cytokine genes. The differentiation to Th2 cells is associated with changes in the chromatin structure of the *IL-4* and the *IL-13* gene

(Agarwal *et al.*, 1998; Agarwal *et al.*, 1998a; Takemoto *et al.*, 1998). Complimentary changes in chromatin occur at the *IFN-γ* locus in the course of Th1 commitment (Agarwal *et al.*, 1998; Agarwal *et al.*, 1998a).

Transcription factors such as GATA-3 and T-bet, the main inducers of Th2 and Th1 differentiation, might control the production of subset specific cytokines also by inducing epigenetic changes in the chromatin structure. T-bet has been shown to specify Th1 effector fate by targeting chromatin remodeling to individual *IFN-γ* alleles (Mullen *et al.*, 2001). After polarisation, the majority of silenced cytokine alleles were repositioned to heterochromatin. Naïve T cells transit through sequential stages of cytokine activation, commitment, silencing and physical stabilization during polarisation into differentiated effector subsets (Grogan *et al.*, 2001).

1.6 Cross-regulation between Th1 and Th2 cells

Th1 and Th2 cells can antagonize each other by blocking both differentiation and effector functions. IL-4, STAT6, GATA-3 and Maf act to maintain the Th2 phenotype of differentiated T cells and downregulate Th1 cytokine expression. The presence of high concentrations of IL-4 and IL-10 inhibits the secretion of IL-12 and IFN-γ, blocking the ability to generate Th1 cells from naïve T cells (Hsieh *et al.*, 1993; Rincon and Flavell, 1997; D'Andrea *et al.*, 1993; Ohmori *et al.*, 1997,Gajewski *et al.*, 1988; Demeure *et al.*, 1994). The downregulation of IFN-γ production by IL-4 appears to be mediated through the STAT6 target gene products GATA-3 and Maf. Ectopic expression of GATA-3 or Maf, even in IL-4-

deficient naïve T cells results in marked inhibition of IFN-γ production by Th1 cells (reviewed in Rao and Avni, 2000). In the mouse, differentiating Th2 cells lose expression of the β2 subunit of the IL-12 receptor, thus dampening their ability to respond to IL-12 stimulation and focusing their cytokine production towards IL-4. Ectopic expression of GATA-3 in murine Th1 cells also results in downregulation of IL-12Rβ2 chain expression, indicating that this downregulation is also mediated by GATA-3. IL-10 which is produced in large amounts by Th2 cells inhibits macrophage functions such as secretion of IL-12, that induces Th1 cell development. IL-10 also antagonizes the upregulation of MHC molecules caused by IFN-γ (reviewed in Moore *et al.*, 1993; Mosmann and Moore, 1991).

Conversely, ectopic expression of the Th1 transcription factor T-bet in differentiating T cells leads to down-regulation of IL-2 and several Th2-specific cytokines, suggesting that the role of this transcription factor in the Th2 lineage is analogous to that of GATA-3 in the Th2 lineage (reviewed in Rao and Avni, 2000). IFN-γ secreted by Th1 cells directly suppresses IL-4 secretion and therefore inhibits differentiation into Th2 cells (Gajewski *et al.*, 1988; Demeure *et al.*, 1994) and blocks the proliferation of Th2 effector cells (Fitch *et al.*, 1993). Th1 cells are unaffected because they do not express the β chain of the IFN-γ receptor (Pernis *et al.*, 1995).

1.7 Definition of Th1 and Th2 cells by differentially expressed cell surface proteins

The identification of novel cell surface markers specific for the Th1 or Th2 cell subset could provide a tool to assess and modulate the Th1/Th2 cell balance in diseases. CD4(+) effector T cells generated *in vitro* express a broad range of chemokine receptors, some of which are preferentially expressed on Th1 cells, such as CXCR3, CCR5 (reviewed in D'Ambrosio *et al.*, 2000). CXCR3 binds to the chemokines IP10 (interferon-inducible protein 10), I-TAC (interferon-inducible T cell alpha-chemoattractant) and Mig (monokine induced by gamma-interferon), while CCR5 binds to MIP1((macrophage-inflammatory protein) (Sallusto *et al.*, 1998; Laurence *et al.*, 2001). The chemokine receptors CCR3, CCR4, CCR8, which bind to eotaxin, TARC (thymus and activation-regulated chemokine) and MDC-1 (macrophage-derived chemokine) respectively (Sallusto *et al.*, 1998; Sallusto *et al.*, 1998a), are preferentially expressed on Th2 cells (Sallusto *et al.*, 1997; Bonecchi *et al.*, 1998; Sallusto *et al.*, 1998; O'Garra and McEvoy *et al.*, 1998). Chemokines which act as agonists for CXCR3, are antagonists for CCR3 (Loetscher *et al.*, 2001). These results suggests that chemokines that attract Th1 cells via CXCR3 can concomitantly block the migration of Th2 cells in response to CCR3 ligands, thus enhancing the polarisation of T cell recruitment. Recently, a novel leukocyte chemoattractant receptor, expressed only on Th2 cells, has been reported, which was designated CRTH2, (chemoattractant receptor-homologous molecule expressed on Th2 cells). Allergen-induced proliferation of peripheral blood mononuclear cells is significantly reduced by antibody-mediated depletion of

CRTH2(+) cells (Nagata *et al.*, 1999). These findings suggest, that distinct chemotactic signals may contribute to the preferential localization of Th1 and Th2 cells in sites of inflammation.

Other Th1 cell-specific cell surface molecules include the IL-12Rβ2 chain, whose expression ensures Th1 type polarisation and IFN-γ production and which is lost from polarising Th2 cells (Szabo *et al.*, 1997). IL-18R is a member of the IL-1R family and its signalling in combination with IL-12R signalling induces a strong IFN-γ production and drives Th1 development (Robinson *et al.*, 1997). IL-18R was found to be selectively expressed on Th1 cells (Xu *et al.*, 1998a). LAG-3, another Th1 cell-specific surface marker, is a MHC class II ligand that has been used *in vivo* to stimulate MHC class II(+) APC to increase tumor-specific immune responses (El Mir *et al.*, 2000). Its expression is strongly increased by IFN-γ (Annunziato *et al.*, 1997; Scala *et al.,* 1998). IFN-γ receptor β chain expression is downregulated in polarising Th1 cells and stays unaffected in polarising Th2 cells, maintaining their susceptibility to IFN-γ induced suppression of proliferation (Pernis *et al.*, 1995). Lymph node homing receptor CD62L (L-selectin) expressing CD4(+) T cells were found to produce IL-4 and IL-5, whereas CD62L negative CD4(+) T cells expressed IFN-γ (Kanegane *et al.*, 1996). Finally, the molecule of interest for this thesis - the orphan receptor ST2L, which has been linked to Th2 cell effector functions, is differentially expressed on Th2 but not on Th1 cells (Xu *et al.*, 1998).

Expression patterns of "stable" cell surface makers for Th1 and Th2 cells should be unique for the respective subset. Chemokine receptors are generally not used

to distinguish Th1 from Th2 cells due to their transient expression and partly overlapping expression patterns. For example, major fractions of circulating CCR4(+) memory CD4 lymphocytes co-express the Th1-associated receptors CXCR 3 and CCR5, suggesting a potential problem in using these markers for Th1 versus Th2 lymphocyte cells (Andrew *et al.*, 2001). In the murine and the human system, ST2L and IL-18R are widely applied to distinguish Th1 and Th2 cells (Liew *et al.*, 1999; Chan *et al.*, 2001). IL-12 receptor β2 and IFN-γ receptor β are also useful markers for Th1 and Th2 cells, respectively (Hamalainen *et al.*, 2000; Rogge *et al.*, 1999; Groux *et al.*, 1997a). Th1 and Th2 responses can be modulated by using anti-ST2L antibodies, anti-IL18R antibodies or anti-IL-12 antibodies *in vitro* and *in vivo* (Xu *et al.*, 1998; Xu *et al.*, 1998a; Heinzel and Rerko, 1999).

1.8 ST2 and ST2L: members of the IL-1 receptor superfamily

1.8.1 IL-1 receptor/Toll-like superfamily

The interleukin-1 (IL-1) receptor/Toll-like receptor (TLR) superfamily is an expanding group of receptors that participates in host responses to injury and infection. The superfamily is defined by the Toll/IL-1 receptor (TIR) domain, which occurs in the cytosolic region of family members and is further subdivided into two groups based on homology to either the Type I IL-1 receptor or the *Drosophila* Toll receptor extracellular domain (Fig.1.3). The former group includes the IL-18 receptor and ST2L, which may have a role in Th2 cell function. The latter group

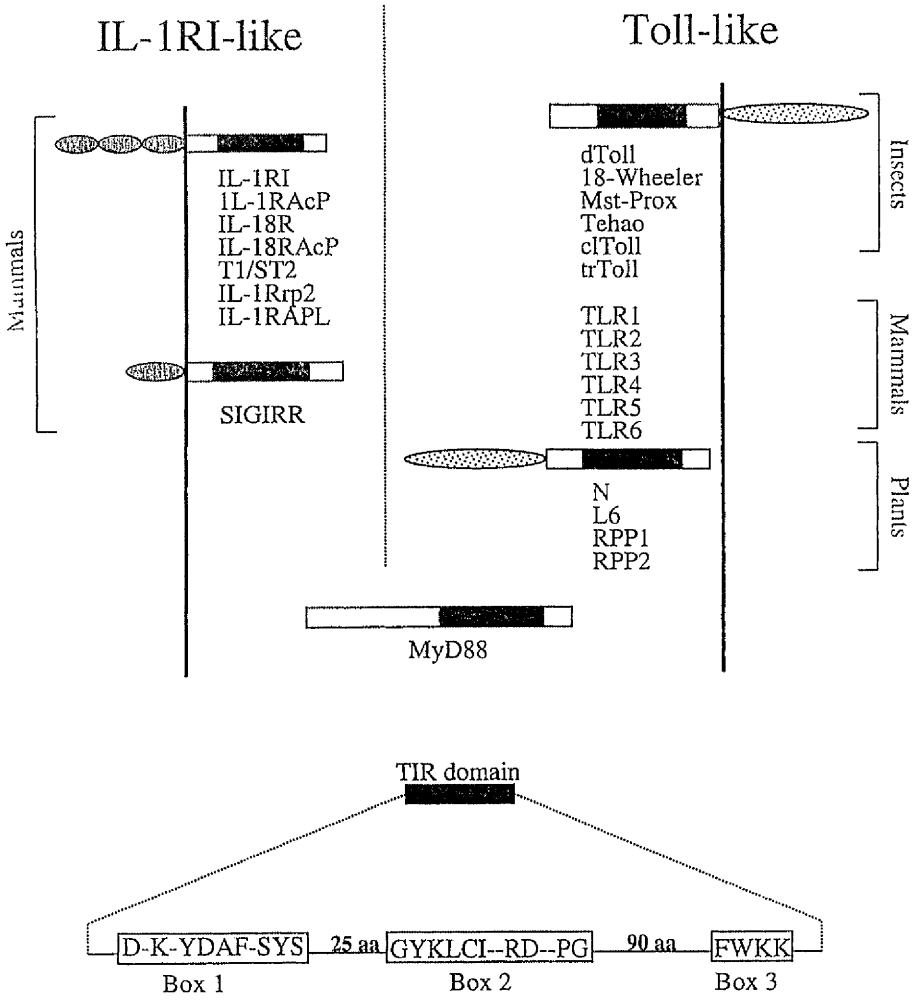


Fig. 1.3 IL-1R/ Toll family

The family is defined by the presence of the Toll/IL-1 receptor (TIR) domain, which is involved in signalling, and is subdivided into two groups based on homology to either IL-1R-like extracellular immunoglobulin loops (shaded elipses) or Toll-like leucine-rich repeats (stippled elipses).The family includes members from mammals, insects and plants (reviewed in Fitzgerald and O'Neill, 2000).

includes at least ten mammalian TLRs (Kaisho and Akira, 2001), including TLR2, binding to gram-positive bacteria and mycobacteria, and TLR4, part of the lipopolysaccharide (LPS) receptor and essential for the recognition of both gram-negative bacteria and respiratory syncytical virus (reviewed in Bowie and O'Neill, 2000; Takeuchi *et al.*, 1999; Brightbill *et al.*, 1999; Poltorak *et al.*, 1998; Kurt-Jones *et al.*, 2000).

1.8.2 IL-1R family members

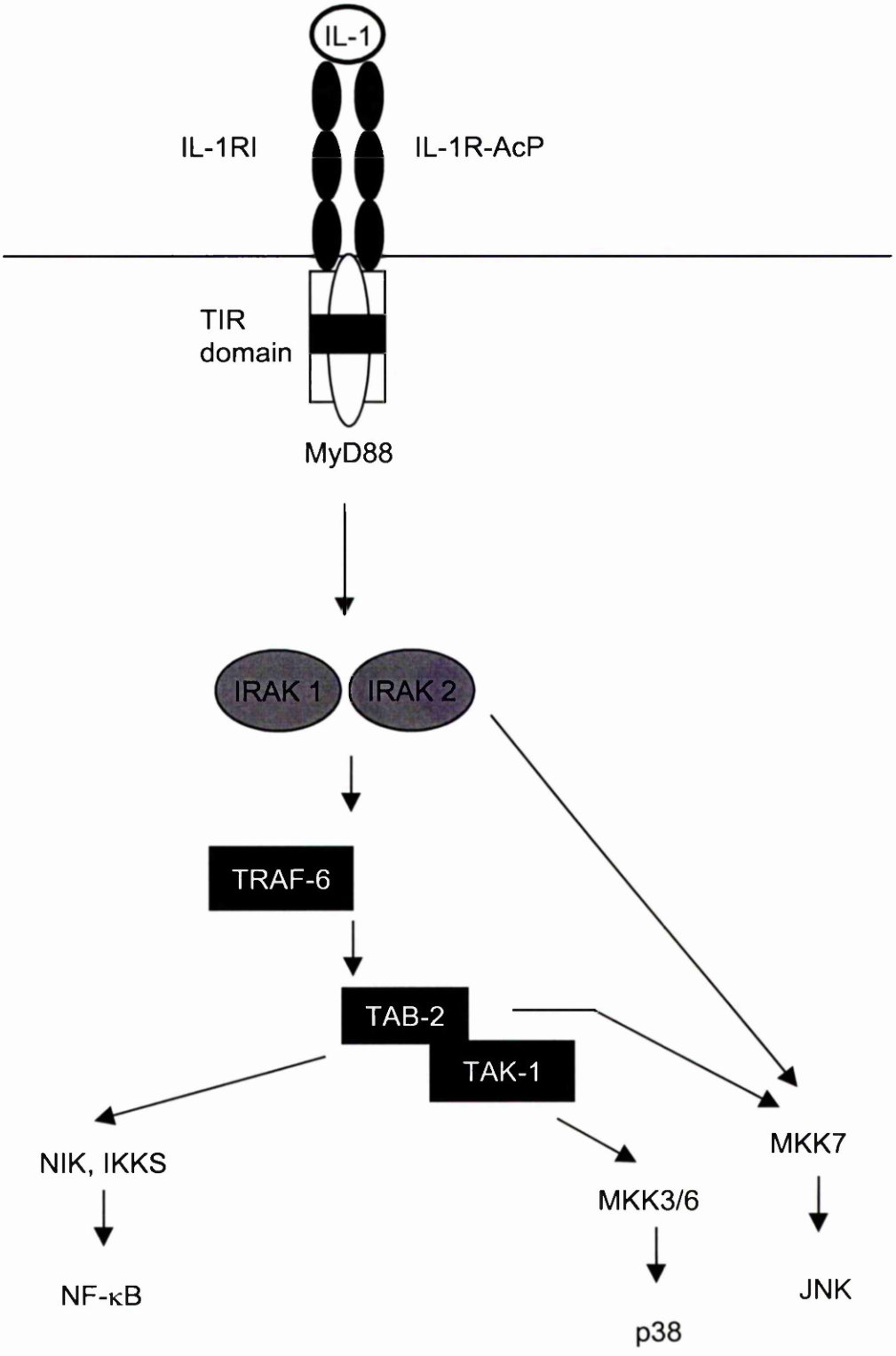
IL-1 (reviewed in Nicola, 1994) is secreted mainly by monocytes, macrophages and keratinocytes. There are three members of the IL-1 gene family: IL-1α , IL-1β and IL-1Ra (IL-1 receptor antagonist). IL-1α and IL-1β are agonists and IL-1Ra acts as a specific receptor antagonist. In most studies the effects of IL-1α and IL-1β in terms of biological activity are indistinguishable. IL-1 is produced in response to microbes and microbial products such as LPS as well as in response to viruses, IFN-γ and TNF-α. It has a central role in inflammatory responses and autoimmune diseases such as rheumatoid arthritis. IL-1 affects a wide range of cells involved in immune and inflammatory responses including fibroblasts, where it causes proliferation and prostaglandin release. It causes for example keratinocyte proliferation and IL-6 release, B cell differentiation and Ig secretion and Th2 cell proliferation.

IL-1RI, IL-1RII and the IL-1 receptor accessory protein (IL-1RAcP) belong to the IL-1R superfamily (reviewed in Dinarello, 1998). The extracellular domains of the IL-1RI, IL-1RII and the IL-1R-AcP are members of the immunoglobulin superfamily

and are heavily glycosylated. Blocking the glycosylation sites reduces the binding of their ligand IL-1 (Mancilla *et al.*, 1992). IL-1R family members have a single transmembrane segment and a cytoplasmic domain of varying length. IL-1RI is the primary signal transducing receptor (Stylianou *et al.*, 1992, Sims *et al.*, 1993), while the type II receptor lacks a signal transducing cytosolic domain and is therefore nonfunctional. IL-1 binds first to the IL-1RI with a low affinity, which may cause a structural change in IL-1, which allows the IL-1R-AcP to join the IL-1RI-IL-1 complex, producing a high-affinity binding complex. (Greenfeder *et al.*, 1995) (Fig. 1.4). After binding of IL-1 to the IL-1RI/IL-1R-AcP complex, the adapter protein MyD88 is recruited and binds to IRAK-1 and 2 (IL-1R-associated kinase-1 and 2) and the signalling complex of the two receptor chains. Thereby it mediates the association of IRAK-1 and 2 with the receptor. IRAK-1 and 2 become heavily phosphorylated and concomitantly activated. Then IRAK-1 and 2 regulate the redistribution of TAB2 (TAK-1 binding protein 2), facilitating the formation of a TRAF6 (TNF receptor-associated factor 6)-TAB2-TAK1 (TGF-β activating kinase 1) complex, which is an essential step to activate TAK1. TAK1 then activates NIK (NF-κB-inducing kinase) leading to the activation of NF-κB via the signalsome (a large multiprotein signalling complex that contains IKKs [I-κB kinases]). MyD88 and IRAK-1 also participate in the activation of p38 and JNK (Jun N-terminal kinase) pathways (reviewed in O'Neill and Dinarello, 2000; Wesche *et al.*, 1997; Knop and Martin, 1999; Takaesu *et al.*, 2001; Takaesu *et al.*, 2000; Ninomiya-Tsuji *et al.*, 1999). In T lymphocytes the addition of IL-1 increases nuclear binding of c-jun and c-fos, the two components of AP-1 (Muegge *et al.*, 1989). The

Fig. 1.4 IL-1 receptor (IL-1R) signalling pathways

IL-1 binds first to the IL-1RI with low affinity. This allows the IL-1R accessory protein (AcP) to join and form a high-affinity binding complex. After binding of IL-1 to the IL-1 RI/IL-1R-AcP complex, the adaptor protein MyD88 is recruited and binds to the IL-1R-associated kinase-1 and 2 (IRAK-1 and 2) and the signalling complex of the two receptor chains. This then mediates the association of IRAK-1 and 2 with the receptor. IRAK-1 and 2 become phosphorylated and concomitantly activated. Then IRAK-1 and 2 regulate the redistribution of TAK-1 binding protein 2 (TAB2) and facilitates the formation of a TNF receptor-associated factor 6 (TRAF6) -TAB2-TAK1 complex, which is an essential step to activate TAK1 (TGF-β activating kinase 1). TAK1 then activates the NF-κB-inducing kinase (NIK) leading to the activation of NF-κB via the signalsome [a large multiprotein signalling complex that contains IKKs (I-κB kinases)]. MyD88 and IRAK-1 also participate in the activation of p38 and JNK pathways. The translocation of the transcription factors NF-κB, p38, JNK and AP-1 to the nucleus leads to the expression of a large number of genes characteristic of inflammation (reviewed in Dinarello, 1998).


IL-1
IL-1RI
IL-1R-AcP
TIR
domain
MyD88
IRAK 1
IRAK 2
TRAF-6
TAB-2
TAK-1
NIK, IKKS
NF-κB
MKK3/6
p38
MKK7
JNK

translocation of the transcription factors NF-κB, p38, JNK and AP-1 to the nucleus leads to the expression of a large number of genes characteristic of inflammation, such as IL-6, IL-8, ICAM-1 and IL-1 itself (reviewed in Nicola, 1994).

The IL-18R was originally identified using degenerate oligonucleotides based on conserved regions in the TIR domain. The amino acid sequence of IL-18R is identical with that of IL-1R-related protein (IL-1Rrp, Yoshimoto *et al.*, 1998), which was initially cloned as an orphan receptor bearing similarity to the type I IL-1R (IL-1RI, Parnet *et al.*, 1996). It was shown to signal given the fact that a chimeric receptor incorporating the extracellular domain of IL-1RI fused to the intracellular domain of IL-18 could bind IL-1 and lead to NF-κB activation (Parnet *et al.*, 1996). Similar to IL-1, IL-18 requires an accessory protein (IL-18R-AcP) to associate with the receptor-ligand for signalling. IL-18 binds to IL-18R and induces the activation of IL-1R-associated kinase, TRAF-6, NF-κB, and c-Jun N-terminal kinase (JNK) in Th1 cells (Okamura *et al.*, 1998; Torigoe *et al.*, 1997; Kojima *et al.*, 1998; Adachi *et al.*, 1998). Further, MyD88 is a critical component in the IL-1 and IL-18 receptor signalling cascade (Adachi *et al.*, 1998). Therefore, IL-1R and IL-18R share common signalling pathways, such as the MyD88/IRAK/TRAF6 signalling pathway leading to the activation of NF-κB and JNK (Kojima *et al.*, 1998).

The member of the IL-1R family this thesis is concerned with is ST2L, an orphan receptor and its shorter soluble variant ST2 (Yanagisawa *et al.*, 1993; Tominaga *et al.*, 1989). ST2L is able signal to NF-κB by using a chimeric receptor incorporating the extracellular domain of IL-1RI fused to the intracellular TIR-containing ST2L domain (Mitcham *et al.*, 1996).

1.8.3 ST2 and ST2L

Murine ST2 mRNA (2.7 kb) was first identified to be expressed in growth-stimulated murine fibroblasts and found to be related to the immunoglobulin superfamily (Tominaga *et al.*, 1989). A 5 kb long mRNA transcript called ST2L, exhibiting 28% sequence similarity to IL-1RI (Yanagisawa *et al.*, 1993), was identified through screening of a fibroblast library with a ST2 cDNA fragment (Yanagisawa *et al.*, 1993). The longer transcript (ST2L) translates to a membrane bound form consisting of three parts: an extracellular region with three immunoglobulin domains and N-glycosylation sites, a putative transmembrane domain and a cytoplasmic domain. The shorter transcript (ST2) translates to the soluble form, which shares the common extracellular domain, but lacks the transmembrane and the intracellular domain (Gaechter *et al.*, 1996; Gaechter *et al.*, 1998) (Fig. 1.5).

The expression pattern of the murine soluble and membrane bound receptor form differ drastically. The ST2L mRNA transcript is restricted to cells of major haemopoietic organs like fetal liver, spleen, bone marrow (Roessler *et al.*, 1995) and to cells of the lung, a non-haemopoietic organ, throughout ontogenesis (Bergers *et al.*, 1994). It was also found in macrophage cell lines, erythroid progenitors and bone marrow-derived mast cells (Roessler *et al*, 1995; Bergers *et al.*, 1994). Further, Xu *et al.* (1998) reported that ST2L is expressed on Th2, but not Th1 cells. The short mRNA transcript is predominantly expressed in non haemopoietic tissues such as embryonic bone, skin and retina. It was also

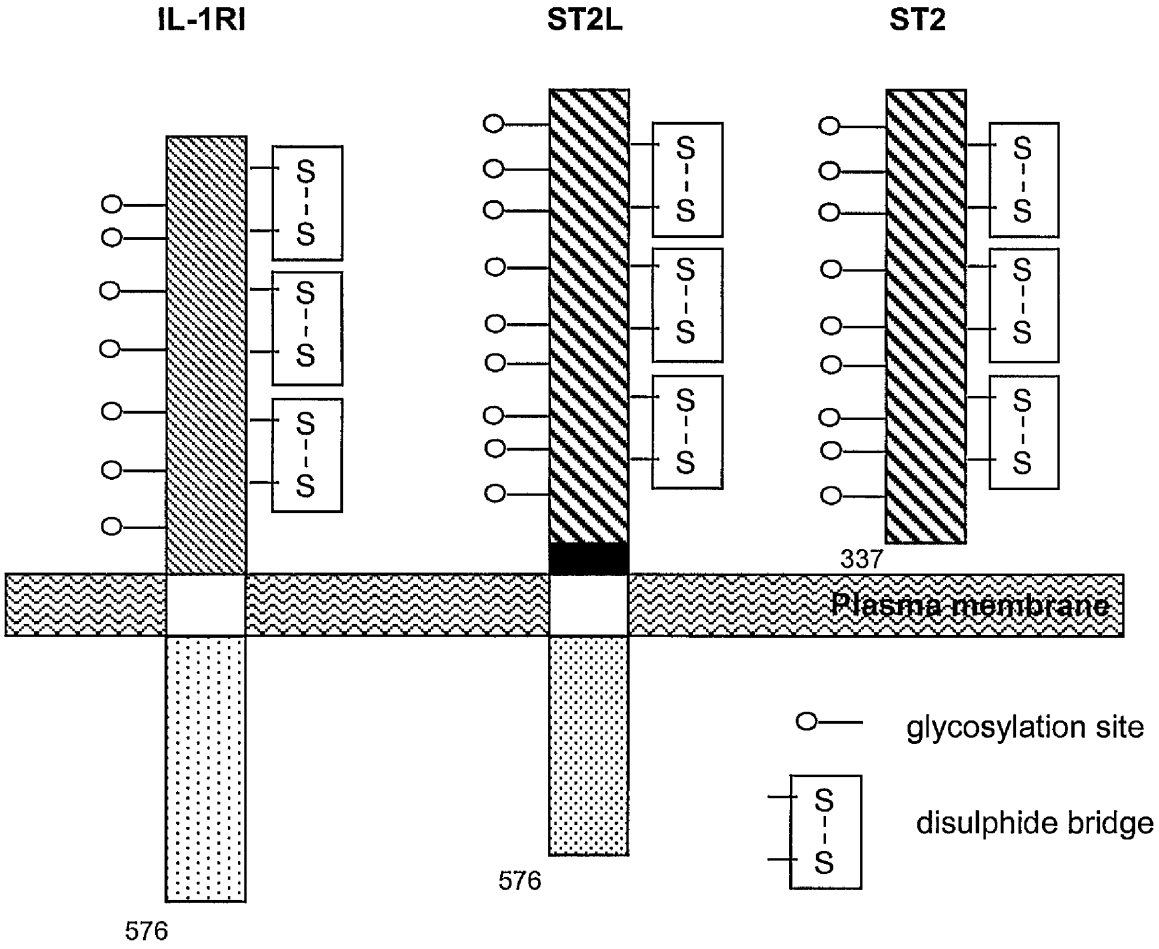


Fig. 1.5 Structure of IL-1RI, ST2L and ST2

ST2L is an a orphan receptor related to IL-1RI, consisting of 3 parts, an extracellular region with three immunoglobulin-like domains and N-glycosylation sites, a putative transmembrane domain and a cytoplasmic domain. ST2, the shorter splice variant, shares the common extracellular domain but lacks the transmembrane and the intracellular domain (taken from Yanagisawa *et al.*, 1993).

identified in developing mammary gland, fibroblast cell lines and some T cell lines (Roessler *et al.*, 1995).

These differential expression patterns suggests that ST2L and ST2 may have different functions on different cell types and that their expression should be controlled at promoter level and/or post-transcriptional level. In rat fibroblasts, alternative promoter usage leads to the generation of ST2 and ST2L from the proximal and distal promoter, respectively. The sequence difference at their 3' end results from alternative 3' processing (Berger *et al.*, 1994). In contrast, in the mouse and human, the mRNA for both ST2 and ST2L is transcribed from one promoter. The murine upstream (distal) promoter directs transcription of ST2 and ST2L in haemopoietic cells such as mast cells, whilst the murine downstream (proximal) promoter directs expression in fibroblasts (Thomassen *et al.*, 1995; Gaechter *et al.*, 1996; Iwahana *et al.*, 1999). Gaechter *et al.* (1996) suggested that transcription initiation is tightly coupled to 3' processing, such that if transcription starts at the distal promoter, the long mRNA is produced, whereas initiation at the proximal promoter instructs the transcription machinery to give rise to the short transcript. Which one of these two promoters is active in murine T cells is yet to be identified.

ST2L expression is upregulated by serum stimulation of Th2 cells (Xu *et al.*, 1998), by overexpression of the GATA-1 transcription factor in mast cells, the expression of Fos-ER in fibroblasts and by addition of LPS to monocytes (Gaechter *et al.*, 1998; Berger *et al.*, 1994; Saccani *et al.*, 1998). Recently, Meisel *et al.* (2001) reported that ST2L is not expressed on naïve Th cells, but Th2

polarising conditions dramatically increase the number of ST2L expressing cells. Several APC or T cell-derived cytokines, such as IL-6, IL-5, IL-1, TNF-α, which are growth and/or survival factors for Th2 cells (Joseph *et al.*, 1998; La Flamme *et al.*, 1999), are able to enhance the expression of ST2L on Th2 cells (Meisel *et al.*, 2001).

ST2 expression is induced by inflammatory stimuli such as LPS (Saccani *et al.*, 1998). Further, IFN-γ, TNF-α, IL-1β, UVB, calcium ionophores as well as oncogenes (Fos and Ha-*ras*) and the growth factors PDGF, FGF and bFGF induce ST2 expression in fibroblasts and mast cells (Kumar *et al.*, 1997; Laursen *et al.*, 1998; Gaechter *et al.*,1998; Werenskiold *et al.*, 1989). However, the role of Th1 and Th2 type cytokines in the differential regulation of ST2 and ST2L gene expression in T helper lymphocytes is unclear.

The function of ST2L is still controversial. Anti-ST2L antibodies have been successfully used to alter the Th1/Th2 type balance in *in vivo* disease models, such as *Leishmania major* infection in BALB/c mice, where an increased resistance to the infection was reported in antibody treated mice (Xu *et al.*, 1998). This treatment also exacerbated collagen-induced arthritis in DBA/1 mice, attenuated eosinophilic inflammation and reduced the lung inflammation caused by respiratory syncytial virus (Xu *et al.*, 1998; Loehning *et al.*, 1998; Walzl *et al.*, 2001). These results showed that inhibition of ST2L and/or complement mediated killing of ST2L-expressing Th cells resulted in decreased Th2 responses. Cross-linking of ST2L with monoclonal anti-ST2L antibody (anti-ST2L mAb) provided a costimulatory signal for Th2 but not for Th1 cells and directly induced proliferation

and type 2 cytokine production (Meisel *et al.*, 2001). This demonstrates that ST2L plays an important role in the activation of Th2 cells. Several groups used a ST2-Fc fusion protein, containing the extracellular part of ST2L, to mimic the effects of ST2. Treatment with ST2-Fc reduced the number of eosinophils in the airway and the secretion of Th2 cytokines during eosinophilic inflammation (Loehning *et al.*, 1998; Coyle *et al.*, 1999) as well as lung inflammation and airway hyperresponsiveness in a murine model of allergic asthma (Coyle *et al.*, 1999). Inhibition of ST2L signalling with either anti-ST2L mAb or ST2-Fc fusion protein selectively inhibited cytokine secretion from Th2 cells (IL-4, IL-5, IL-6) without modifying IFN-γ secretion by Th1 cells (Coyle *et al.*, 1999). Presuming that ST2 and ST2L compete for the same ligand and therefore ST2 can block ST2L signalling, the results suggest that ST2L signalling is required for optimal production of Th2, but not Th1 cytokines. In contrast, ST2-Fc fusion protein impaired inflammatory cytokine responses in LPS-stimulated macrophages, which do not express ST2L (Sweet *et al.*, 2001). ST2-Fc was shown to mediate this function by binding to an unidentified cell surface molecule (Sweet *et al.*, 2001).

ST2L is an orphan receptor, so the identification of its ligand is essential to extend the knowledge about its function. Binding studies using ST2L revealed affinity to membrane-bound molecules of B cell lines (myeloma-derived RPMI8226 cells) (Yanagisawa *et al.*, 1997) and activated B cells (Lambrecht *et al.*, 2000), dendritic cells and activated macrophages (unpublished data from Coyle, 1999; Sweet *et al.*, 2001). Gayle *et al.* (1996) reported the cloning of a putative ligand, a cell surface protein from a neuroblastoma cell line, which was unable to initiate signal

transduction by ST2L receptor. ST2L also bound to a putative ligand secreted from BALB/c 3T3 fibroblasts and human umbilical vein endothelial cells (Kumar *et al.*, 1995).

One out of three created ST2L knockout mice demonstated the importance of ST2L signalling for Th2 development and effector function. Townsend *et al.* (2000) found, that in a primary pulmonary granuloma model induced by *Schistosoma mansoni* eggs eosinophil infiltration was abrogated in ST2L-/- mice and the level of Th2 cytokine was impaired. Even in a secondary pulmonary granuloma model, draining lymph nodes cells from the ST2L-/- animals produced significantly reduced levels of IL-4 and IL-5, despite developing granulomas of a magnitude similar to those of wild type mice and comparable antigen-specific Ig isotype production. ST2L expression does not play a role in the *in vitro* generation of Th2 cells and Th2 cytokines in cells derived from naïve animals, but its presence can affect the onset of Th2 cytokine responses to antigenic stimulation *in vivo*. However, Hoshino *et al.* (1999) and Senn *et al.* (2000) reported no effects of ST2L deficiency on Th2 cell development and effector functions induced by infection with the helminthic parasite *Nippostrongylus brasiliensis* and in the mouse model of allergen-induced airway inflammation. In summary, ST2L signalling is essential for Th2 cell effector functions but not for Th2 cell development, which was confirmed by studies with anti-ST2L antibodies, ST2-Fc fusion protein and ST2L knockout mice. Binding of ST2L to cell surface molecules and soluble molecules has been found, but no functional ligand has yet been cloned.

In ST2L expressing cells, ST2 might compete for the ligand and therefore prevent ST2L signalling. In cells which do not express ST2L, ST2 might mediate its function by binding to a membrane bound ST2 binding protein (Fig. 4.1), which then can signal and cause changes in effector functions as seen in macrophages (Sweet *et al.,* 2001). Part of this current work was designated to establish an effect of ST2 on ST2L(-) naïve T helper cells and to address the question whether this effect is mediated by binding of ST2 to a Th cell surface molecule.

1.9 Function of Th1 and Th2 cells in disease

The cytokine profiles determine the effector functions of the Th cell subset during immune response.

Table 1.2 Th1/Th2 cell protective/pathogenic effects in diseases

	Th1 effector cells	**Th2 effector cells**
Protective against	• intracellular bacterial infections, e.g. mycobacteria • fungal infections • intracellular parasites, e.g. *L. major*	• most extracellular bacteria • metazoan parasites, e.g. helminth infections
Pathogenic in	• organspecific autoimmune diseases, e.g. experimental allergic encephalomyelitis (EAE), insulin-dependent diabetes, rheumatoid arthritis	• *L. major* infections • chronic graft-versus host disease • progressive systemic sclerosis • systemic lupus erythematosus • allergies, e.g. asthma

(reviewed in Spellberg and Edwards, 2001; Lafaille, 1998; Romagnani, 1997)

1.9.1 Type 2 protective and pathogenic responses

Th2 cell mediated responses make BALB/c mice inherently resistant to infections by helminths (Else *et al.*, 1991). Type 2 responses correlate with diminished worm burden, whereas type 1 responses allow chronic infection and scarring to develop (Else *et al.*, 1993). Abrogation of IFN-γ production or administration of exogenous IL-4 in mice susceptible to helminths reversed their normal type 1 immunity and the resultant type 2 responses mediated expulsion of the parasite from the gut (Else *et al.,* 1994). Th2 cell responses are pathogenic during allergic responses such as asthma, which is a chronic disorder of the airways characterised by airway narrowing, mucus hypersecretion, and infiltration of the airway wall with eosinophils. It is now believed that asthma is controlled by Th2 lymphocytes producing cytokines such as IL-4, IL-5, IL-9, and IL-13 (reviewed in van Rijt *et al.*, 2001). The infiltration of the tissues with increased numbers of eosinophils is the result of the co-ordinated action of cytokines, particularly IL-5 as well as CCR3 binding chemokines and the adhesion molecules P-selectin and VCAM-1, acting in concert to cause selective trafficking of eosinophils into allergic tissue. This process is orchestrated by allergen-specific Th2 lymphocytes (Wardlaw *et al.*, 2000).

1.9.2 Type 1 protective and pathogenic responses

Pathogenic intracellular bacteria such as *Salmonella typhimurium* directly induce IFN-γ production in infected mice. Strains of mice producing higher levels of IFN-γ clear the microbes up to 10-fold more efficiently than lower IFN-γ producing strains

(Pashine *et al.*, 1999). Therefore, type 1 reponses are used here by the immune system for protection against acute bacterial infection. Further, type 1 immunity has been shown to be protective against mycobacteria, such as *Mycobacterium leprae*. Mice inherently resistant to *M. leprae* produce IL-12 early on at the site of infection (Kobayashi *et al.*, 1998; Sugawara *et al.*, 1999), but mice susceptible to *M. leprae* infection fail to produce early IL-12 (Kobayashi *et al.*, 1998).

Th1 responses are pathogenic in autoimmune diseases such as diabetes mellitus. Type I (insulin-dependent) diabetes mellitus (IDDM) is an autoimmune disease that results from the destruction of insulin-secreting pancreatic islet beta-cells by autoreactive cells and their mediators. It is well established that IDDM is associated with dysregulated humoral and cellular immunity, exemplified by altered production of and response to macrophage and T cell-derived cytokines and a shift in T helper (Th) cell differentiation in favour of a pathogenic Th1 pathway. Th1 cytokines, including IL-2 and IFN-γ, induce islet beta-cell destruction directly by accelerating activation-induced cell death (apoptosis) and by up-regulating the expression of selected adhesion molecules, Th1 cytokines facilitate the pancreatic homing of autoreactive leukocytes, hence enhancing beta-cell destruction (reviewed in Almawi *et al.*, 1999).

1.10 Antagonistic Th1 and Th2 cell influence on *L. major* Infections

The classic model of type 1/type 2 immunity is *Leismania major* infection of BALB/c mice (reviewed in Spellberg and Edwards, 2001; Sher, 1992).

1.10.1 Common forms of leishmaniasis

Leishmaniasis is a widespread and destructive protozoal disease of humans and animals. There is a range of clinical forms such as visceral, cutaneous and mucocutaneous leishmaniasis (Table 1.3). The *Leishmania* parasite is a protozoa subtype mastigophora/flagellate, which alters its host between a vertebrate (human) and the sandfly of the genera *Phlebotomus* and *Lutzomyia*, which is the vectors for the disease.

Table 1.3 Classification of Leishmaniasis

Disease	Species
Visceral	*L. donovani*
	L. chagasi
Cutaneous	*L. major*
	L. tropicana
	L. mexicana
	L. amazonensis
Mucocutaneous	*L. brasiliensis*

Cutaneous leishmaniasis (CL) caused by *L. major* (Oriental sore) is found mainly in the Middle East and Africa. Cutaneous lesions begin as small erythrematous papules on exposed areas of the body where infected sandfly vectors have fed. The incubation period may be as short as 1-2 weeks up to as long as 1-2 months. The early lesions may be pruritic, but the ulcer is not painful. The ulcer can remain relatively dry, or may exude seropurulent material. Cutaneous leishmanial lesions

will eventually heal spontaneously, but they can persist for up to a year or more without treatment. Another form of cutaneous leishmaniasis, diffuse cutaneous leishmaniasis (DCL), is a striking but uncommon complication of cutaneous disease associated with immunological unresponsiveness to leishmanial antigens. This causes widespread thickening of the skin lesions that ultimatively resemble those of lepromatous leprosy and fail to heal spontaneously.

Visceral leishmaniasis (VL, Kala azar or Chaga's disease) caused by *L. donovani* or *L. chagasi* is found in Africa, South Europe, Asia and South America. The parasite replicates in mononuclear phagocytic cells of the spleen, liver, lymph glands and bone marrow and produces a chronic disease. The incubation period of VL is usually long, 1-3 months. Common symptoms include fever, weight loss, anaemia, skin darkening and hepatosplenomegaly. In untreated cases deaths are common due to secondary bacterial infections such as pneumonia or tuberculosis. Mucocutaneous leishmaniasis, found in Middle and South America, is almost never self-healing. It occurs as a result of the parasite spreading from an initial lesion at the sandfly bite to the mucosal membranes. The lesions that develop can restrict airways and be grossly disfiguring, often requiring plastic surgery to repair.

1.10.2 Morphology and life cycle of the parasites

The leishmanial parasites exhibit only two forms in their life cycles (reviewed in detail by Chang *et al.,* 1990). In the alimentary tract of their insect vectors, the parasite exists extracellularly as the flagellate, motile promastigote. In the phagolysosomal vesicles of host mononuclear phagocytes, the parasite occurs

intracellularly in the nonmotile amastigote form. The amastigote, when ingested by the sandfly during a blood meal taken from an infected vertebrate host, migrates to the midgut of the sandfly, where it transforms into the promastigote in a process that takes about 3 days. As a result of replication by means of binary fission and subsequent migration to the foregut of the insect, the promastigote partially obstruct the digestive tract of the insect. When the infected sandfly takes a second blood meal, it regurgitates promastigotes from its pharynx into the bloodstream of the vertebrate. Once inside the bloodstream of the vertebrate, such as humans, canines or rodents, the promastigotes are phagocytosed by the mononuclear phagocytic cells of the host, whereupon they transform into amastigotes and begin replication within modified phagolysosomes designated parasitophorous vacuoles. Eventually, the host cells lyse, releasing free parasites. The newly released amastigotes then infect other cells or are taken up by sandflies, thereby completing the life cycle of the parasite (Fig.1.6).

It seems that the amastigote has adapted to survive and multiply in an acid environment (pH4.5-5.5), because the environment of the phagosome becomes acidified after parasites or bacteria are ingested by macrophages. *L. major* amastigotes express a surface proton-translocating ATPase that can regulate the intracellular pH of the parasite (Zilberstein *et al.*, 1985). In contrast, promastigotes survive maximally when the pH of the incubation medium is near 7.0 (Mukkada *et al.,* 1985). The three clinical forms of leishmaniasis result from different affinities of the various species of *Leishmania* for macrophages located in different part of the body. Temperature may be one of the major factors involved in this tropism.

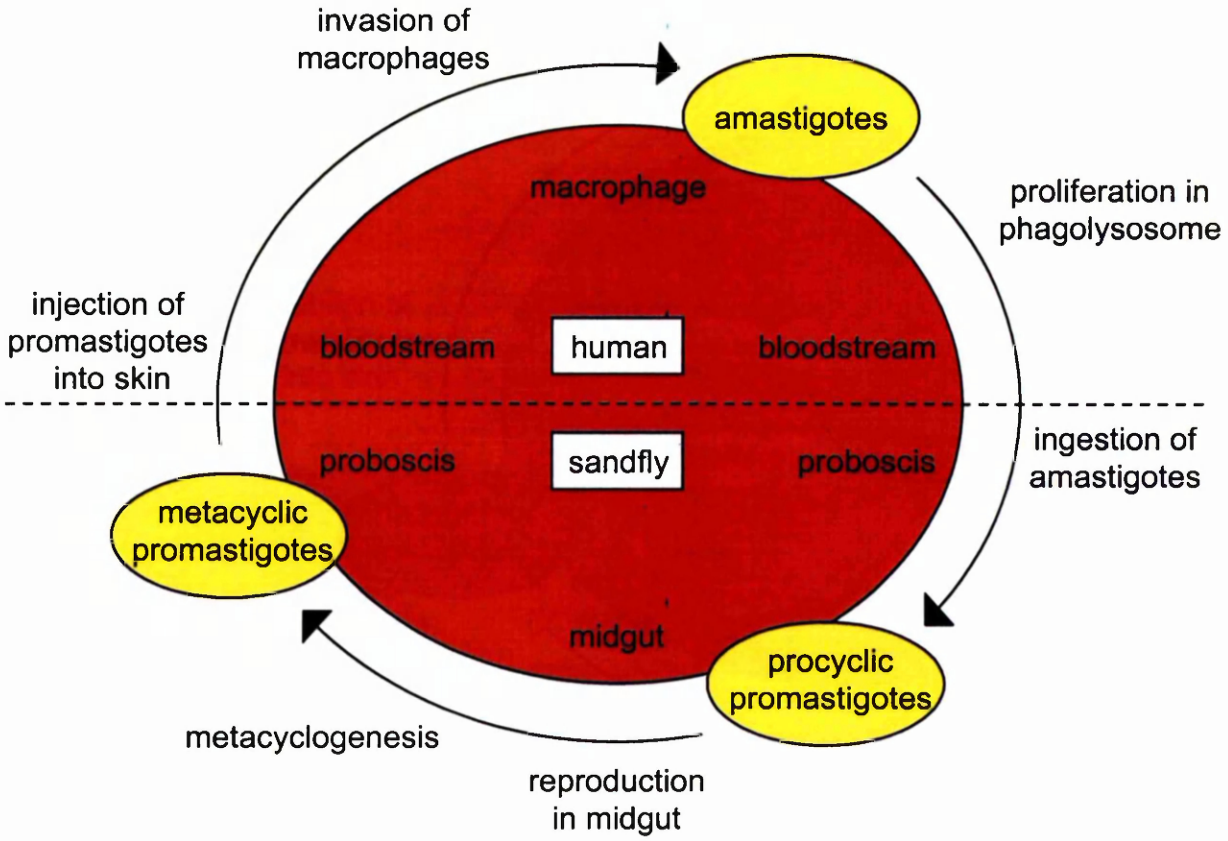


Fig. 1.6 *Leishmania* lifecycle

Typical lifecycle of *Leishmania* parasites. Extracellular flagellated promastigotes reproduce in the midgut of *Phlebotomus* and *Lutzomyia* sandflies. In a process called metacycligenesis they cease dividing, detach from epithelial cells and migrate to the insect mouthparts. When the sandfly feeds, metacyclic promastigotes are injected into the skin of the mammalian host (e.g. human) and enter the bloodstream. Parasites invade macrophages and differentiate into non-flagellated amastigotes in the phagolysosome. In this hostile environment, amastigotes are able to proliferate and are released by lysis of the host cell to infect other macrophages. Infected macrophages are ingested by feeding sandflies and parasites transform to the promastigote stage again to continue the cycle (reviewed in Chang *et al.*, 1990).

The *Leishmania* species that produce cutaneous and mucocutaneous diseases grow better at the slightly cooler temperature (35°C) of the skin. In contrast, *L. donovani*, the cause of visceral leishmaniasis, prefers the slightly higher temperature of 39°C for growth (Berman and Neva, 1981).

1.10.3 Immunology of leishmaniasis

It is essential to investigate the immunology of leishmaniasis to be able to develop treatments against this destructive disease.

1.10.3.1 Animal models of leishmaniasis

The disease pattern produced in animals infected experimentally wih *Leishmania* differs widely according to both the animal and the *Leishmania* species involved. Experimental infections of hamsters and mice with *L. donovani* have been used as experimental models for human visceral leishmaniasis. In particular, studies of the murine model of infection with *L. donovani* have provided important information regarding the genetic aspects of resistance and susceptibility to infection by this parasite (Bradley, 1977).

Upon infection of mice with *L. major*, almost the entire spectrum of the disease pattern seen in human leishmaniasis can be observed (Behin *et al.*, 1979). The severity of disease depends on the genetic backround of the mouse (Howard *et al.*, 1980). For example, BALB/c mice exhibit severe lesions at the site of inoculation, followed by disseminated disease and death. Mice of other strains (e.g. CBA, C57BL) are resistant to infection, developing only small lesions at the

site of inoculation which heal after a few weeks, followed by solid immunity to reinfection.

1.10.3.2 Protective mechanisms in leishmaniasis

i) Role of macrophages

In humans infected with the promastigote form of *L. major*, the parasite is an obligate intracellular parasite of macrophages, in which it transforms into its amastigote form. Amastigotes are found in phagolysosomes in the macrophage, where they not only survive, but replicate, until parasite-specific T cell-mediated immunity intervenes and activates the macrophages sufficiently so that they can destroy the parasite (Titus *et al.*, 1992; Alexander *et al.*, 1992).

There are different macrophage defence strategies to kill the intracellular parasite. An oxygen burst, producing reactive oxygen intermediate, is induced in nonactivated macrophages after phagocytosis of *L. major*, but the parasite can produce catalase and glutathione peroxidase in response (Murray, 1982; Murray, 1981; Murray, 1981a). A second mechanism for killing the parasite is nitric oxide (NO) produced by inducible NO synthase (iNOS) (Green *et al.*, 1991; Liew *et al.*, 1991; Liew and Cox, 1991). Mice resistant to *L. major* infection produce significantly more NO and iNOS than susceptible mice (Liew *et al.*, 1991a). Inducible NOS-deficient mice showed susceptibility to *Leishmania* infection, confirming that reactive nitrogen intermediates are necessary and sufficient for control of visceral leishmaniasis infection (Murray *et al.*, 1999; Wei *et al.,* 1995).

Additionally, TNF-α production in macrophages can destroy *L. major* parasites by increasing the NO production (Liew *et al.*, 1990). These macrophage resistance mechanisms are also induced by IFN-γ released by Th1 cells (see below) (Titus *et al.*, 1984; Nacy *et al.*, 1985).

ii) Role of humoral immunity

Healing of an established infection in mice is independent of the specific anti-leishmanial antibody response (reviewed in Liew, 1986) and, indeed, mice with progressive disease have significantly higher levels of specific antibodies than mice that have recovered from the infection (Colle *et al.*, 1983). Furthermore, mice are not protected by the passive transfer of large amounts of specific antibody from mice effectively immunised with killed promastigotes (Howard *et al.,* 1984). However, if present at the start of infection, specific antibodies may hamper the initiation of infection by preventing the attachment of promastigotes to macrophages. Monoclonal antibodies specific for lipid containing glycoconjugates of *L. major* have been shown to prevent parasitization of macrophages *in vitro* by inhibiting their binding to the macrophage membrane (Handman and Goding, 1985). Parasites that fail to be internalized are destroyed within a few minutes (Handman and Mitchell, 1985).

iii) Role of T cells

It is now generally accepted that *L. major*-specific T cells play a central role in determining whether the host will develop a healing or progressing disease with the parasite.

a) CD4(+) T cells

As mentioned above, some strains, such as BALB/c, are highly susceptible to *L. major* infection and fail to develop a T cell-mediated response to the parasite. In contrast, other strains of mice, such as C3H, C57BL/6 and CBA, develop self-healing lesions associated with strong cell-mediated immunity (Reiner *et al.*, 1995; Locksley *et al.*, 1991). Howard *et al.* (1981) found that the extreme susceptibility of BALB/c mice to *L. major* could be abrogated if the animals were sublethally irradiated and this resistant state could be overcome by transfer of CD4(+) T cells from normal BALB/c mice. Conversely transfer of CD4(+) T cells from animals that had healed *L. major* infection enhanced resistance. Since then, CD4(+) parasite-specific T cells have been shown to have both beneficial (Scott *et al.*, 1988; Scott *et al.*, 1990; Muller *et al.*, 1989; Holaday *et al.*, 1991) or detrimental (Holaday *et al.*, 1991; Titus *et al.*, 1984; Titus *et al.*, 1991) effects in *L. major* infected mice, with Th2 cells mediating susceptibility and Th1 cells mediating resistance (Scott *et al.*, 1988). Furthermore, susceptibility to infection in mice is associated with preferential production of IL-4, while cells from animals that are resistant to *L. major* produce high levels of IFN-γ and little IL-4 (Heinzel *et al.*, 1989; Scott 1991; Chatelain *et al.*, 1992). As noted above, IFN-γ is thought to act by its ability to

activate macrophages to destroy parasites through oxygen burst activity (Murray 1982; Murray 1981; Liew *et al.*, 1990a; Green *et al.*, 1990). On the other hand, IL-4 can block the ability of IFN-γ to activate macrophages to destroy *Leishmania* (Lehn *et al.*, 1989; Liew *et al.*, 1989).

Early events

Consistent with what is known of the factors that regulate Th1/Th2 cell polarisation, depletion of either IL-12 or IFN-γ prior to infection of normally resistant mice abrogates Th1 development, leading to Th2 cell dominance and susceptibility to *L. major* (Scott 1991; Scharton-Kersten *et al.*, 1995). Conversely, neutralization of IL-4 in susceptible BALB/c mice blocks Th2 development, allowing mice to develop a Th1 response and self-heal (Heinzel *et al.*, 1989). To mediate their influence on Th cell development, these cytokines must be present early after infection. During the first days of infection, lymph node cells from C3H mice produce IFN-γ, but not IL-4, while cells from BALB/c mice produce less IFN-γ and substational amounts of IL-4 as early as 16 h after infection (Scott 1991; Louis *et al.*, 1998). NK cells are the source of IFN-γ produced at this early stage of infection in C3H mice (Scharton and Scott 1993). *L. major* infection in C3H mice is also associated with increased level of IL-12 production that is required both for NK cell activation and Th1 cell response (Scharton-Kersten *et al.*, 1995). Consistent with these results are findings demonstrating that treatment of *L. major*-infected BALB/c mice with IL-12, given at the time of infection, enhances Th1 cell development and healing (Sypek *et al.*, 1993; Heinzel *et al.*, 1995). The

most stringent test for the importance of IL-12 comes from studies with IL-12 p40 knockout mice, which have been shown to be susceptible to *L. major* (Mattner *et al.*, 1996). The likely source of IL-12 are macrophages stimulated by the parasite, but there are strong indications that dentritic cells may also be responsible for the IL-12 production as shown following infection with *L. donovani* (Gorak *et al.*, 1998).

The early burst of IL-4 production influences the outcome of the disease in infected BALB/c mice. Louis *et al.* (1998) showed that a burst of IL-4 mRNA, peaking in the draining lymph nodes of BALB/c mice 16h after infection, occurs within CD4(+) T cells that express V beta 4-V alpha 8 T cell receptors. V beta 4-deficient BALB/c mice are resistant to infection, demonstrating the role of these cells in Th2 development. The early IL-4 response is absent in these mice, and Th1 responses occurs following infection. The LACK (*Leishmania* homologue of receptors for activated C kinase) antigen of *L. major* induces comparable IL-4 production in V beta 4-V alpha 8 CD4(+) T cells. Thus, the IL-4 required for Th2 development and susceptibility to *L. major* is produced by a restricted population of V beta 4-V alpha 8 CD4(+) T cells after cognate interaction with a single antigen from this complex parasite. IL-4 produced rapidly by these CD4(+) T cells induces within 48 hours a state of unresponsiveness to IL-12 among parasite-specific CD4(+) T cell precursors by downregulating the IL-12 receptor β2 chain expression.

There is some evidence that immunity to *Leishmania* is much more complex than can be explained by a simple Th1/Th2, IFN-γ/IL-4-dichotomy. *L. major*-specific T

cell lines and clones have been described that produce large amounts of IFN-γ and mediate parasite-specific delayed-type hypersensitivity, but can exacerbate the course of cutanous leishmaniasis in either susceptible BALB/c or normally resistant CBA mice (Titus *et al.*, 1984; Titus *et al.*, 1991). In addition, it has been reported that a $CD45RB^{high}$ subset of CD4(+) cells from infected BALB/c mice contains a population of Th1-type cells, which produce IFN-γ *in vitro* and transfer resistance to *L. major* infection in SCID mice (Powrie *et al.*, 1994). Moll and Rollinghoff (1990) showed that while protective CD4(+) T cells in *L. major* infection were Th1 cells, defined by their IFN-γ production, excacerbative T cells were not Th2 cells, but rather showed overlapping Th1 and Th2 type cytokine pattern. Boom *et al.* (1990) showed that when BALB/c mice were vaccinated against *L. major*, CD4(+) *L. major*-specific T cells isolated from the animals no longer produced IL-4, but, suprisingly, did not produce IFN-γ. Instead, the cells produced large amounts of TNF-α, which is known to increase NO and iNOS (inducible NO synthase) production in macrophages (Liew *et al.*, 1990).

Regulation of established immune responses to L. major

Most current treatments of leishmaniasis in BALB/c mice, such as IL-12 administration, must be given prior to, or at the time of infection, suggesting that they act by influencing events in initial T cell differentiation, rather than by reversing established T-cell responses. The failure of various cytokine or anti-cytokine therapies to alter the course of the disease when treatment is delayed until the 2^{nd} or 3^{rd} week after infection suggests that once established, polarised

Th2 type responses may not be amenable to immune manipulation. However, there is considerable evidence from *in vitro* studies that a variety of factors have the potential to regulate the effector function of differentiated T helper cell subsets. For example, treatment of IFN-γ producing $CD45RB^{high}$, normally protective T cells with antibodies to IFN-γ before transfer into SCID mice render these cells ineffective (Powrie *et al.*, 1994). Conversely, normally susceptible $CD45RB^{low}$ cells, when treated with anti-IL-4 antibodies, conferred resistance to *L. major* infections when transferred to SCID mice (Powrie *et al.*, 1994). In summary, agents, able to decrease *L. major*-specific Th2 type cytokine expression during an established immune response, might still be able to cure the infection.

b) CD8(+) T cells

CD8(+) T cells are involved in the resolution of lesions induced in mice by *L. major*, although it appears that their role might be less than that of CD4(+) T cells. MHCII-deficient mice of C57BL/6 backround, lacking CD4(+) T cells, become susceptible to infection with *L. major* (Erb *et al.*, 1996). Further, CD8-deficient mice exhibit an effective and long-lasting immunity against the *L. major* infection (Huber *et al.*, 1998), confirming that CD8(+) T cells are not required to control the infection. However, in comparison to susceptible mice, resistant mice produce three times more parasite-specific CD8(+) T cells and these can transfer specific DTH reactions to naïve recipients (Titus *et al.*, 1987). Depletion of CD8(+) T cells by treatment of infected mice with anti-CD8 mAb also exacerbates skin lesions and abrogates the induction of resistance which is normally seen following

immunization with killed promastigotes (Farrell and Louis, 1989). As CD8(+) T cells produce IFN-γ as well as TNF-α (Fong and Mosmann, 1990), the production of these cytokines by CD8(+) T cells may help macrophages to kill *Leishmania*. Therefore, CD8(+) T cells might contribute to the CD4(+) T cells mediated resistance to the infection by killing *Leishmania*-infected macrophages, both by production of lymphokines and by direct lysis (da Conceicao-Silva *et al.*, 1994).

1.10.4 Treatment of *L. major* infections in mice and humans

Extensive research has been carried out using murine leishmaniasis models such as BALB/c mice, that are highly susceptible to the disease, which led to the identification of a number of treatments that are now being investigated in humans. Treatments used in the murine model are generally based on inhibiting the *L. major* parasite directly (Iniesta *et al.*, 2001; Selzer *et al*, 1999) or on increasing Th1 cell responses, e.g. through anti-IL-4 antibodies or IL-12 administration (Heinzel *et al.*, 1989; Sypek *et al.*, 1993). The standard clinical treatments for cutaneous leishmaniasis (reviewed in Berman, 1997) is pentavalent antimony (sodium stibogluconate), but this can cause significant pancreatic, cardiac, musculoskeletal and hematologic morbidity. Paromomycin, an aminoglycoside, in combination with methylbenzethonium chloride (MBCL) have also been used topically (Herwaldt and Berman, 1992; El-On *et al.*, 1985, 1986, 1992). The latter shows toxic effects when administed to mice (Neal *et al.,* 1994) and can produce local inflammation (El-On *et al.*, 1986). Due to the side effects of MBCL, Grogl *et al.* (1999) tested other paromomycin-based formulations and

found that topical treatment with WR 279.396 (15% paromomycin + 0.5% gentamicin) healed *L. major* infections successfully without toxic side effects. Among the aminoglycosides, paromomycin is the only one with clear antiprotozoal and antileishmanial activity (El-On and Greenblatt, 1983), but early work by El-On *et al.* (1984) demonstrated that 12% gentamicin had a modest activity in *L. major* infections when used topically. Nevertheless, the mechanism of gentamicin in *L. major* infections remains unclear. In this thesis, the *in vitro* ability of gentamicin to modulate the Th1/Th2 type balance was discovered. Whether this feature of gentamicin is causing its beneficial effect in *L. major* infections will be addressed.

Specific Aims of Study

In infections like *Leishmania major* the balance between Th1 and Th2 cells determines the disease outcome. Detection and modulation of this balance may become possible by exploiting newly discovered cell surface markers specific for Th1 cells (e.g. IL-18R) and Th2 cells (e.g. ST2L). This thesis focuses on the modulation of the Th1/Th2 cell balance through ST2L, which have been closely linked to Th2 cell effector functions, and its splice variant soluble ST2 (ST2). In addition, the ability of gentamicin, an aminoglycoside that suppresses Th2, but not Th1 type responses, to modify *L. major* infections in susceptible BALB/c mice was also examined.

Chapter 2 Materials and Methods

2.1 Animals

Female, 6 weeks old, BALB/c mice were purchased from Harlan Olac (Bichester, UK). DO.11.10 TCR transgenic mice homozygous for the chicken OVA (cOVA) peptide$_{323-339}$/I-A^{d} on the BALB/c background were bred in the University of Glasgow's facilities.

2.2 Aminoglycosides

G418 (geneticin sulphate, Gibco BRL, Life Technologies, Paisley, UK) was prepared as a 20 mg/ml stock solution in 100 mM HEPES buffer, pH 7.3. Gentamicin sulphate (Sigma-Aldrich Company Ltd., Poole, England) was prepared as a 50 mg/ml stock solution in PBS. Both reagents were sterile filtered and kept at –20°C until use.

2.3 Estimation of gentamicin contents in serum

Serum of mice injected with 50 mg/kg gentamicin was collected at different time points and pooled. The serum concentration of gentamicin was assessed in collaboration with the Dep. of Pathology (Western Infirmary, Glasgow) using the TD$_x$ system (Abbott Laboratories, Abbot Park, Illinois), which applied fluorescence polarisation immunoassay technology (Nielsen *et al.*, 2000). This technology is based on antigen/antibody reactions (Gentamicin/antibody against Gentamicin). The added surplus of antibody is labelled with a fragment of a fluorescence dye molecule and is able to freely rotate as long as it is unbound in solution. Antigen/antibody complexes stabilise the plain of polarisation, which is emitted. This plain of polarisation can then be measured and gives information about the amount of antigen in solution. The sensitivity of this assay is 10 ng/ml.

2.4 Preparation of spleen and lymph node cells

To obtain spleen and lymph node cells, mice were sacrificed by dislocation of the neck, a schedule 1 method. Spleen and lymph nodes were dissected under semi-sterile conditions. A single cell suspension was obtained by gently pressing spleen and lymph nodes through a sterile tea strainer and then filtered through sterile nylon mesh (Cadisch Precision Meshes, London, UK) and washed twice with RPMI 1640 medium (Gibco BRL). The cell number was counted in a 1:10 dilution in 0.1% Trypan Blue (Sigma) in 1x PBS buffer, using a counting chamber (Improved Neubauer, Weber Scientific International Ltd., Teddington, UK).

2.5 Cell culture – general conditions

All cells types described below were grown in complete RPMI (RPMI 1640 medium, 10% (v/v) FBS, 2 mM L-glutamine, 50 U/ml Penicillin and 50 μg/ml Streptomycin, 50 μM 2-Mercaptoethanol) at 37°C supplemented with 5% CO_2 unless otherwise stated. Cells were centrifuged at 400 x g for 5 min at 4°C unless otherwise stated.

2.6 Preparation of antigen presenting cells (APC)

For use as APC, BALB/c spleen cells were either irradiated with X-ray (110kV, 2500 rads) at the Beatson Radiation Unit, Western Infirmary, Glasgow or 1 x 10^7 BALB/c spleen cells were treated with 50 μg of mitomycin C (Sigma) for 45-60 min at 37°C followed by 4 washes.

2.7 Cell culture of Th cell clones

Th cell clones were grown in complete RPMI medium. Dorris (specific for egg lysozyme, Sigma) and D10 (specific for conalbumin, Sigma), both on CBA

backround, were obtained from American Type Culture Collection (ATCC, Atlanta, Georgia). X4, X9 and X12, all on BALB/c backround, were specific against group A streptococcal M protein (cells and antigen were a gift from Dr JH Robinson, University of Newcastle upon Tyne, UK). The cell lines were maintained by monthly stimulation with irradiated APC in a ratio of 1:10 and an appropriate amount of their specific antigen (Table 2.1). After 3 days, 10 ng/ml IL-2 (gift from Genosys) was added to stimulate the proliferation. The cells were split 1:5 every 7-10 days and further stimulated with IL-2.

Table 2.1 Cell culture of T helper cell clones

Th cell clones	Antigen	Concentration used
D10 (Th2)	Cab (conalbumin)	100 μg/ml
Dorris (Th1)	HEL (hen egg lysozyme)	500 μg/ml
X4 (Th1)	Peptide 15-33	12.5 μg/ml
X9 (Th1)	Peptide 300-319	25 μg/ml
X12 (Th2)	Peptide 300-319	25 μg/ml

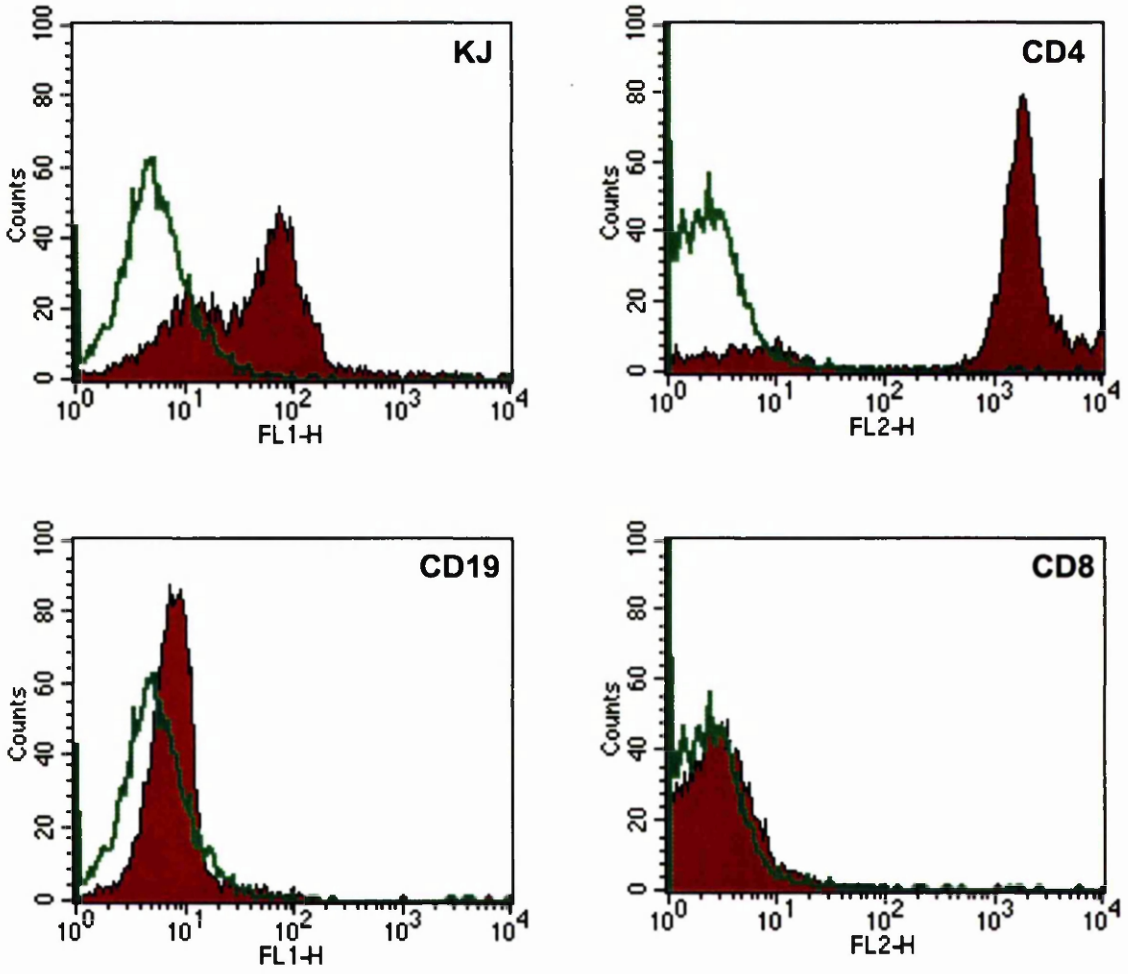
2.8 Negative selection of CD4(+) T cells

The negative selection of CD4(+) T cell was performed by magnetic cell sorting (MACS) with magnetic beads bound antibodies and CS columns as described by the manufacturer (Miltenyi Biotec Inc., Bergisch Gladbach, Germany). Single cell suspension was prepared from spleen cells or lymph nodes from DO11.10 TCR transgenic mice as described in 2.2. To lyse the red blood cells, 1ml of Red Blood Cell Lysis Buffer Hybri-Max® (Sigma) was added to a pellet of 10^8 cells and incubated for 1 min at room temperature. Two washes removed Lysis buffer and cell

debris. The adherent cells were removed by incubating the cells in complete RPMI 1640 medium for 45-60 min in a 75 cm^2 flask at 37°C followed by collection of non-adherent cells with the supernatant. The cells ($2x10^8$) were washed with sterile MACS buffer (1 x PBS, 2% (v/v) FBS) and resuspended in a final volume of 1 ml MACS buffer containing rat antibodies against CD8 (12.5 μg/ml), CD11b (100 μg/ml), CD19 (90 μg/ml) and CD16/32 (30 μg/ml). After a 20 minute incubation on ice, the unbound antibodies were washed off with 2 washes of 15 ml MACS buffer at 200 x g for 10 minutes at 4°C. The cells were then resuspended in 1 ml MACS buffer and 200 μl anti-rat IgG conjugated beads (Miltenyi Biotech Inc.) was added for a further 20 minute on ice. After 2 washes with MACS buffer the cells were resuspended in 800 μl MACS buffer and applied through a nylon mesh filter to a MACS CS separation column which had been washed previously with 65 ml MACS buffer. To remove the unbound CD4(+) cells, the column was washed with 35 ml MACS buffer and the cells were collected and washed twice in RPMI 1640. Flow cytometry analysis indicated that these cells were 85-90% CD4(+), 65-90% KJ1.26(+) with ≤ 5% of CD8(+) T cells and CD19(+) B cells (Fig. 2.1).

2.9 Derivation of polarised CD4(+) T cells *in vitro*

As described by Xu *et al.* (1998), CD4(+) T cells from OVA-specific TCR transgenic mice were cultured with 300 nM OVA peptide (OVA 323-339) and irradiated BALB/c spleen cells (1:10 - APC : Th cells). To drive CD4(+) T cells under neutral conditions (Coyle *et al.*, 1999), the cells were grown in the presence of 10 ng/ml IL-2 from day 2 after antigenic stimulation. Th1 cells were established by the addition of 10 ng/ml IL-12 (Gentech, USA) and 1 μg/ml monoclonal anti-IL-4 antibody (R&D), given at the time of antigenic stimulation. Th2 cells were grown in the presence of 10 ng/ml IL-4



Marker	% expression
CD4	75
KJ	65
CD19	2
CD 8	1

Fig. 2.1 Test of successful negative selection of CD4(+) T cells by flow cytometry

T cells were purified from spleen of DO11.10 mice as described in section 2.4. The success of the negative selection of CD4(+) T cells (section 2.8) was checked by staining for cell surface markers CD4, KJ, CD8 and CD19 (section 2.34). The table above shows the percentage of living cells which stained positive for each of the markers.

(Gentech, USA) and 3 μl/ml polyclonal rabbit anti-IL-12 serum given at the time of antigenic stimulation. Both Th1 and Th2 cells were expanded with 10 ng/ml IL-2, which was added on day 3. Antigenic stimulation under polarising conditions was repeated every 7-10 days after the previous stimulation to enhance the number of polarised Th1 or Th2 cells. The success of polarisation was assessed by ELISA and Real time PCR (Fig. 2.2). IL-4, IL-5 and the Th2 cell marker ST2L, but no IFN-γ were expressed in polarised Th2 cells, whilst Th1 cells expressed only IFN-γ and very low amounts of ST2L.

2.10 Culture of macrophage cell line RAW264

The murine macrophage cell line, RAW264, was obtained from ATCC (Atlanta, USA). The cells were grown on 10 cm bacteriological plastic plates (Bibby Sterilin, Staffordshire, UK) and, once confluent, were detached by injecting in and out of a syringe before being split at 1:5 or 1:10 ratio.

2.11 Culture of bone marrow macrophages

Bone marrow macrophages were obtained from the femurs of BALB/c mice by gently flushing out the marrow with a syringe containing RPMI 1640 medium. After 2 washes with RPMI 1640 the single cell suspension was cultured with complete RPMI 1640 medium containing 10 ng/ml (10^3 U/ng) recombinant human CSF-1 (a gift from Professor David Hume, Brisbane, Australia). Cultures were fed with CSF-1 on days 0, 3 and 6. At day 7 the adherent macrophages were harvested by scraping.

For *in vitro* experiments the cells were stimulated with 100 ng/ml LPS from *Salmonella minnesota* (Sigma, Poole, UK).

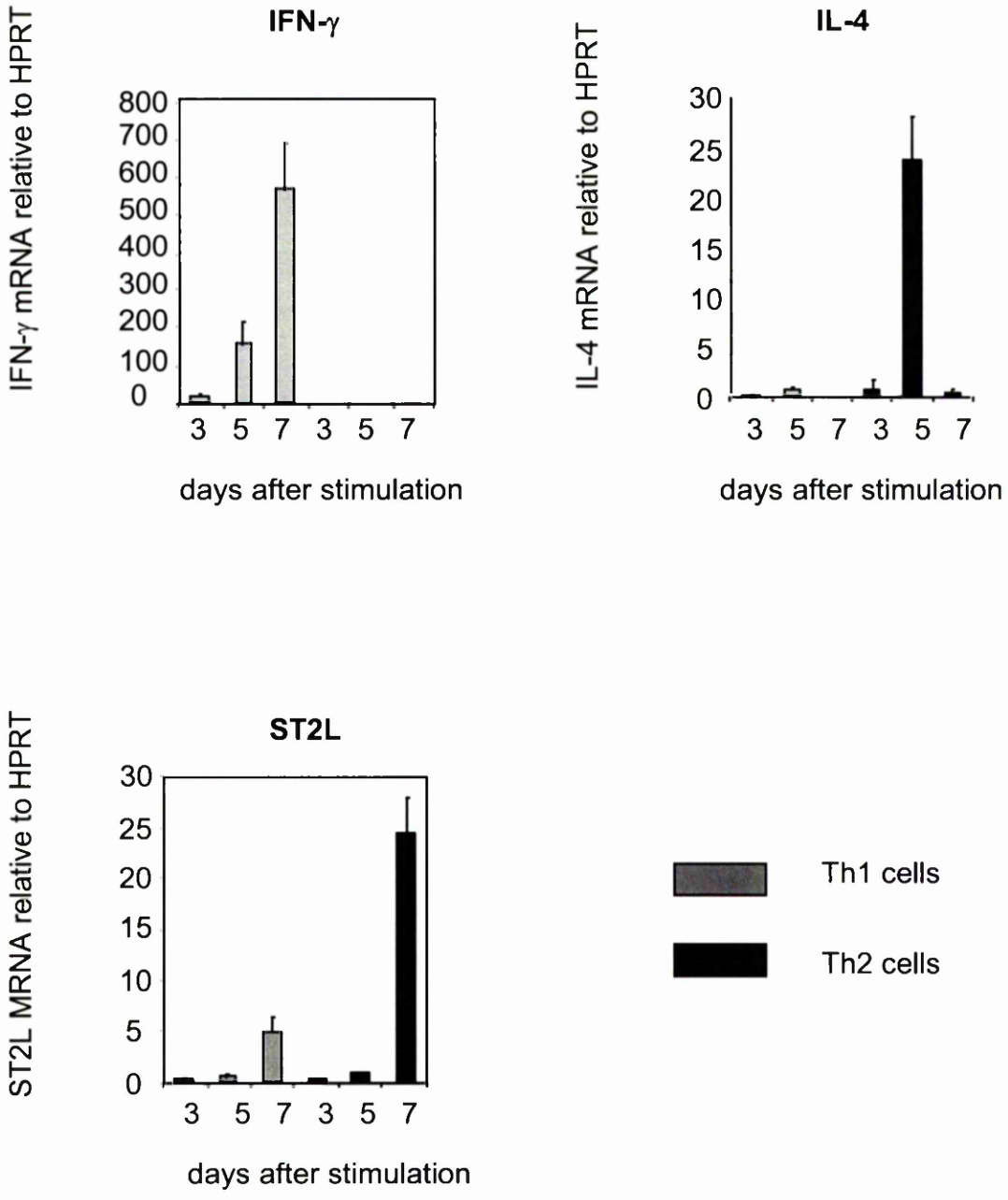


Fig.2.2 Real-time PCR of polarised Th1 and Th2 cells

CD4(+) T cells were purified by negative selection and polarised towards Th1 and Th2 cells as described in section 2.4 and 2.8. The gene expression of IFN-γ, IL-4 and ST2L were assessed by Real time PCR (section 2.31 and 2.32). The error bars represent the standard deviation between triplicate cDNA samples.

2.12 Culture of peritoneal macrophages

BALB/c mice (6-8 week old) were injected intraperitoneally with 2 ml thioglycollate broth (40.5 g/L, DIFCO, East Molesey, UK) and after 3 days the peritoneal macrophages were removed by washing the peritoneum out with 2 x 5 ml PBS. Macrophages were stimulated with 100 ng/ml LPS.

2.13 Culture of *L. major* parasites

Leishmania major parasites were obtained from frozen culture or from the footpad of infected BALB/c mice. The footpad was cut into small pieces, filtered through nylon mesh and the remaining suspension was washed twice with saline at 1200 x g for 10 min at 20°C. The parasites were then cultured in 5 ml Schneider's medium (Gibco BRL) supplemented with 20% (v/v) FBS, 2 mM L-glutamine, 50 U/ml Penicillin and 50 μg/ml Streptomycin at 27°C for 3 days in air. 100 μl of this culture were then passaged into 40 ml of Schneider's medium and cultured for a further 6-8 days to obtained parasites in the stationary phase.

2.14 *L. major* infection of BALB/c mice

BALB/c mice, used for in vivo trials, were 6 weeks of age, female and around 20 g in body weight. The footpad was injected with $1x10^5$-$5x10^5$ *L. major* promastigotes, growing in late stationary phase culture, diluted in 50 μl sterile 1x PBS. The footpad swelling was measured weekly with a caliper (Kroeplin, Germany) and the difference between uninfected and infected foot was calculated. When the footpad swelling increased above 4 mm the animals were sacrificed by dislocation of the neck (schedule 1 method).

2.15 Serial limiting dilution assay for *L. major* parasites

L. major parasites, which were extracted from infected footpads, were diluted in serial dilutions ($1:10^1$-$1:10^{12}$) in complete Schneider's medium and incubated for 2-3 days at 27 °C in air. The relative parasite numbers were then analysed for the end dilution, which does not contain parasites.

2.16 Preparation of *L. major* antigen

Lag phase parasites were spun down at 1200 x g for 10 min at 20°C and washed twice with sterile saline. Parasites were counted in 1% formaldehyde in PBS. The parasite number was adjusted to 1 x 10^8 parasites/ml in complete RPMI medium. The parasite culture was put through three "freeze/thaw" cycles (-70°C for 20 min, 37°C for 5 min) to obtain non-infectious *L. major* antigen, which was stored at –20°C until use.

2.17 Infection of bone marrow macrophages with *L. major* parasites

Mature bone marrow macrophages maintained in complete RPMI medium were infected at a ratio of 1:10 (macrophages : parasites) with late lag phase *L. major* parasites. After 3 days incubation, the remaining parasites were washed off with pre-warmed medium and the macrophages lysed as described by Huang *et al.* (1998). SDS (0.01% (w/v)) in 100 µl of pre-warmed medium were added to infected cells and incubated for up to 30 min at 37°C, assisted with repeated pipetting of the cells. The released amastigotes were resuspended in 600 µl Schneider's medium containing 30% (v/v) FBS and a serial limiting dilution assay was performed by adding 200 µl aliquots in triplicate to 96 well flat bottom plates and analysing them after 3 days.

2.18 MTT assay

Lymphocytes were cultured in complete RPMI 1640 in round bottom 96-well plates. The supernatant was removed by centrigugation at 400 x g for 5 min at 4°C. Cell viability was assessed by adding 1 mg/ml MTT reagents (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Thiazolyl blue; Sigma) in complete RPMI 1640. After 2 h at 37°C the medium was removed by centrifugation of the plate and the precipitate dissolved in isopropanol. The solution was transfered into a flat bottom 96 well plate and the absorbance was determined at 570 nm with the MRX plate reader (Dynex).

2.19 Cell Proliferation Assay

Spleen and lymph node cells, obtained from single cell suspensions (section 2.4) and Th cell clones were grown in flat bottomed 96-well plates at $4x10^5$ - $1x10^5$ cells per 200 µl complete RPMI 1640. The cells were stimulated with the appropriate antigen or 4 µg/ml anti-CD3 or 2 µg/ml ConA or 1 µg/ml LPS. They were pulsed with 1 µCi ^{3}H-Thymidine/well (West of Scotland Radionucleotide Dispensary, Western Infirmary, Glasgow) and incubated for 4 h or overnight at 37°C in 5% CO2. The cells were harvested onto glass filter mats using a Betaplate™96-well Harvester (LKB Wallac, Turku, Finland). The incorporated ^{3}H-Thymidine was determined by scintillation counting, using EcoScint, in a Liquid scintillation counter (LKB Wallac).

2.20. Griess reaction

To assess the production of NO by macrophages the nitrite concentration in culture supernatants was determined by Griess reaction (Ding *et al.*, 1998). Griess solution A (0.1% (v/v) α-naphtyl-amine in distilled H_2O) and Griess solution B (1% (v/v) sulfanilamide in 5% (v/v) phosphoric acid) were mixed in a 1:1 (v/v) ratio. The mixture

was prepared immediately before every use and 100 µl were added to 100 µl sample in a flat bottomed 96-well plate and then incubated for 10 min at room temperature. The colour change was assessed at 570 nm and the sample concentrations were calculated from the standard curve using serial dilutions of sodium nitrite. The detectable concentration ranged from 80 ng/ml to 10 µg/ml (145 µM to 1 µM).

2.21 Measurement of cytokines by ELISA

Culture supernatant was analysed for cytokine levels by sandwich ELISAs. Immunolon4 plates (Dynex Technologies, Ashford, UK) were coated overnight with the appropriate concentration of rat-anti-cytokine antibody (see Appendix, table A.2.1) diluted in 0.1 M $NaHCO_3$ buffer. The plates were washed twice with 1x PBS/0.05% (v/v) Tween 20 (wash buffer) and blocked for 1 h at 37°C with 200 µl of 10% (v/v) FBS (Gibco-BRL) in PBS (ELISA buffer). After 2 washes with wash buffer, 50-100 µl sample were added in triplicate to the plate. Serial dilutions of recombinant cytokine in duplicate were also added as standards (see Appendix, table A.2.2). The plates were then incubated for 2 h at 37°C or overnight at 4°C to allow binding between cytokine and antibody. Biotinylated-detection antibody (50 µl of the appropriate concentration, see Appendix, table A.2.3) in ELISA buffer were added after 4 washes and incubated for 1 h at 37°C. The plates were washed 6 times, before 100 µl of a 1:1000 dilution of Extravidin Peroxidase (Sigma) in ELISA buffer were added to each well. After incubation for 30-60 min at 37°C, the plates were washed 6 times and then 100µl of the chromophore TMB and H_2O_2 (Dynatech Laboratories Ltd., Billinghurst, UK) were added and incubated until the colour reaction was visible. The plates were read at 630 nm on an MRX microplate reader

(Dynex). The level of cytokine present in test wells was calculated from the standard curve.

2.22 Activation of TGF-β1

TGF-β1 in supernatants is produced in an inactive precursor form, acid treatment allowed the precursor form to be cleaved, allowing the antibody to detect the cytokine. Supernatants for determination of TGF-β1 levels were treated with 1/25 volume 1 N HCl for 15 minutes at room temperature or for 1 h at 4°C. The supernatant was neutralised with 1/25 volume 1N NaOH and used immediately in ELISA or stored at –80°C.

2.23 ST2 ELISA

100 µl of ST2-containing supernatant, in triplicate, were allowed to coat an Immunolon4 plate overnight at 4°C. After two washes with PBS/Tween20 the plates were blocked for 1 h at 37°C with ELISA buffer. Monoclonal biotinylated anti-ST2L antibody was diluted to 2µg/ml in ELISA buffer and 100 µl added to each well. After incubation for 2 h at 37°C the plates were washed 6 times, before 100 µl of a 1:1000 dilution of Strepavidin Peroxidase (Diagnostic Scotland, Carluke, Scotland) in ELISA buffer were added to each well. After 1 h at 37°C, the plates were washed 8 times and the colour reaction revealed using 100 µl TMB. The plates were read at 630 nm.

A similar method was applied to assess the amount of serum IgG1 and IgG2a. The amount of anti-IgG antibody is stated in section A.3.3.

2.24 Protein quantification

Two methods were applied to estimate the amount of ST2 protein, purified from insect or CHO cells. Firstly, the OD at 280nm of the solution was measured by spectrophotometer (Pharmacia-Biotech, Bucks, UK) and the quantity of protein was calculated using the following formula: 1.4 (OD280nm) ≅ 1.0 mg/ml.

Secondly, the Coomassie® Protein Assay (Pierce, Rockford, USA) was used according to manufacture's instructions. Briefly, 960 μl of Coomassie Protein Reagent was added to 40 μl of sample and the colour change was read at 600 nm in the spectrophotometer. The OD values were then compared to bovine serum albumine (BSA) standards, ranging from 12.5-200 μg/ml.

2.25 SDS-PAGE

Separation of proteins was achieved under reducing conditions by SDS-PAGE (Laemmli, 1970). All gels had a ratio of 29:1 of acrylamide to bis-acrylamide (Biorad). Resolving gels were made at 10% in 1.5 M Tris-HCl buffer, pH 8.8; 1% (w/v) SDS; 1% (w/v) ammonium persulphate (BDH) and 0.1% (v/v) N,N,N',N'-tetramethylenediamine (TEMED) (Sigma). Stacking gels (5%) were similarly prepared using 0.5% 1.5 M Tris-HCl buffer, pH 6.8. All gels were run using a standard electrophoresis buffer containing 25 mM Tris, 0.25 M glycine (BDH) and 0.1% (w/v) SDS. Samples to be electrophoresed, were mixed with 50% (v/v) of 2x concentrated sample buffer (1 M Tris-HCl, pH 6.8, 0.2 M DTT, 4% (w/v) SDS, 0.4% (w/v) bromophenol blue and 20% (v/v) glycerol (BDH), heated to 100°C for 5 min and cooled on ice prior loading on gels. The protein standards (See Blue™ Plus 2 Pre-stained Standard, 3-188 kDa, Novex ™; 'Rainbow' molecular weight marker, 10-250 kDa, Amersham) were treated similarly and run on all gels. Electrophoresis was

carried out at a constant current of 5 mA/cm gel length until the bromophenol blue marker reached the bottom of the gel. The gel was then used for Western Blotting or stained with Coomassie blue.

2.26 Coomassie blue staining of SDS-PAGE gels

At the end of electrophoresis, the gels were stained for 1 h with 0.5% (w/v) Coomassie brilliant blue R250 (Sigma) in 40% (v/v) methanol (BDH) and destained over 4-5 h in several changes of 40% (v/v) methanol, 10% (v/v) glacial acetic acid until the background was colourless. Stained gels were transferred to 3 MM filter paper (Whatman) and dried under vacuum at 80°C.

2.27 Western Blotting

Protein, run on a SDS gel, was transferred to nitrocellulose using a transblot system (Hoeffer Scientific SE600, San Francisco, USA). Four pieces of 3 MM filter papers were cut to a size slightly bigger than the gel selected for Western blot analysis and were then soaked in transfer buffer (20 mM Tris, 40 mM glycine, 20% (v/v) methanol, BDH). Two of the filter papers were placed on the cathode of the electroblotter. The gel, soaked in transfer buffer, was placed on the filter papers. A sheet of nitrocellulose (Biorad), cut to the same size as the gel and pre-wetted with transfer buffer, was placed on top of the gel. The remaining two sheets of filter paper were placed on top of the nitrocellulose. Proteins were transferred to the nitrocellulose by applying a constant current of 0.8mA/cm^2 of gel area overnight. Nitrocellulose filters were then incubated with wash buffer 1 (0.01 M Tris-HCl buffer, pH 7.5; 0.1 M NaCl and 0.1% (v/v) Tween-20) containing 2% (w/v) BSA (SIGMA, Poole, UK) for at least 1 h to block non-specific protein binding. Primary antibodies were diluted in wash buffer 1 containing 1% (w/v) BSA, and applied to the filter for 1 h at room

temperature or overnight at 4°C. Blots were washed twice in wash buffer 1, followed by two washes in wash buffer 2 (0.01 M Tris-HCl, pH 7.5; 0.5 M NaCl and 0.1 % (v/v) Tween-20) and finally twice with wash buffer 1 again. Each wash lasted 10 min. The blots were then incubated with the appropriate HRP-conjugated secondary antibody (diluted up to 1:5000 in wash buffer 1 containing 1 % (w/v) BSA) for 1 h at room temperature. After washing again, as described above, immuno-reactive bands were visualised by the Enhanced Chemiluminescence (ECL) system (Amersham, Little Chalfont, UK) and exposed to X-OMAT film (Kodak, New York, USA).

2.28 Dot Blotting

Protein containing supernatants were applied to nitrocellulose membrane and dried for 1 h at room temperature. The membrane then was blocked with 2 % (w/v) BSA-containing wash buffer 1 and further treated as described in section 2.27 (Western Blotting).

2.29 RNA extraction

Total cellular RNA was extracted by using the RNAZOL^{TM}B method, adapted from Chomczynski and Sacchi (1987). All steps were performed at 4°C with RNAse-free equipment. Approximately $2x10^6$ cells were harvested from cell cultures and transferred into 1.5 ml microcentrifuge tube. After centrifugation at 400 x g for 5 min, the supernatants were removed and the pellets kept on ice. 400 µl RNAzol™ B (Biogenesis) was then added to lyse the cells. The RNA was solubilized by passing the lysate a few times through the pipette. Then 40 µl chloroform were added and the tube shaken for 15 s and rested on ice for 5 min. The suspension was centrifuged at 12000 x g for 15 min and the upper aqueous phase transferred to a fresh tube. 1 volume isopropanol was added to precipitate RNA in 15 min on ice,

which was spun down for 20 min at 12.000 x g (4°C). The pellet was washed with 2 volumes pre-cold 75% ethanol and centrifuged at 10.000 x g for 8 min. The pellet was semi-dried at room temperature and resuspended in 30 µl water. To determine the total RNA content, the OD at 260nm and 280nm were measured and the concentration (µg/ml) was calculated by following formula: [(OD_{260nm} x 62) – (OD_{280nm} x 36) x dilution]

RNA samples were stored at -70°C until used.

2.30 Agarose gel electrophoresis

Appropriate amounts of agarose powder (1 g in 100 ml buffer for a 1% gel) were dissolved in 0.5 x TBE electrophoresis buffer (0.045 M Tris-borate, 1 mM EDTA) in a glass bottle and heated in a microwave oven until the agarose dissolved. The solution was cooled to 60°C and mixed with ethidium bromide (SIGMA) to a final concentration of 0.5 µg/ml. The warm agarose was poured into a mould with a comb. After the gel had completely set, the comb was carefully removed and the gel was mounted in an electrophoresis tank (Pharmacia LKB. GNA 100) filled with 0.5 x TBE. The DNA samples and a DNA 1kb marker (BRL, Scotland) were mixed with loading buffer (0.25% (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 15 % (v/v) Ficoll in water) and loaded into the slots of the gel. The gel was run at 1-5 V/cm for an appropriate time before being the examined by ultraviolet light and photographed (Polaroid DS-34 Direct Screen Instant Camera) if necessary.

2.31 First strand synthesis with DNAse treatment

To avoid amplification of genomic DNA, RNA (1-5 µg) was incubated for 15 min at 37°C with 1 µl 10 x PCR buffer, 1 µl $MgCl_2$ (50 mM) and 1 U DNAse free RNAse (Gibco-BRL) in a total volume of 10 µl. The enzyme was heat inactivated at 65°C for

10 min, 1 μl random primers (100 ng/μl) was then added and the mix heated to 70°C for 10 min and then cooled to 4°C. The synthesis of the cDNA was initiated by adding 2 μl 10 x PCR buffer, 1 μl dNTP (10mM), 2 μl DTT (0.1 M) and 100 U Superscript II RT-Polymerase (GIBCO-BRL) in a total volume of 20 μl. The synthesis was performed using the following conditions: 15 min at 25°C to allow the random primers and polymerase to bind, 50 min 42°C to extend the parallel cDNA strand and 10 min 70°C to inactivate the enzyme and degrade remaining RNA.

2.32 TaqMan real-time PCR

TaqMan real-time PCR (Gibson *et al.* 1996) was performed according to the manufacturer's instructions (PE Biosysystem, Foster City, USA) and as described by Overbergh *et al.* (1999). The principle of this method is outlined in Figure 2.3.

Primers and fluorogenic probes (see table 2.2) were designed using the PrimerExpress™v1.0 program purchased from PE Biosystem. 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 performed in the ABI-prism 7700 Sequence Detector, which contains a Gene-Amp PCR system 9600 (PE Biosystems). PCR amplifications were performed in a total volume of 25 μl, containing 0.5 μl cDNA sample, 50 mM KCl, 19 mM Tris-HCl (pH 8.3), 10 μm EDTA, 200 μM dATP, dCTP, dGTP and 400 μM dUTP, 5 mM $MgCl_2$, 300 nM each primer, 0.625 U AmpliTaqGold™ and 0.25 U AmpErase Uracil N-Glycosylase (PE Biosystems). Each reaction also contained 200 nM fluorogenic probe. Each PCR amplification was performed in triplicate wells, using the following conditions: 2 min at 50°C and 10 min at 94°C, followed by a total of 40 two-temperature cycles (15 s at 94°C and 1 min at 60°C).

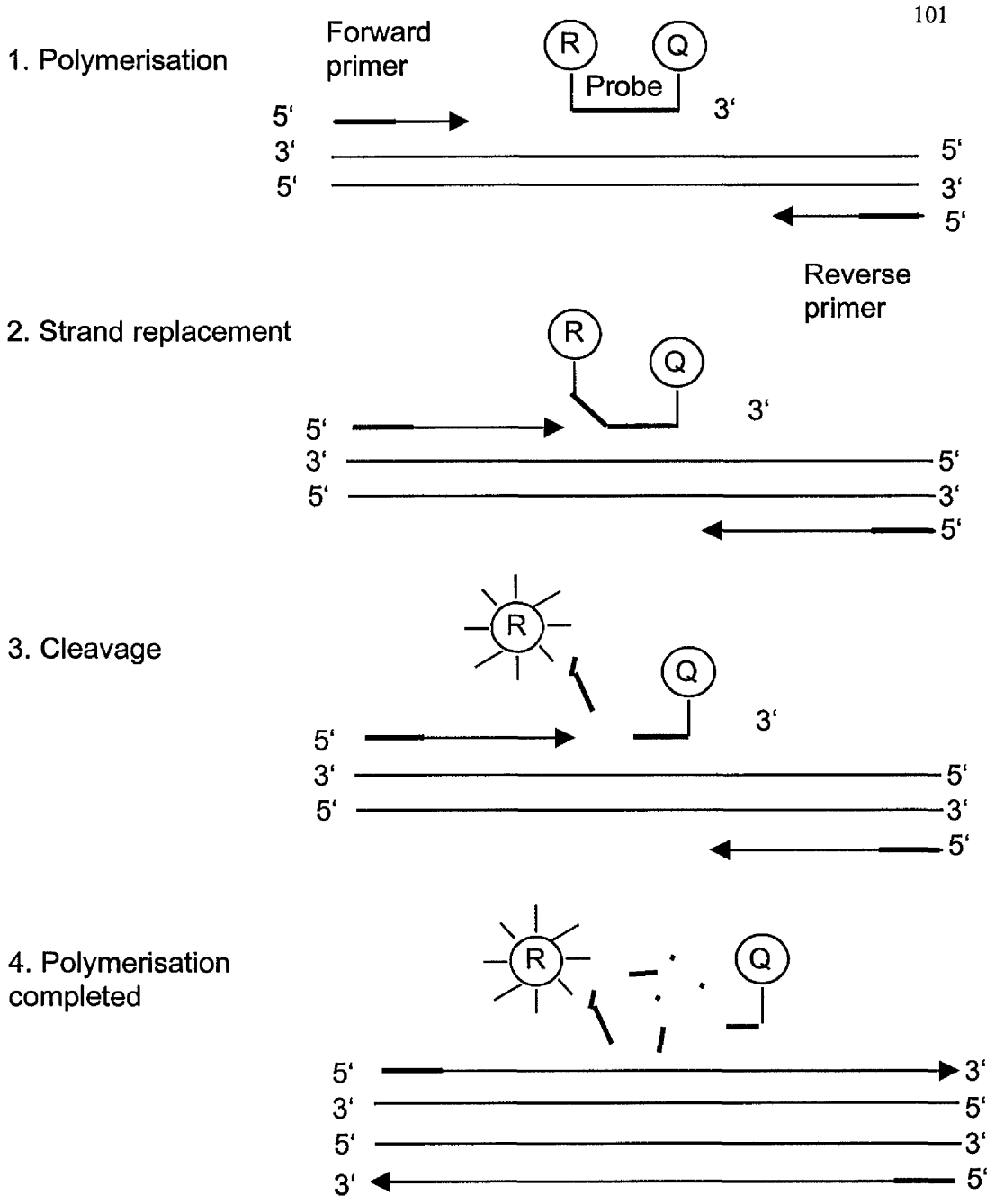


Fig 2.3 TaqMan real-time PCR

The TaqMan probe consists of an oligonucleotide with a 5' reporter dye (R) and a 3' quencher (Q). When the probe is intact the proximity of the quencher to the reporter results in suppression of reporter fluorescence. As PCR proceeds the polymerase enzyme cleaves the probe with it's 5'-3' nuclease activity. The reporter and quencher become separated, resulting in increased reporter fluorescence. The 3' end of the probe is blocked to prevent extension of the probe during PCR.

Table 2.2 Real time PCR probes and primers

Gene	Species	Forward primer	Probe	Reverse primer
ST2	murine	5`d(TGGTCTTTCCAGAATGTTTCTGTCT)3`	5`FAM (CCCCTCTCTGTGTCCCCTCCTCCTT)TAMRA 3`	5`d(GGAGGATGCCGGGAGAA)3`
ST2L	murine	5`d(CCAGAATAGCAGAAGACAGGTGTTT)3`	5`FAM d(TTCTGGCCCCTCACATGSTGCACA)TAMRA 3`	5`d(CCTGCTCGTAGGCAAATTCCT)3`
HPRT	murine	5`d(GCAGTACAGCCCCAAAATGG)3`	5`FAM d(TAAGGTTGCAAGCTTGCTGGTGAAAAGGA)TAMRA 3`	5`d(AACAAAGTCTGGCCTGTATCCAA)3`

Data analysis was performed using the Sequence Detection software (PE Biosystems) which calculates the threshold cycle, Ct (see Figure 2.4). This represents the PCR cycle at which an increase in fluorescence above a threshold can be first detected. Samples were normalised by their reference reporter (HPRT) by subtracting the Ct value of HPRT from the Ct value of the gene of interest: this value is ΔCt. To obtain a value for fold increase relative to HPRT the formula $2^{-\Delta Ct}$ was used. Multiplication of this value by 100 gives expression of the gene of interest as a percentage of HPRT. The positive error is the standard deviation of the difference , $s=\sqrt{(s_1^2+s_2^2)}$, where s_1 and s_2 are the standard deviations of the Cts of HPRT and the gene of interest.

2.33 Biotinylation of monoclonal anti-mouse ST2L antibody

1 mg/ml of antibody was dialysed against 1L of 50 mM Na_2CO_3 (pH 8.5) overnight at 4 ^{o}C before 37 µl of a 1 mg/ml solution of Sulfo-NHS-Biotin (Pierce) was added for 2 h on ice. The mixture was then dialysed against 1x PBS, 0.1% (w/v) NaN_3 overnight

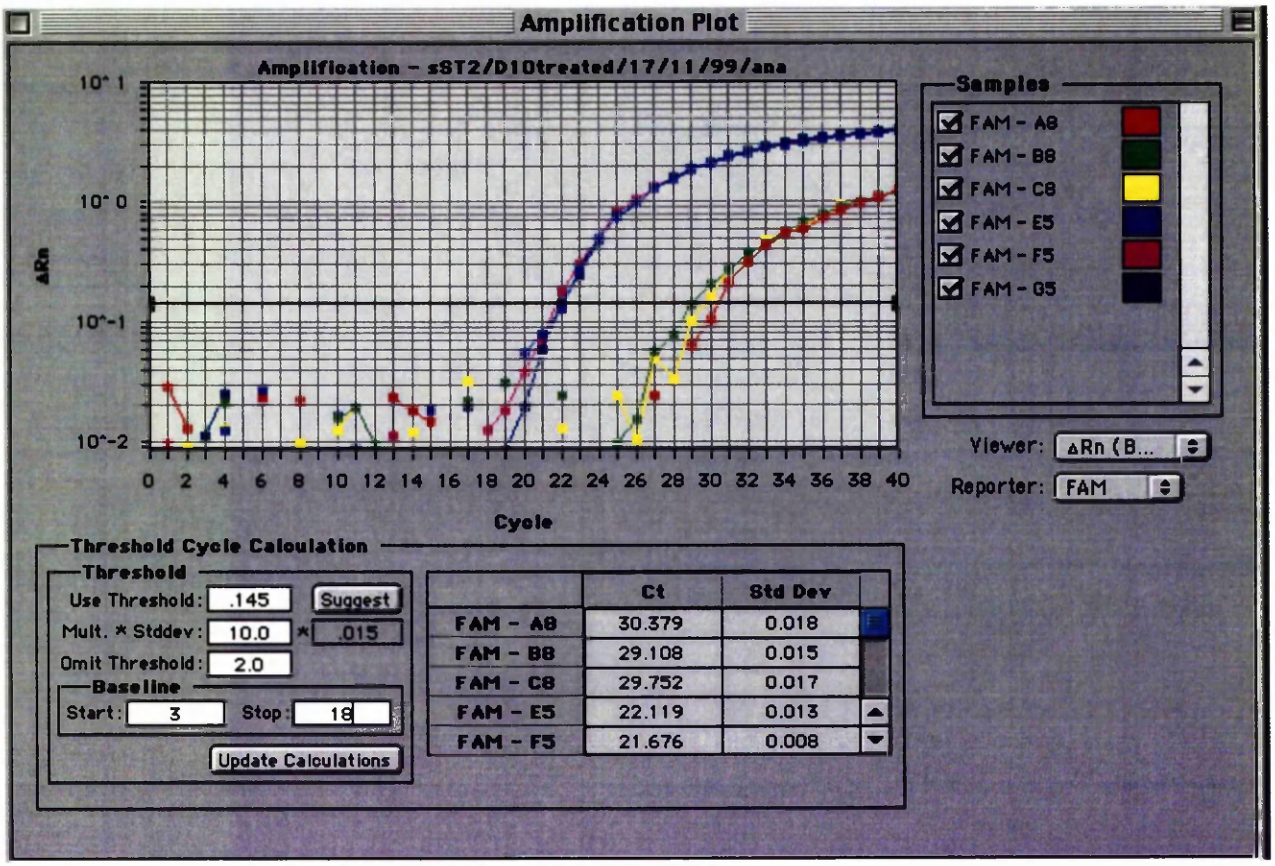


Fig. 2.4 Typical TaqMan amplification plot

The amplification plot shows the fluorescence emission (normalised Reporter, RN) varies with the PCR cycle number. Initially, the fluorescent signal is below the detection limit of the Sequence Detector. In the second stage, the signal can be detected as it continues to increase in direct proportion to the amount of specific amplified product. As PCR continues, the ratio of polymerase enzyme to PCR product decreases and hence the amount of PCR product ceases to increase exponentially, until eventually the RN reaches a plateau. The Ct (threshold cycle) is determined during the exponential phase (threshold indicated by black line). Two triplicate samples are shown (A-C8, E-F5).

and aliquots of 100 µl were stored at 4°C until use. The antibody was tested by showing that Th2 cells expressed ST2L on their surface whereas Th1 cells did not (Fig. 5.1, 5.2).

2.34 Staining of cell surface markers

Aliquots of $5x10^5$ - $1x10^6$ cells were washed with FACS buffer (1x PBS, pH 7.5; 5% (w/v) FBS and 0.1% (w/v) sodium azide) and non-specific binding was blocked by incubating with 2.5 µg/ml Fc block (see Appendix, table A.1) for 10 min on ice. Then the first anti-mouse antibody (conjugated with Biotin, FITC, PE or unconjugated) at the appropriate concentration was added to the cells and incubated for 30 min on ice. The samples were washed twice with 2 ml FACS buffer at 400 x g for 5 min and if necessary a secondary antibody was added to the cells and incubated for 30 min on ice (concentrations in Appendix, table A.1). The cells were then washed three times and analysed immediately using the Flow cytometer FACSALIBUR ® (Becton Dickinson FACS Brand Analyzer, San Jose, USA) and CELLQuest Software or fixed in Fixation buffer (1% (v/v) formaldehyde in FACS flow (Becton Dickinson, San Jose, USA)) and kept for up to 24 h at 4°C in the dark before being analysed. The emitted wavelength for FL1 (FITC-labelled antibodies) was 530 nm and for FL2 (PE-labelled antibodies) 585 nm.

To enumerate the positively stained white cells in a spleen cell population, red blood cells and cell debris were removed using FACS Lysing solution (Becton Dickinson, San Jose, USA). 2 ml of 1x FACS Lysing solution were added to the cell pellet containing 10^8 cells, gently mixed and stored for 10 min at room temperature in the dark. A centrifugation at 300 x g for 5 min followed by a resuspension of the cells in 1 ml FACS buffer and a second centrifugation at 200 x g for 5 min ensured the removal of all debris. The leukocytes were then analysed by flow cytometry.

2.35 Binding studies with ST2-Fc on Th cells

After blocking nonspecific binding with 2.5 μg/ml Fc block for 10 min on ice, 100 μg/ml ST2-Fc or hIgG (Sigma) as a control were added to the cell aliquots and incubated for 30 min on ice. The unbound protein was removed by two washes with 2 ml of ice-cold FACS buffer (400 x g, 5 min, 4°C). Monoclonal FITC-labelled anti-human hIgG1 antibody (2.6 μg/ml, Sigma) or the FITC-isotype control (FITC-mouse IgG2a) and 2.5 μg/ml Fc block were added and incubated for 30 min on ice. The cells were then washed twice and analysed for positive staining by flow cytometry.

2.36 Propidium iodide/ annexin V staining

Propidium iodide (PI) will intercalate into DNA of cells, whose plasma membrane has been ruptured due to necrosis or in the state of late apoptosis (Lecoeur *et al.*, 2001). Annexin V marks cells that show early signs of apoptosis by binding to phosphatidylserine, which externalises in apoptotic cells (BD Pharmingen, 1999).
Cells ($1x10^6$) were resuspended in 100 μl 1x Binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) containing 5 μl FITC-annexin V (Pharmingen) plus 10 μl of a 50 μl/ml stock solution PI (Pharmingen) and incubated at room temperature for 30 min. After one wash with 1x Binding buffer the cells were analysed by flow cytometry for positive staining within 1 h.

Table of flow cytometry antibodies (Appendix, table A.1)

2.37 Expression and purification of ST2

2.37.1 Cell culture of insect cell lines Sf9 and High5

Insect cell lines Sf9 and High5 were purchased from Invitrogen Corp. (Leek, The Netherlands). Sf9 cells were cultured in IPL41 medium (GIBCO-BRL, Eggstein, FRG) containing 10% (v/v) FCS, while High5 cells were grown in serumfree SF900 medium (GIBCO-BRL). The cell lines were grown as monolayers at 27°C in the dark under air and split 1:5 when confluent.

2.37.2 Amplification of baculoviruses in Sf9 cells

A stock of $1x10^8$ plaque-forming units (pfu) of recombinant baculovirus was received from Dr. Werenskiold, Munich. To infect Sf9 cells, the cells were split to obtain a semi-confluent layer and 0.1 plaque-forming units (pfu) per cell of baculovirus were added for 1 h and then washed off. After 4-5 days cultures were confluent with 10-20 % dead cells and had released baculovirus. The supernatants were harvested and examined for the presence of recombinant baculoviruses by PCR.

Infected Sf9 cell supernatants were boiled at 100°C for 5 min and 5 µl supernatant were then incubated with 5 µl 10x PCR buffer (0.5 M KCl, 0.1M Tris-HCl, pH 8.3), 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTP mix (10 mM), 0.5 µl of each primer (10 µM stock concentration) and 1.25 U of Taq-Polymerase (Gibco-BRL). Water was added to give a total volume of 50 µl. The amplification was performed under following conditions: 1 min at 94°C, followed by 40 cycles at 94°C for 40 s, 50°C for 30 s and 72°C for 40 s, followed by 10 min at 72°C. Specific for the extracellular domain of the ST2 gene the following PCR primers, designed by Dr. Xu (Westerm Infirmary, Glasgow), were used to detect ST2 in Sf9 cell supernatants.

(1a) Forward primer: 5' d(TTACAAATCCTCCATACAAC) 3'

(3') Reverse primer: 5' d(TCAAGCAATGTGTGAGGGACACTCCTT) 3'

In a semi-quantitative PCR, three dilutions (1:1, 1:5 and 1:10) of each supernatant were tested in the PCR and compared with supernatant containing $1x10^8$ pfu/ml virus. If the titre of baculovirus in the tested supernatants was found lower than 10^8 pfu/ml, then fresh Sf9 cells were infected with the analysed supernatant to increase the virus titre.

2.37.3 Infection of High5 cells with baculovirus and release of ST2 into the supernatant

High5 cells were grown to a confluent layer in tissue culture flasks before being infected with 10 pfu /cell of baculovirus. Supernatant was harvested after 3-4 days and analysed by Western Blot for the expression of ST2 using 1 μg/ml polyclonal rabbit anti-mouse ST2L antibody (Xu *et al.*, 1998).

2.37.4 Purification of baculovirus-derived ST2

Supernatant from baculovirus-transfected High5 cells was concentrated 5-10 fold in a Stirred Ultrafiltration Cell 8010 (Millipore Corp Ltd., Watford, UK). The YM30 membrane (Millipore) in the Stirred Ultrafiltration Cell excluded all molecules smaller than 30 kD. The concentrated supernatant was dialysed against 1 L PBS, pH 7.5 overnight at 4°C and then added to a column containing anti-FLAG M2 affinity gel (Sigma), which had been pre-equilibrated with PBS, pH 7.5. Non-specifically bound protein was removed by washing the column with 50 ml PBS, pH 7.5 and ST2 was then eluted by competition with 10 ml of 100 μg/ml FLAG peptide (Sigma) in PBS to avoid protein inactivation. Any remaining protein was eluted using 10-20 ml of 0.1 M

glycine-HCl, pH 3.5. The success of the purification was confirmed through Western Blots with anti-FLAG antibodies (Sigma) used at a 1:1000 dilution of the stock solution.

2.38 Expression and purification of ST2-Fc from stably transfected CHO cells

2.38.1 Cell culture of CHO cells

Stably transfected CHO cells, overexpressing a fusion protein of the extracellular part of ST2L with human IgG1 (ST2-Fc), were obtained from Dr. Daiwu Kang (Astra Zeneca, Lund, Sweden). Cells were cultured in DMEM w Glutamaxl (Gibco-BRL) supplemented with 1% MEM (1x), 14 μg/ml G418 and 10% FBS (Gibco-BRL). When the CHO cells became confluent, the medium was washed off and replaced by DMEM w Glutamaxl with 10 % FBS with low IgG (GIBCO-BRL). Thus, the CHO cells released ST2-Fc into medium with a low IgG content. The cells were cultured in this way for 5-6 days, before the supernatant was collected.

2.38.2 Purification of ST2-Fc

5 ml Protein A Sepharose (Amersham-Pharmacia-Biotech, Bucks, UK) were packed on a column and washed with at least 10 column volumes of binding buffer A (20 mM sodium-phosphate pH 7.0). 1-2 L supernatant of ST2-Fc-transfected CHO cells were loaded onto the Sepharose A column and the column was washed extensively with buffer A (20 mM sodium-phosphate pH 7.0). The amount of protein in the eluate was monitored by OD_{280nm} and buffer A was added until the OD_{280nm} indicated that no protein remained in the eluate. Protein A-bound material was then eluted with 0.1 M glycine-HCl, pH 2.5 into 1/10 volume neutralisation buffer (1 M Tris-HCl pH 9.0, 0.2% (v/v) Tween 20). The flow rate was 5 ml/min and 3.5 ml eluate per tube were

collected. The OD_{280nm} of all fractions was monitored during the elution and protein contents was confirmed by running the supernatants on a denaturing SDS gel and staining with Commasie blue. The fractions with the highest protein contents were pooled and dialysed overnight with two changes of 1 L PBS, 0.02% (v/v) Tween 20, pH 7.4 at 4°C. The protein was concentrated to a total volume of 2-3 ml in the Stirred Ultrafiltration Cell 8010 (Millipore), containing the YM30 membrane. The total protein content was determined by using the Coomassie Plus assay (Pierce). To determine the protein purity, the purified ST2-Fc was run on a Coomassie blue-stained 10% SDS gel.

2.39 Cell contact experiments between fixed Th cells and macrophages

To investigate whether Th2 cell expressed ST2L could mimic the suppressive effect of ST2-Fc on activated macrophages, $5x10^6$ cells of Th2 clone X12 and Th1 clone X9 were resuspended in 1 ml sterile Fixing buffer ((1% (w/v) paraformaldehyde in 1x PBS, pH 7.0) and incubated for 30 min on ice. The cells were washed 4 times and resuspended in complete RPMI 1640. CSF-1 matured macrophages were added to the fixed Th cells in a ratio of 10:1 (macrophages : Th cells), stimulated after 1 h with 100 ng/ml LPS and incubated for a further 18 h. Cytokine expression (IL-6, IL-12) in macrophage supernatant and in fixed Th cell clones (IFN-γ, IL-5) was assessed by ELISA.

2.40 Photography of cells

2.40.1 Th cell clones treated with G418

Th clone cells were cultured with 10 ng/ml IL-2 and 0, 400 and 800 μg/ml G418 for 2 days. A wet preparation of the cells on slides was performed and they were

examined under the Phase contrast microscop (Labophot, Nicon, Japan). The photos were taken at x 40 magnification.

2.40.2 L. major parasites treated with gentamicin

Using the software IP lab in connection with a Zeiss Axioskop (Jena, Germany) *L. major* parasites treated with 0 and 100 μg/ml gentamicin for 3 days were filmed. Every 2 seconds a picture was taken at x 63 magnification.

2.41 Statistics

To ensure that two data sets, e.g. ELISA, proliferation, footpad measurement data sets, were significantly different, groups of means were compared using the 2-sample Student's t-Test or the Mann-Whitney test applying the MINITAB program (MINITAB Inc., Lancaster, UK). A p-value of <0.05 was considered significant.

Differences in ST2-Fc and hIgG binding to T helper cells, detected by anti-human IgG1 antibody (section 2.35), were analysed using the K-S two-sample test (CELLQuest Software, Becton Dickinson). This test analyses the statistical significance of differences between overlaid histograms. The calculation obtains the greatest difference between the curves. D/s(n) is an index of similarity for two curves. If D/s(n)=0, then the curves are identical. D represents the greatest difference between the two curves.

Chapter 3 Expression and purification of ST2

To gain a tool, which can modulate ST2L mediated responses, ST2 was expressed and purified from baculovirus-infected insect cells and stably transfected chinese hamster ovary (CHO) cells. While baculovirus-derived ST2 had no detectable biological function on Th cells and macrophages, 50-100 μg/ml of CHO cell-derived ST2-Fc significantly inhibited IL-6 and IL-12 production from LPS-stimulated macrophages. All purified batches of ST2-Fc protein showed similar levels of suppression and were used in chapter 4 to investigate the effect of ST2 on CD4(+) T cells. The inhibition of macrophage cytokine production by ST2 could be mimicked with fixed Th2, but not fixed Th1 cells. In this chapter, the observation that ST2 has an anti-inflammatory action on macrophages was confirmed and extended. A detection system to assess the biological activity of ST2-Fc has also been developed and successfully used.

Introduction

The diversity in effector function of Th1 and Th2 cells is caused by the differential expression of molecules such as cytokines, chemokines and cell surface molecules. ST2L, an IL-1 receptor family member, was shown to be a cell surface marker for Th2 cells (Xu *et al.*, 1998). The functions of ST2L and its splice variant ST2, which lacks the transmembrane and intracellular domains, are still unclear. Administration of anti-ST2L antibodies or ST2-Fc fusion protein impaired Th2 cell effector functions in inflammatory diseases, suggesting that ST2L has an important functional role on Th2 cells (Xu *et al.*, 1998, Loehning *et al.*, 1998). A current hypothesis suggests that in murine T helper cells, ST2 is an antagonist to membrane-bound ST2L. ST2 potentially binds the as yet unidentified ligand of ST2L and prevents signalling (Loehning *et al.,* 1998; Senn *et al.*,

2000). Therefore, ST2 is a valuable tool to investigate the *in vivo* and *in vitro* function of ST2L. In this investigation, viable cell expression systems were selected and established to produce and purify functional ST2, which was then tested *in vitro* and *in vivo* in different disease models. The expression of ST2 in an *E.coli* expression system produced insoluble protein, which therefore could not be used for functional assays (Xu D., unpublished results). A mammalian cell expression system, which would produce soluble, fully glycosylated protein was not available at the time. As another group (Rupp *et al.*, 1995) had already established a successful protocol for purification of ST2 using the baculovirus expression system, the same system was initially adopted in these studies. This group provided the expression construct for ST2. Later, a mammalian cell expression system (stably transfected chinese hamster ovary cells) for the ST2 fusion protein (ST2-Fc) was applied (Kang D, Astra, Sweden). Dr. Kang provided the stably transfected CHO cells and the purification protocol. Methods were developed to detect successful expression and for purification of baculovirus-derived-ST2 and CHO cell derived-ST2-Fc. The biological function of ST2 and ST2-Fc was assessed by treatment of macrophages, based on the finding that ST2 suppresses inflammatory cytokine production by LPS-stimulated macrophages (Sweet *et al.*, 2000).

Baculovirus expression system

Baculoviruses belong to a diverse group of large double-stranded DNA viruses that infect many different species of insects as their natural hosts (Matthews, 1982). Infectious baculovirus particles enter susceptible insect cells by facilitated endocytosis or fusion, and viral DNA is uncoated in the nucleus. The baculovirus genome is replicated and transcribed in the nuclei of infected host cells where the large

baculovirus DNA is packaged into rod-shaped nucleocapsids (Burgess, 1977). Since the size of these nucleo-capsids is flexible, recombinant baculovirus particles can accommodate large amounts of foreign DNA. The infected insect cells release extracellular virus particles by budding off from the cell membrane. In the late stage, occluded virus particles (OV) are assembled inside the nucleus and released when the infected cells lyse. The OV are embedded in a homogenous matrix made predominantly of a single protein, called polyhedrin (Rohrmann, 1986). Expression of the highly abundant polyhedrin gene is non-essential in tissue culture and its strong promoter can be used for the synthesis of foreign gene products. Insertional inactivation of the polyhedrin gene in baculovirus results in the production of occlusion body-negative viruses.

Rupp *et al.* (1995) described a ST2 expression system, based on recombinant baculovirus infection of insect cells. The complete open reading frame of the mature ST2 protein lacking its leader peptide was amplified by PCR from the cDNA clone pT1.10 (Klemenz *et al.,* 1989). The amplified product of 1 kb was inserted into the baculovirus transfer plasmid pAcGP67B (BD Pharmingen) as a BamHI/XbaI fragment. The baculovirus transfer vector contained, beside the sequence of the mature secretory ST2 gene, an *E.coli* origin of replication, an ampicillin resistance marker, a promoter for polyhedrin and a large tract of baculovirus sequence flanking the cloning region to facilitate homologous recombination (Fig. 3.1). Purified recombinant vectors containing ST2 were co-transfected with baculovirus DNA into insect cells and recombinant viruses, which arose by homologous recombination between the transfer vector and baculovirus DNA, were selected. The recombinant ST2 produced in insect cells was slightly less glycosylated than the endogenous gene product of mammalian cells. To

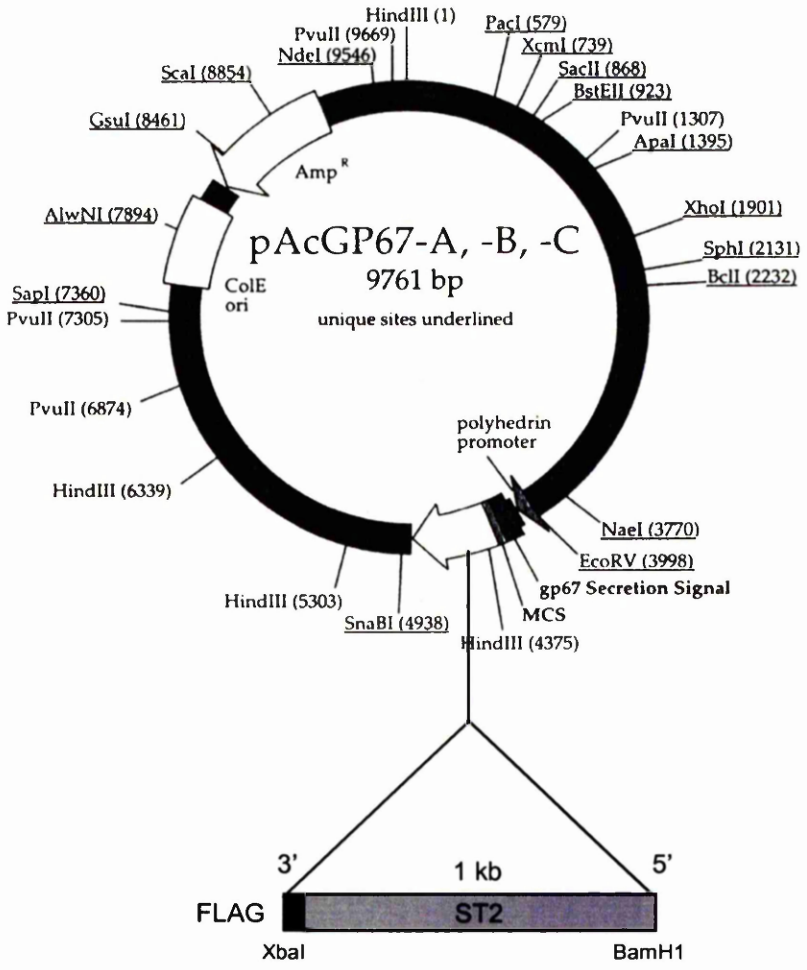


Fig.3.1 Map of pGP67T1S-Flag plasmid

The complete open reading frame of the mature ST2 lacking its leader peptide sequence was cloned (Rupp *et al.*, 1995). At the 3' terminal coding region a sequence for the FLAG peptide was fused and the 1 kb fragment was cloned via XbaI/BamHI site into the Baculovirus transfer vector pAcGP67-B.

increase the yield of secretion of glycosylated protein in this system, the coding region of ST2 was fused in frame with the leader sequence of the highly expressed baculovirus protein GP67 glycoprotein. The recombinant ST2 protein was now directed into the secretory pathway. For facilitated purification, the sequence for an epitope of eight amino acids (Flag peptide) was added to the 3' end of ST2.

CHO cell expression system

The extracellular part of ST2L fused to the constant region of human IgG1/Fc was stably transfected into a chinese hamster ovary (CHO) cell line (Coyle *et al.*, 1999; Fig.3.2). The cells secreted a protein with a molecular weight of 80-90 kDa. The expression in CHO cells ensured full O- and N- glycosylation. The ST2-Fc fusion protein has been reported to inhibit the production of Th2 cytokines and eosinophilic inflammation of the airways in Th2 driven mucosal response model (Coyle *et.al.*, 1999). Further, ST2-Fc treatment of OVA challenged Th2 recipient mice led to suppression of the development of airway hyperresponsiveness (Coyle *et.al.*, 1999).

Results

3.1. Expression and purification of baculovirus-derived ST2

3.1.1. Amplification of recombinant baculovirus in Sf9 cells

As summarized in Fig. 3.3 and as described in detail in section 2.37.1 and 2.37.2, Sf9 cells were expanded in serum-containing medium and infected with recombinant baculovirus, which amplified in the cells and concentrated in the supernatant. To

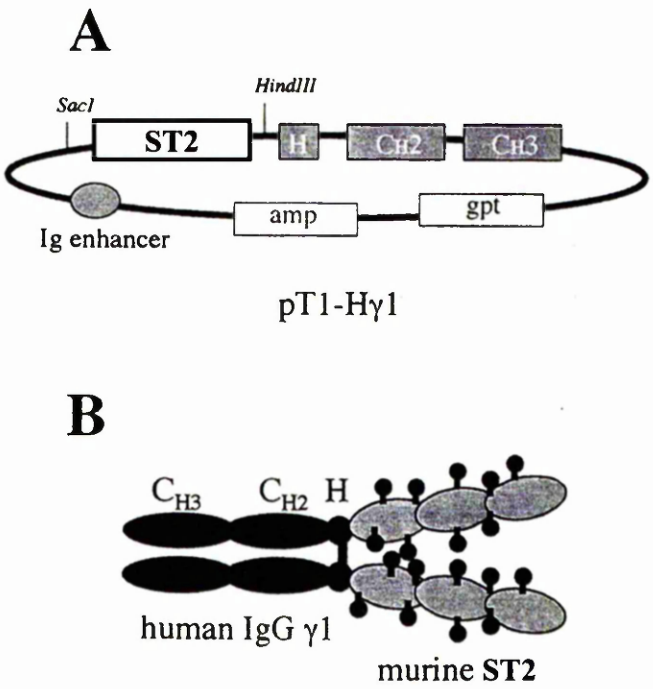


Fig.3.2 **Eukaryotic expression of the ST2-Fc fusion protein in Cos7 cells representative for ST2-Fc expression in CHO cells**

(A) Schematic representation of the pT1-Hγ1 expression vector. The vector bears the 1kb fragment of ST2 and the constant chain 2 and 3 (C_H2 and C_H3) domains.

(B) Schematic representation of the homodimeric disulphide-linked ST2-Fc fusion protein. Potential N-linked glycosylation sites are indicated by knobs. (Moritz *et al.*, 1998a)

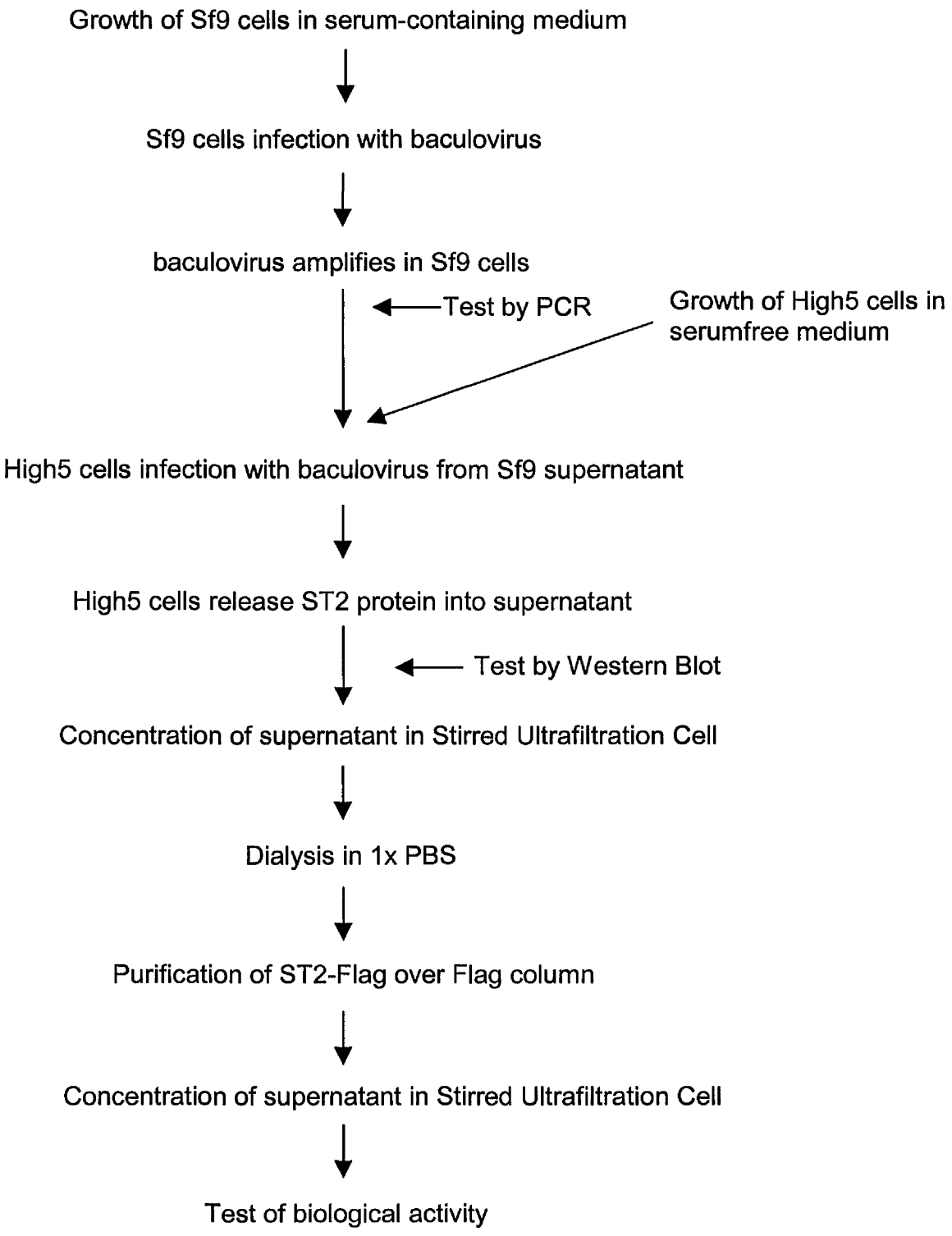


Fig. 3.3 Production and purification of ST2 from baculovirus-infected insect cells

estimate levels of recombinant baculovirus, supernatant of infected Sf9 cells was assessed by PCR. Both PCR primers used bound within the sequence of the ST2 gene. The brightness of PCR products from the tested supernatant in three different dilutions was compared to that of PCR products from supernatant with known virus contents of 10^8 pfu/ml (Fig. 3.4). If the amount of recombinant baculovirus in the tested supernatants was found to be lower than 10^8 pfu/ml, fresh Sf9 cells were infected with this supernatant to increase the virus titre. Infected Sf9 cells produced only small amounts of ST2 protein; therefore another insect cell line, High5, was introduced to maximize the amount of expressed ST2 protein.

3.1.2. Infection of High5 cells and expression of ST2 protein

High5 cells were grown in serum-free medium to avoid later contamination of ST2 with other protein. High5 cells infected with virus containing Sf9 supernatant, released more 55-65 kDa protein than Sf9 cells. ST2 expression was detected by Western Blot using a polyclonal anti-mouse ST2L antibody (Fig. 3.5). The yield of expressed ST2 and the timepoint of harvest of ST2 from High5 supernatant were optimized (Fig. 3.6). Maximal ST2 expression was found 3 to 4 days after infection with 10 pfu/ml recombinant baculovirus.

3.1.3. Purification of ST2 from High5 supernatants

The purification process was performed as described in section 2.37.3. Briefly, infected High5 supernatant was concentrated 5-10 fold in the Stirred Ultrafiltration Cell and samples of the supernatant before and after the concentration were run on a SDS gel and stained with Coomassie blue to show the success of the concentration. The main

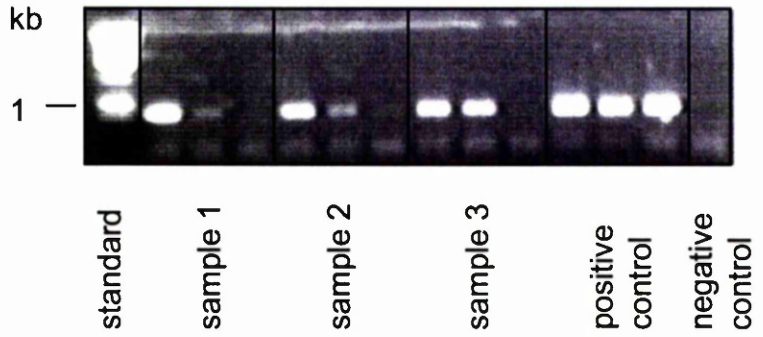


Fig 3.4 ST2-PCR to estimate the recombinant baculovirus titre in supernatants of infected Sf9 cells

Sf9 cells were infected with recombinant baculovirus at 0.1 pfu/cell (section 2.37.1 and 2.37.2). After 4 days the supernatant was analysed by PCR for ST2 gene containing viruses as described in section 2.37.2). The PCR was run on a 1% agarose gel. The three lanes run for each sample represent a 1:1, 1:5 and 1:10 dilution of supernatant. The positive control is supernatant with 10^8 pfu/ml and the negative control is supernatant of uninfected High5 cells.

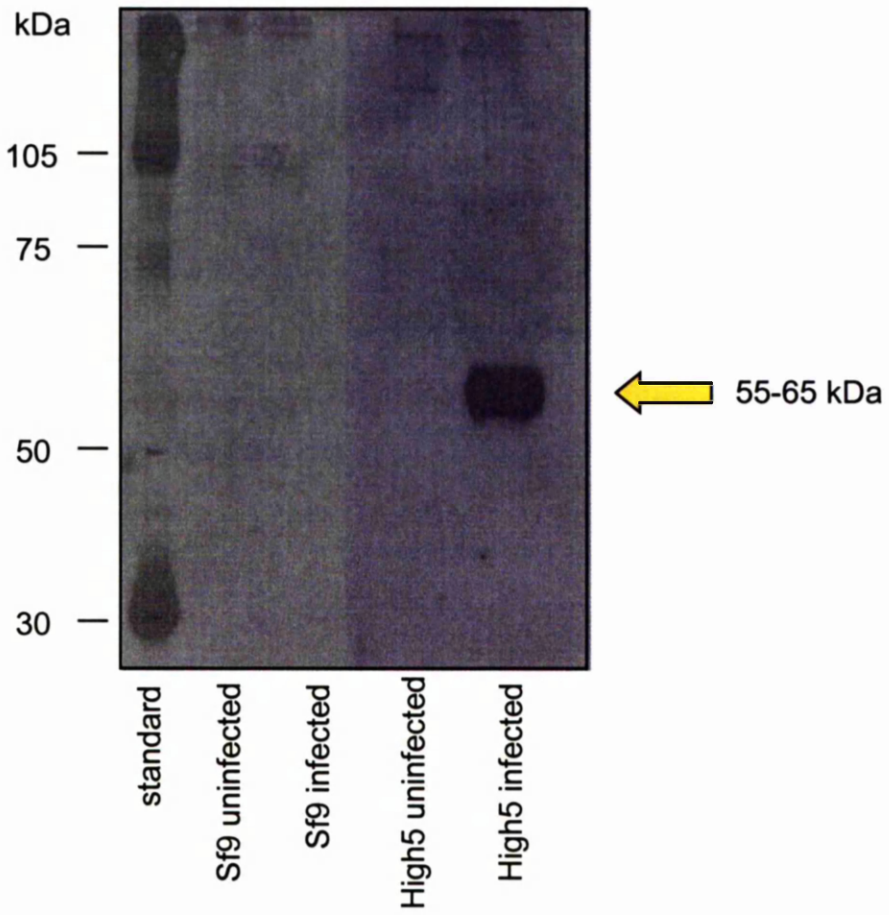


Fig 3.5 Western Blot demonstrating ST2 expression in baculovirus-infected High5 cells

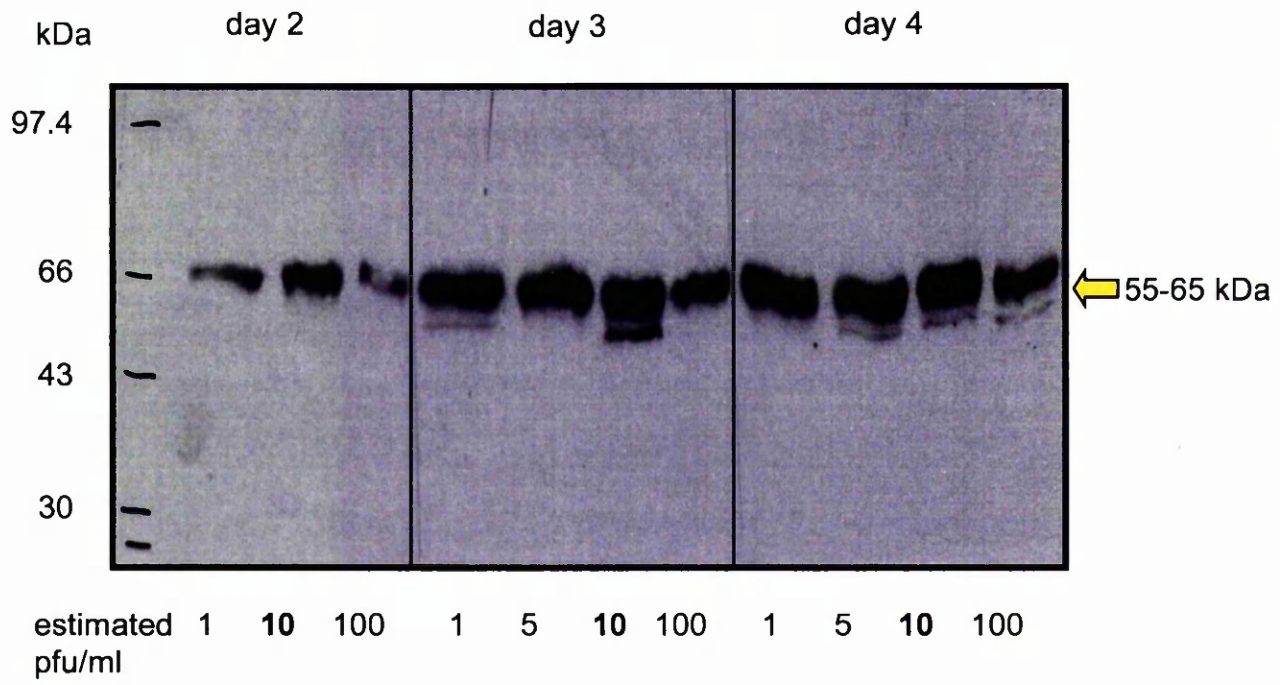
High5 cells were infected with 10 pfu/cells of recombinant baculovirus (section 2.37.3) and the supernatant harvested after 4 days. Western Blot for ST2 detection was performed using 1 μg/ml polyclonal anti-ST2L antibody.

Fig. 3.6 Optimisation of the infection of High5 cells with recombinant baculovirus

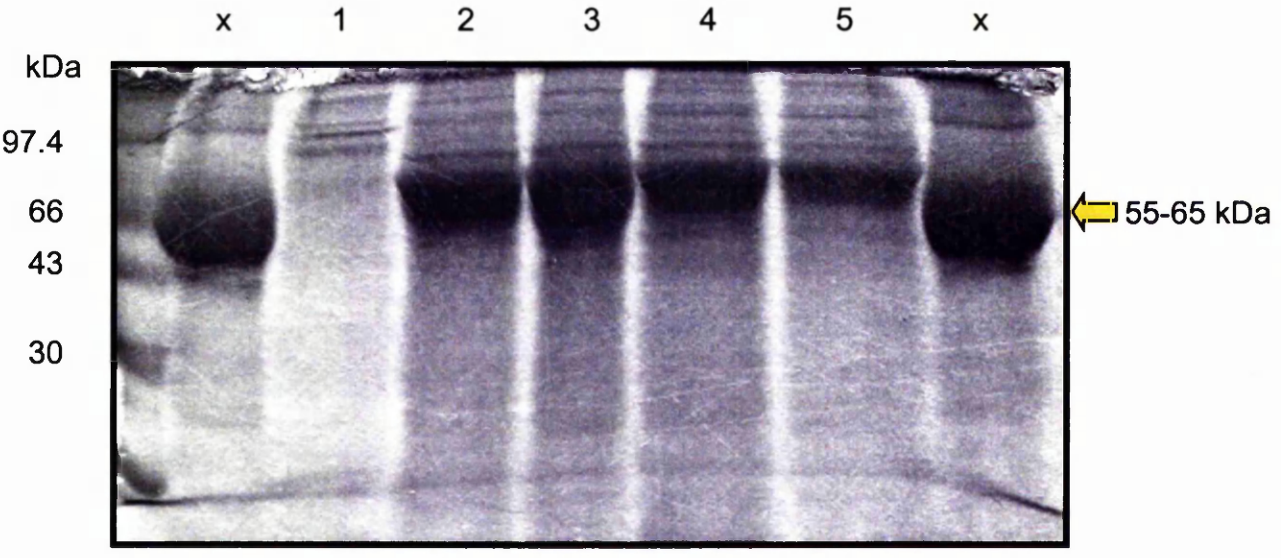
A) Western Blot of recombinant baculovirus-infected High5 cell supernatants. High5 cells were grown to a confluent layer (section 2.37.1) before being infected with different amounts of recombinant baculovirus (section 2.37.3). Supernatant was taken after 2, 3 and 4 days and the amount of ST2 was assessed by Western Blot using anti-Flag antibodies
(1:1000 dilution).

B) Coomassie blue-stained SDS gel of supernatants from baculovirus-infected and uninfected Sf9 and High5 cells. High5 cells were grown to confluency before being infected with different amounts of recombinant baculovirus and incubated for 4 days. Equal amounts of supernatant were run on a SDS gel and stained with Coomassie blue.
1: uninfected; 2: 100 pfu/ml; 3: 10 pfu/ml; 4: 1 pfu/ml; 5: 0.1 pfu/ml; X: not relevant sample

A)


kDa
day 2
day 3
day 4
97.4
66
43
30
55-65 kDa
estimated pfu/ml
1 10 100 1 5 10 100 1 5 10 100

B)


x 1 2 3 4 5 x
kDa
97.4
66
43
30
55-65 kDa

fraction of the concentrated protein ran at approximately 60 kDa, suggesting that ST2 was one of the main products of baculovirus-infected High5 cells. Only a marginal loss of protein due to binding to the filter membrane was observed (Fig. 3.7). ST2 protein was then dialysed against PBS and loaded onto an anti-FLAG column, washed and eluted by competition with Flag peptide. The volume was minimized again to ensure a high concentration of ST2. Samples of all purification steps were assessed for ST2 enrichment by Western Blot, using a monoclonal anti-Flag antibody in a dilution of 1:1000 (Fig. 3.8 A and B). The detected Flag containing protein ran at 50-60 kDa and was also detectable with polyclonal anti-ST2L antibody (data not shown). The protein bound specifically to the anti-FLAG column and the majority of it was eluted by Flag peptide competition. Only a small fraction remained bound to the column and this was subsequently released by Glycin/HCl treatment.

Only 300-400 μg ST2 per litre supernatant produced by $4x10^8$ High5 cells could be purified. The culture of High5 cells in a spinner culture would allow the production of sufficient amounts of the protein, but was not available. The purification process worked out to be time and cost consuming. Hence, another ST2 expression system in CHO cells was chosen (chapter 3.3).

3.2. Biological activity of baculovirus-derived ST2

3.2.1 The effect of baculovirus-derived ST2 on polarised Th1 and Th2 cells and BALB/c spleen cells

Since ST2L is expressed on Th2 cells and ST2 may inhibit ST2L sinalling by binding to the as yet unknown ligand, ST2 was tested on purified polarised T helper cells.

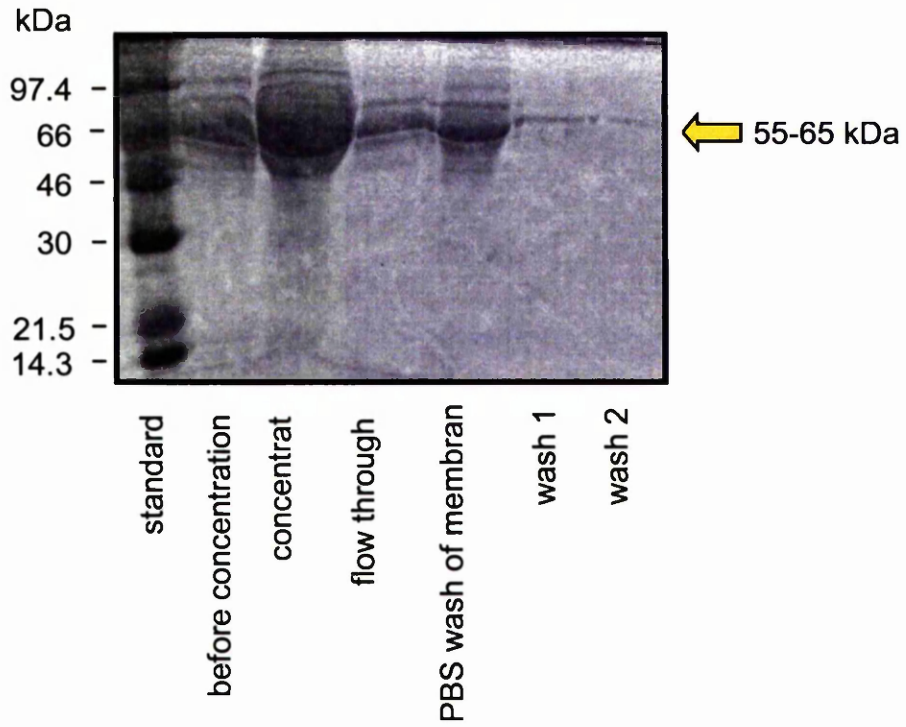


Fig 3.7 Concentration of High5 supernatant in the Stirred Ultrafiltration Cell

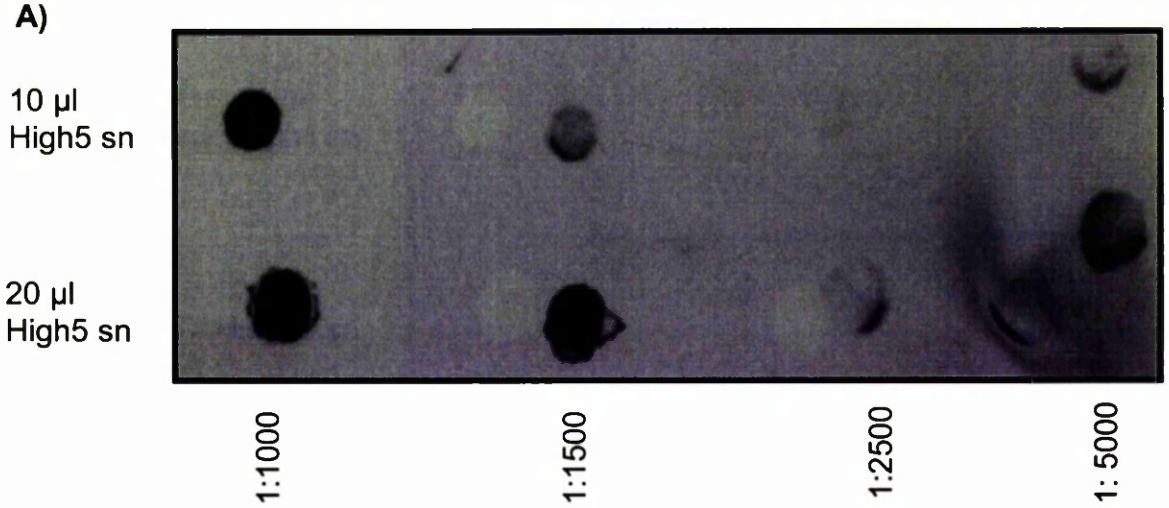
High5 supernatant was concentrated with the Stirred Ultrafiltration Cell containing a 30kDa membrane (section 2.37.4). After the concentration, the membrane was washed with PBS and water (wash 1,2) and all the protein solutions were run on a 10% SDS gel and stained with Coomassie blue.

Fig 3.8 Anti-Flag antibody detection of ST2 in High5 supernatant and during the purification process

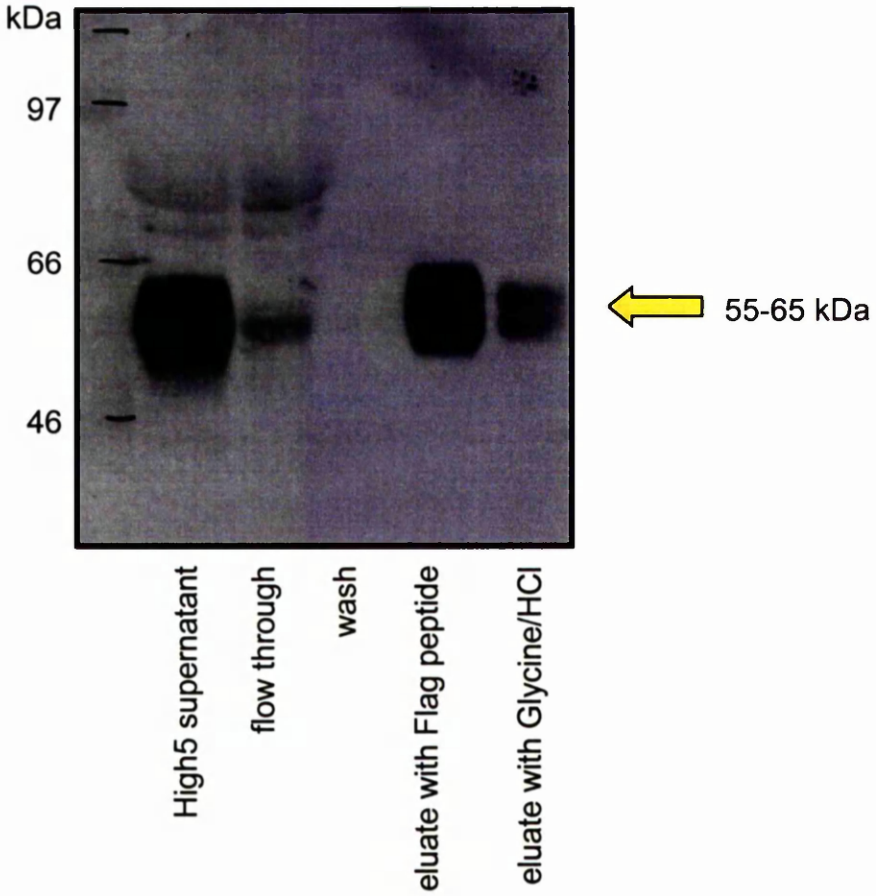
A) Dot Blot of titration of anti-Flag antibody binding to ST2-Flag in baculovirus-infected High5 supernatant.

B) Purification of ST2 over an anti-FLAG column. Western Blotting was performed on samples obtained during the purification process and ST2 was detected by anti-Flag antibody used at a 1: 1000 dilution.

A)


10 µl
High5 sn
20 µl
High5 sn
1:1000
1:1500
1:2500
1: 5000
dilution of anti-Flag antibody stock solution

B)


kDa
97
66
46
55-65 kDa
High5 supernatant
flow through
wash
eluate with Flag peptide
eluate with Glycine/HCl

Furthermore, the possibility that other spleen cell types, including activated B cells and macrophages, may interfere with the function of ST2 on T cells e.g. by producing the ligand, was assessed. In all experiments Flag peptide was added as a control to determine the effect these eight amino acids had on the ST2 function. To exclude the possibility that LPS contamination might contribute to any observed effect, heat-denatured ST2 protein was added as a control. Since LPS is heat stable but proteins are generally not, any effect seen with the denatured ST2 preparation is likely to reflect effects of contaminating products such as LPS.

CD4(+) T cells were purified as described in section 2.37.4, stimulated with antigen, and either polarised towards Th1 or Th2 cells, or left unpolarised (Fig. 3.9) for 3 days. In a second experiment, BALB/c spleen cells were stimulated for 3 days with either 5 μg/ml anti-CD3 or 2 μg/ml ConA to activate T cells, or 1 μg/ml LPS to activate B cells and macrophages. ST2 (10 pg/ml - 1 μg/ml) or the mentioned controls were added to both experiments at the time of stimulation. Proliferation and Th1 and Th2 cytokine profiles were assessed after 3 days and found to be unchanged between ST2-treated cells and control cells.

3.2.2. The effect of baculovirus-derived ST2 on macrophages

Sweet *et al.* (2001) demonstrated binding of Cos7 cell-derived ST2-Fc fusion protein to primary bone marrow-derived macrophages and an inhibition of LPS-induced pro-inflammatory cytokine production, e.g. IL-6 and IL-12 but not IL-10, when ST2-Fc was added in concentrations of 50 - 100 μg/ml. Therefore, the effect of higher concentrations of baculovirus-derived ST2 on LPS-stimulated bone marrow macrophages (BMM) was tested. CSF-1-matured BMMs were treated with up to 50

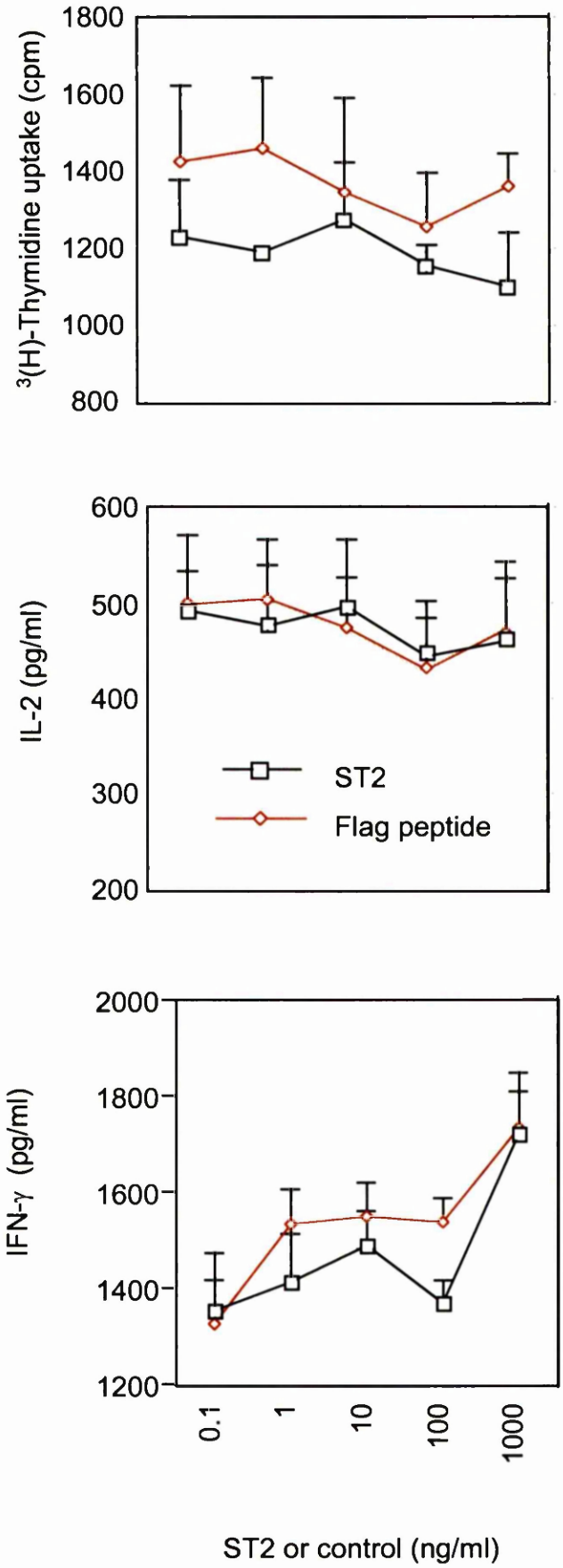


Fig 3.9 The effect of baculovirus-derived ST2 on antigen stimulated CD4(+) T cells

CD4(+) T cells were purified from spleens of DO11.10 mice as described in section 2.4 and 2.8. Cells ($1x10^6$) were then stimulated with irradiated APC (1:10) (section 2.6) and 300 nM OVA antigen for 3 days. A concentration range of baculovirus-derived ST2 or Flag peptide was added at the time of stimulation. Cell proliferation was assessed by ^{3}(H)-Thymidine incorporation. The cytokine patterns were assessed by ELISA. IL-4 and IL-5 levels were below detection. The results (n=3) are shown as the mean ± 1 SD.

μg/ml ST2 or PBS as control for 1 h, and 100 ng/ml LPS were then added for a further 8 h. ELISA analysis of the harvested supernatants revealed that baculovirus-derived ST2 was not able to suppress IL-6, IL-12 or TNF-α from LPS-stimulated macrophages and therefore did not show the biological activity that was apparent with the ST2-Fc fusion protein from Cos7 cells (data not shown).

3.3. Expression and purification of ST2-Fc from chinese hamster ovary (CHO) cells

3.3.1 Expression of ST2-Fc in CHO cells

As described in section 2.38.1 and in Fig. 3.10, ST2-Fc expressing CHO cells were expanded in medium, supplemented with serum containing high IgG, which provided optimal growth conditions. Upon confluency, CHO cells were transferred into medium containing serum with low IgG to minimize IgG interference during the ST2-Fc protein purification process via protein A sepharose. The release of ST2-Fc into culture medium was confirmed through Dot Blot and ST2-ELISA using monoclonal anti-ST2L antibodies. As shown in Fig. 3.11 A and B, both of these methods confirmed the presence of the ST2-Fc protein in supernatant of transfected CHO cells.

3.3.2 Purification of ST2-Fc protein

The purification of ST2-Fc protein from transfected CHO cell supernatants was based on the strong affinity of protein A to the Fc region of immunoglobulins, including IgG. The ST2-Fc protein was bound by a protein A sepharose column and eluted by lowering the pH, which protonated carboxylic acid groups and allowed for release of the

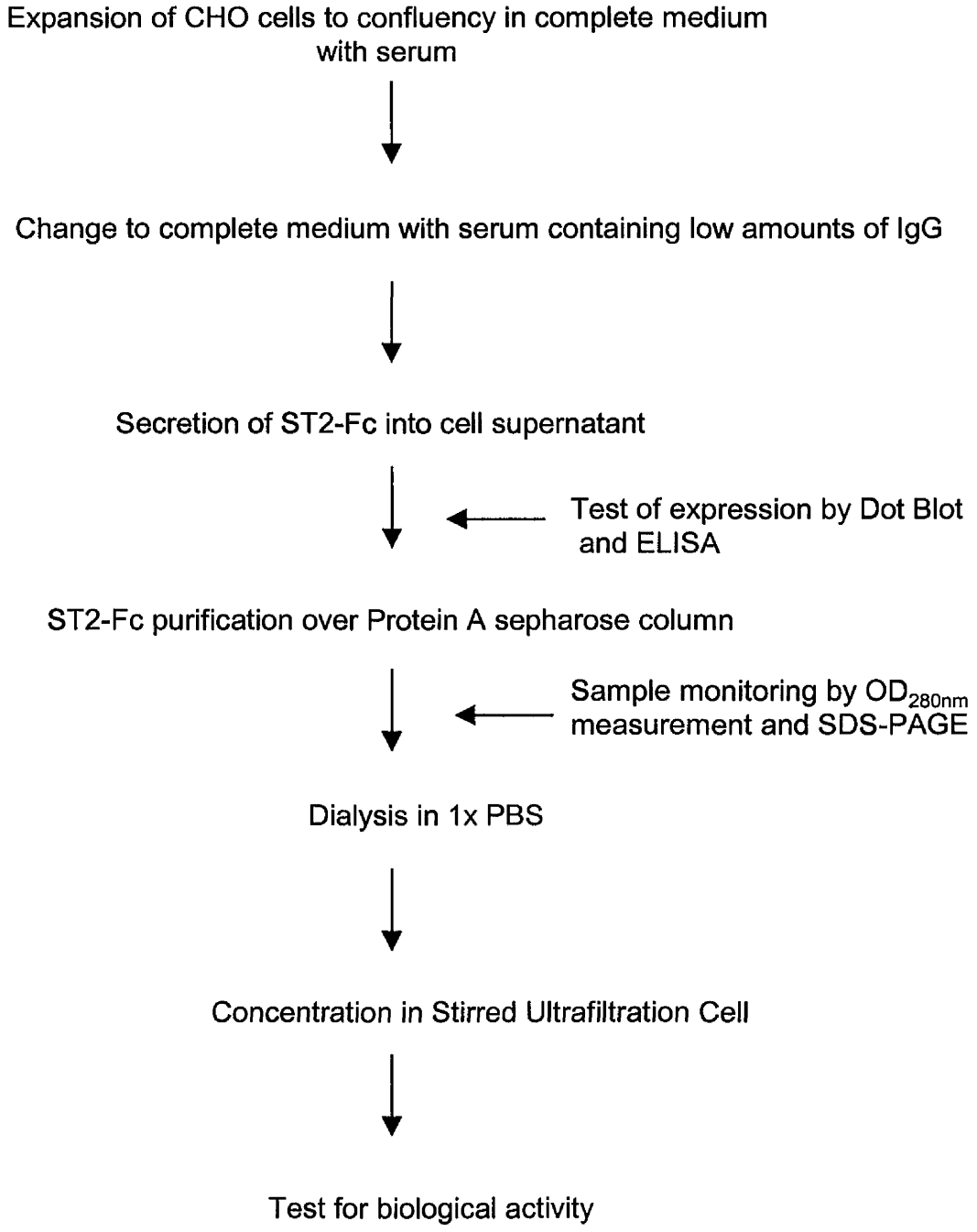


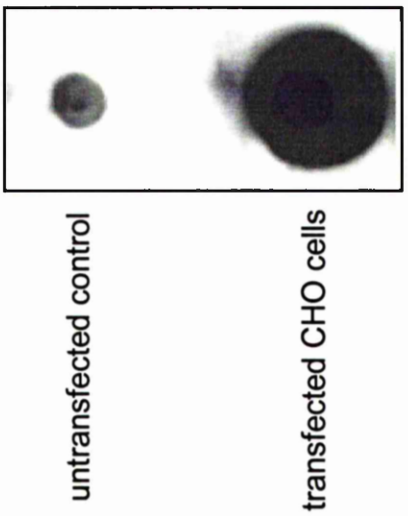
Fig. 3.10 **Production and purification of ST2-Fc from stably transfected CHO cells**

Fig 3.11 Determination of successful ST2-Fc production in CHO cells supernatant

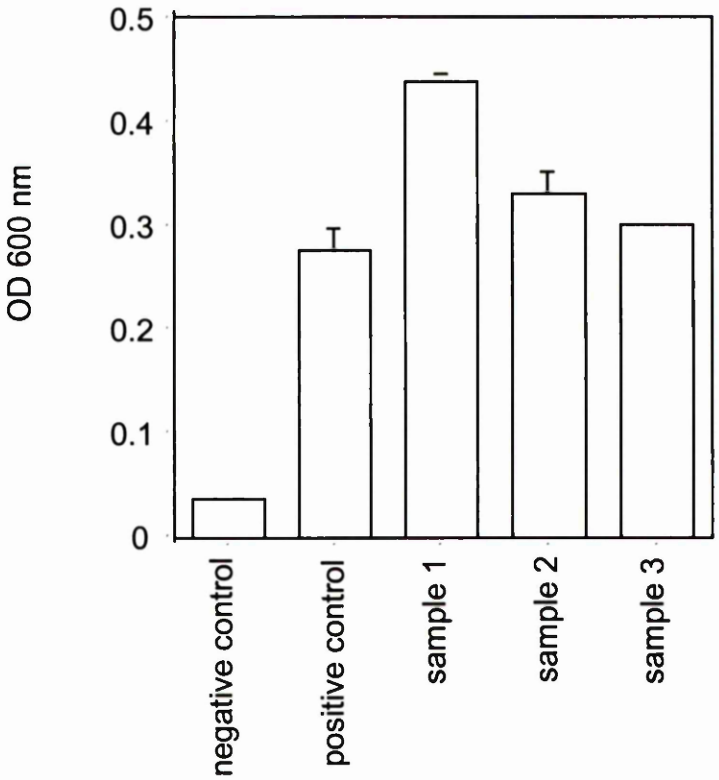
A) A Dot Blot of supernatants of transfected and untransfected CHO cells was performed. ST2-Fc was detected by 1 μg/ml of monoclonal biotinylated anti-ST2 antibody.

B) ST2-Fc was detected by ELISA using monolonal biotinylated anti-ST2 antibody (section 2.23). The supernatant of untransfected CHO cells was used as negative control, the positive control consisted of ST2-Fc received from Dr. Kang, Astra. Sample 1, 2 and 3 were untested supernatants of transfected CHO cells. The error bars represent the standard deviation between the ELISA triplicates of one sample.

A)


untransfected control
transfected CHO cells

B)


OD 600 nm
0.5
0.4
0.3
0.2
0.1
0
negative control
positive control
sample 1
sample 2
sample 3

fusion protein. Protein concentration in fraction eluates was monitored by OD_{280nm} measurement (Fig. 3.12 A). Aliquots of fractions with the highest OD_{280nm} were run on a denaturing SDS gel and stained with Coomassie blue (Fig. 3.12 B). A single band appeared around 90 kDa. Bands with molecular weights of 60-70 kDa, as seen in fraction 1-4 (Fig. 3.12 B), were minimized by more stringent washes (Fig. 3.12 C). The fractions with the highest protein contents were pooled, dialysed and concentrated. The purified protein preparation contained a single major band at 80-90 kDa (Fig. 3.13). One or two faint smaller bands were also observed that may have resulted from incomplete glycosylation or degradation of ST2-Fc protein. hIgG, the control used in *in vitro* experiments, ran with three bands at 25 kDa, 60 kDa and 80-90 kDa. The 25 kDa band represented the light chain, the 60 kD band represented the heavy chain and the large band may have been be due to incomplete denaturation of the protein.

3.4 Assessment of the biological activity of CHO cell-derived ST2-Fc protein

3.4.1 The effect of ST2-Fc on macrophages

Cos7 cell-derived ST2-Fc protein, obtained from Astra, was able to suppress pro-inflammatory cytokines including TNF-α, IL-6 and IL-12 on LPS-stimulated BMMs (Sweet *et al.,* 2001). To test the biological activity of CHO cell-derived ST2-Fc protein, the protein was cultured with LPS-stimulated BMMs as described in 3.2.2. Human IgG was added as a control to determine the effect of the Fc part of the ST2 fusion protein. Every batch of ST2-Fc protein suppressed production of pro-inflammatory cytokines at a concentration range of 50-100 μg/ml (Fig.3.14). 50 μg/ml of ST2-Fc reduced IL-12 by 72%, IL-6 by 80% and TNF-α by 61%. The expression of IL-10 was not affected. The

Fig 3.12 Purification of ST2-Fc from supernatant of transfected CHO cells

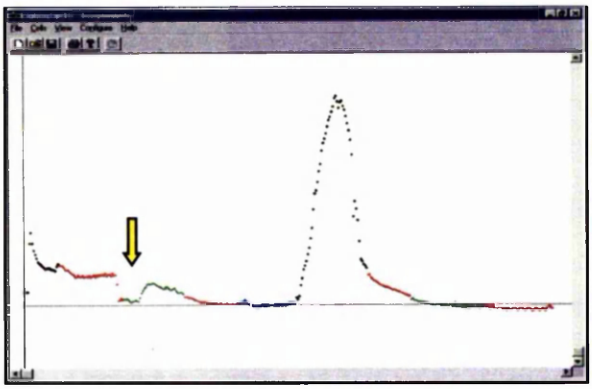
The purification was conducted as described in section 2.38.2.

A) $OD_{280\ nm}$ profile of purification. Different colours represent separately collected fractions of a volume of 3.5 ml. Fraction 4 contains the majority of the eluted ST2-Fc.

B) Run of fractions on a Coomassie blue-stained SDS gel. Fraction 4 contains the majority of the eluted ST2-Fc.

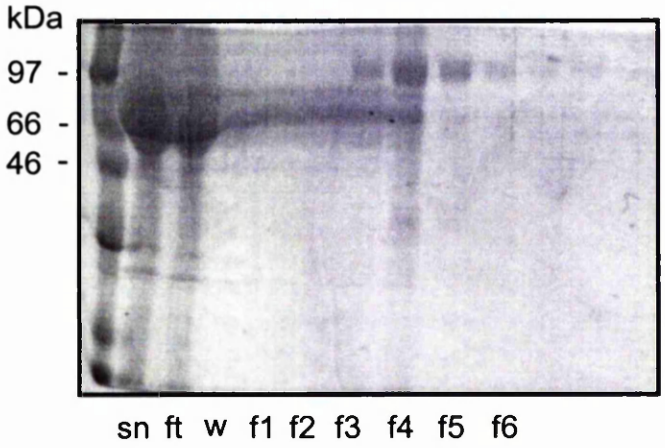
C) Coomassie blue-stained SDS gel of the collected fractions after prolonged washing with buffer A (20 mM sodium-phosphate pH 7.0). This figure shows a separate experiment. Here, fraction 3 contains the majority of the eluted ST2-Fc. (sn =supernatant, ft=flow through, w=wash, f=fraction)

A)



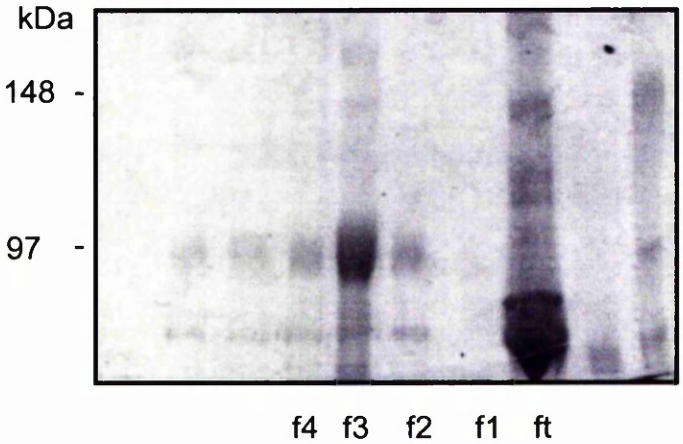
start of elution

B)



≈ 90 kDa

C)



≈ 90 kDa

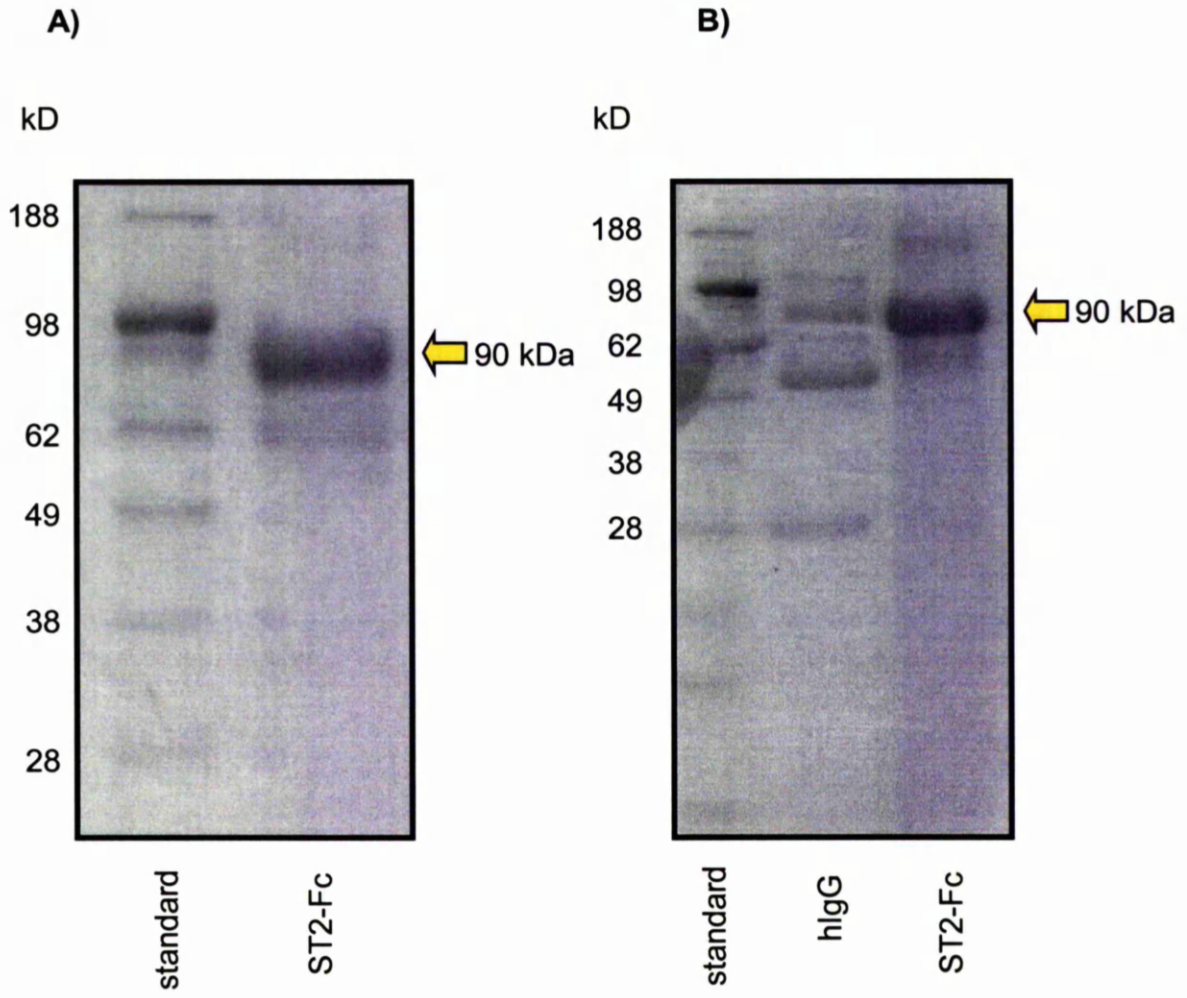


Fig. 3.13 Coomassie blue-stained SDS gel of purified ST2-Fc and the control hIgG

A) Purified ST2-Fc was run on a 10% SDS gel and stained with Commassie blue.

B) Purified ST2-Fc and hIgG (purified over the Protein A sepharose column, described in section 2.38.2) were run on 10% SDS gel and stained with Coomassie blue.

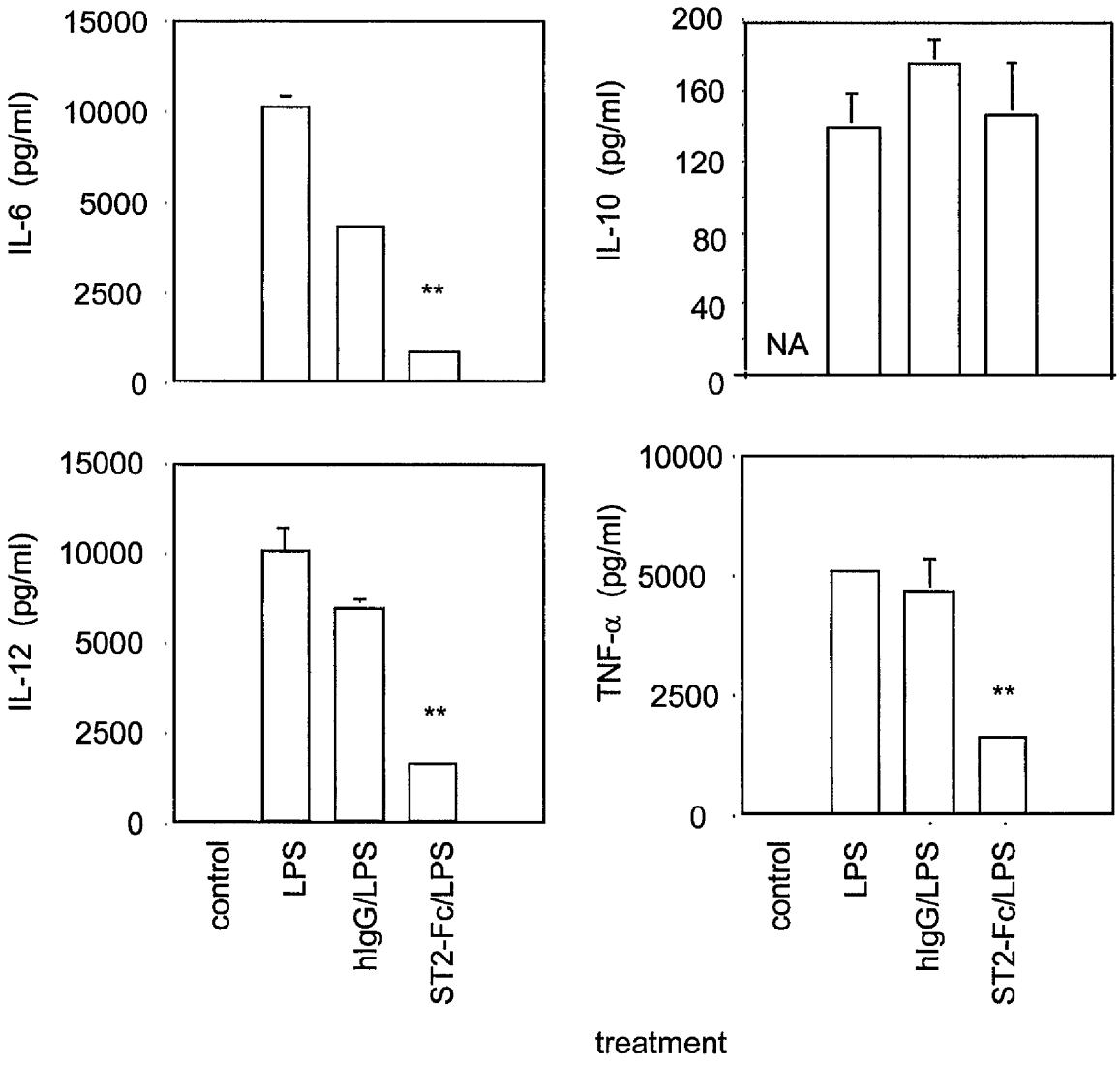


Fig 3.14 ST2-Fc suppresses IL-6, IL-12 and TNF-α production by LPS-stimulated BMMs

BMMs were obtained as described in section 2.11. Matured BMMs ($5x10^5$) were incubated with 50 µg/ml ST2-Fc or hIgG for 1h, then stimulated with 100 ng/ml LPS for 8 h. Control cells were left unstimulated. The cytokine production was measured by ELISA. The results (n=3) are shown as the mean ± 1 SD (**$p<0.005$) and are representative for 4 experiments.

(NA: not analysed)

suppression of pro-inflammatory cytokines by ST2-Fc was not due to toxicity as it had no effect on the viability of RAW264 cells and BMMs as assessed by MTT assay, despite the fact that IL-6 and IL-12 levels decreased dramatically in these cells (Fig. 3.15 and data not shown).

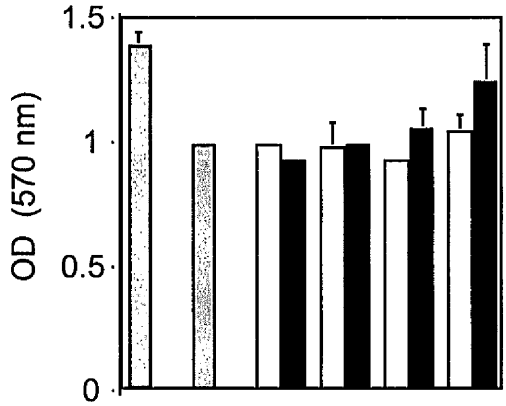
3.4.2. The effect of ST2L on macrophages

The suppressive effect of ST2-Fc might be mediated by its binding to the cell surface of macrophages (Sweet *et al.*, 2001). The binding could be further enhanced by treatment of macrophages with LPS, indicating the upregulation of the putative ST2-binding protein. ST2L and ST2 share a common extracellular region, raising the question of whether membrane-bound ST2L functions in the same way on macrophages as its shorter splice variant. To address this question, the ability of fixed Th2 cells (which express ST2L) to inhibit LPS-stimulated macrophages to produce pro-inflammatory cytokines was assessed. Th1 and Th2 clones were fixed with paraformaldehyde to inhibit their cytokine production and cultured with macrophages as described in section 2.39. Neither of the fixed Th clones produced cytokines or showed signs of proliferation. However, fixed Th2 cells were able to decrease IL-6 expression to 39% and IL-12 expression to 48% of the control level (Fig. 3.16). Fixed Th1 cells, which do not express ST2L, did not alter the IL-6 release, and actually increased IL-12 production from LPS-stimulated macrophages.

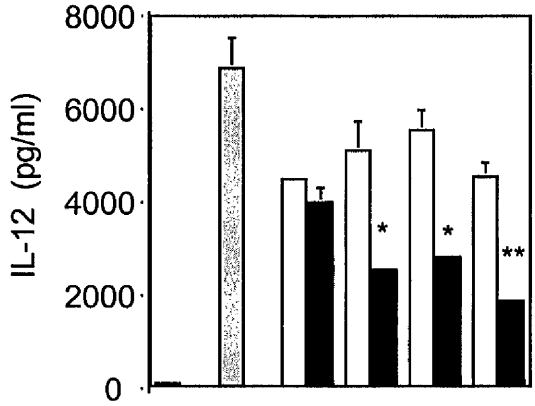
Fig 3.15 Dose-dependent effect of ST2-Fc on LPS-stimulated RAW cells

RAW264 cells ($5x10^5$/well) were treated with a concentration range of ST2-Fc and hIgG for 1h before being stimulated with 100 ng/ml LPS. The cells were incubated for a further 8h. Control cells were not stimulated with LPS. The cells were tested for changes in mitochondrial activity (A) and the supernatants were analysed for IL-12 and IL-6 production by ELISA (B,C). The results (n=3) are shown as mean ± 1 SD (**$p<0.005$, *$p<0.05$) compared with the control (hIgG).

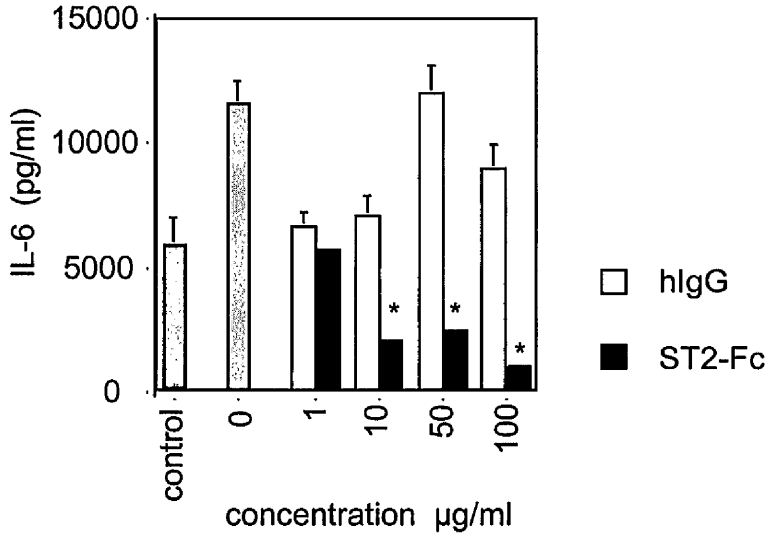
A)


1.5
1
0.5
0
OD (570 nm)

B)


8000
6000
4000
2000
0
IL-12 (pg/ml)
*
*
**

C)


15000
10000
5000
0
IL-6 (pg/ml)
*
*
*
control
0
1
10
50
100
concentration µg/ml
hIgG
ST2-Fc

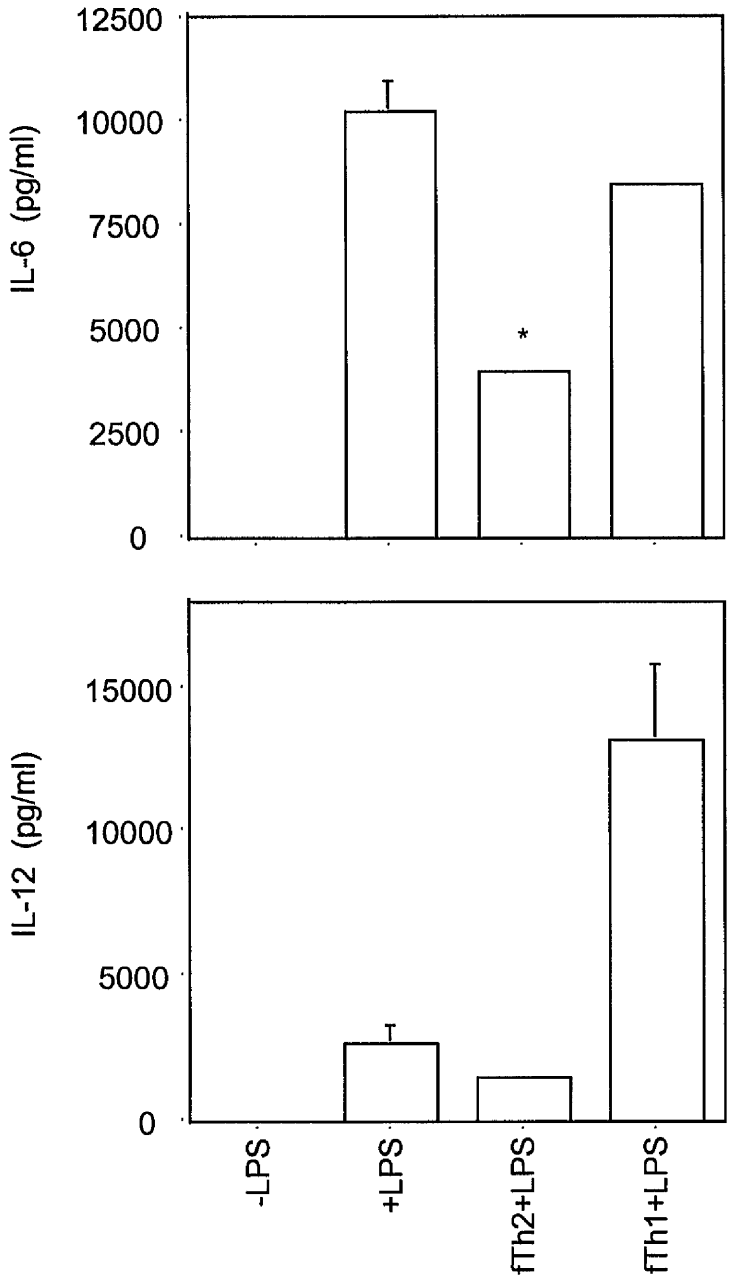


Fig. 3.16 Fixed Th2, but not Th1 cells reduce IL-6 and IL-12 production by LPS-stimulated BMMs

Th1 and Th2 cell clones ($5x10^6$) were fixed in 1% paraformaldehyde (section 2.39) and incubated for 1h with matured BMMs (10:1) before 100 ng/ml LPS were added and the cells were incubated for a further 18h. Cytokine production was analysed by ELISA. The results (n=3) are shown as mean ± 1 SD (*$p<0.05$) and represent 2 experiments.

Discussion

ST2 derived from baculovirus-infected insect cells did not show suppressive activity on LPS-stimulated macrophages, whilst ST2-Fc protein decreased pro-inflammatory cytokine production by LPS-stimulated macrophages. Rupp *et al.* (1995) reported the expression of the secreted, modified ST2 protein by baculovirus-infected insect cells, but no biological function of the protein had been shown. Meisel *et al.* (2001) demonstrated recently that Flag-ST2 could neutralize the effects of immobilized anti-ST2L antibody on Th2 cells but did not show an effect of Flag-ST2 itself on Th2 cells which is consistent with the results presented here. There may be several reasons for the lack of bioactivity of baculovirus-derived ST2. Firstly, the protein function may suffer from a lack of glycosylation and other post-translational modifications, which might be crucial for the biological function of ST2, e.g. for the interaction with its ligand/receptor. Several post-translational modifications such as N- and O-linked glycosylation, phosphorylation and proteolytic cleavage have been reported to occur in the baculovirus-infected insect cells (Hoss *et al.*, 1990; Kloc *et al.*, 1991). However, the baculovirus expression system can express the gene of interest at a high rate, which may overwhelm the ability of the cell to modify the protein product (Crossen and Gruenwald, 1997). Additionally, baculovirus-infected insect cells are impaired in their ability to sialylate proteins (Marchal *et al.*, 2001). Secondly, ST2-Fc protein is likely to dimerise in solution (Gayle *et al.*, 1996), due to the forming of two disulphide bridges. By using a non-denaturing gel, a number of bands above 150 kDa were detected, which could indicate the existence of ST2-Fc dimers (data not shown). Fc fusion proteins have been shown to dimerise and exhibit stronger biological activity than monomeric

native receptors, e.g. the soluble TNF-α receptor fusion protein (Kuerschner *et al.*, 1992; Mohler *et al.,* 1993). Baculovirus-derived ST2 carrying the Flag peptide was found to be monomeric (Gayle *et. al.*, 1996). Whether dimerisation is critical for ST2 function and whether natural ST2 is dimerising is still unclear and needs to be further investigated. Due to the lack of functional anti-ST2 antibodies for Western Blotting of non-denaturing gels, the question of whether natural ST2 forms dimers could not be fully addressed during this study. However, Kuroiwa *et al.* (2001) using gel filtration and SDS PAGE under non-reducing conditions suggested the existence of ST2 multimers in human serum.

The suppressive effect of ST2-Fc on macrophages could be mediated by binding to a cell surface protein, which functions as a receptor (Fig. 4.1 B). Indeed, strong binding of ST2-Fc but not baculovirus-derived ST2 to macrophages was found (Sweet *et. al.*, 2001; data not shown). Fixed Th2 cells, expressing ST2L, but not fixed Th1 cells were also able to reduce pro-inflammatory cytokine production on LPS-stimulated macrophages, indicating a role for ST2L similar to ST2. Monoclonal anti-ST2L antibodies were unable to neutralize the effect of fixed Th2 cells on macrophages, but induced a reduction of pro-inflammatory cytokines (data not shown). Testing fixed Th2 cells, isolated from ST2L knockout mice (Townsend *et al.,* 2000), should prove whether ST2L is important for altering IL-6 and IL-12 production by activated macrophages. It remains unclear, which of the Th1 cell expressed molecules was responsible for the observed increase of IL-12 production by activated macrophages. For example, IFN-γ is able to increase IL-12 production (Ma *et al.*, 1996; Hayer *et al.*, 1995), but IFN-γ production by fixed Th1 cells was not detected.

The effect of the ST2-Fc fusion protein on T helper cells was next investigated and is described in chapter 4.

Chapter 4 The effects of ST2 on naïve but not on committed CD4(+) T cells

Naïve CD4(+) T cells and polarised Th1 cells do not express the orphan receptor ST2L, whilst most CD4(+) T cells become ST2L(+) upon repeated antigenic stimulation under Th2 polarising conditions. The blockade of ST2L function on Th2 cells through ligand competition with its shorter spliced variant, soluble ST2 (ST2), has been extensively studied. An analysis of the effect of ST2-Fc fusion protein, mimicking ST2, on ST2L(-) naïve and antigen-stimulated CD4(+) T cells is presented in this chapter. *In vitro* studies revealed that ST2-Fc suppressed cytokine expression by CD4(+) T cells, which had been primed with antigen. However, antigen-restimulated CD4(+) T cells were resistant to the suppressive effect of ST2-Fc. ST2-Fc binding studies using these cells showed that only naive and freshly stimulated CD4(+) T cells bound the fusion protein, whilst restimulated CD4(+) T cells were unable to do so. This suggests that ST2 functions through binding to an unidentified receptor and suppressing the effector functions of the target cell. These data collectively demonstrate that ST2-Fc is unlikely to act on unpolarised CD4(+) T cells as an ST2L antagonist by competition for a soluble ligand but rather probably functions by binding directly to T cells and triggering signalling.

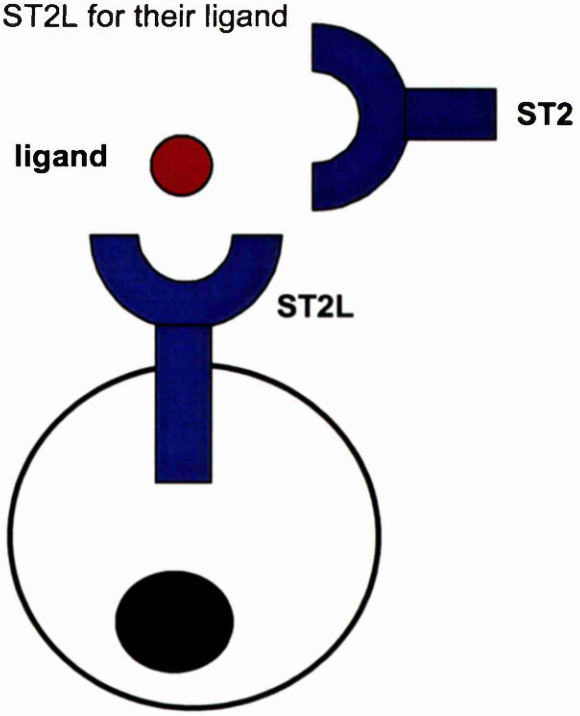
Introduction

The two ST2 gene products, a 5 kb mRNA coding for a membrane-bound receptor (ST2L) and a shorter 2.7 kb mRNA coding for the soluble receptor (ST2), were found to be expressed in several T cell lines (Roessler *et al.,* 1995). The role of

ST2L, an orphan receptor, in Th2 cells has been extensively examined. This molecule is stably expressed on Th2, but not Th1 cells (Xu *et al.*, 1998; Loehning *et al.*, 1998). Blockade of ST2L by anti-ST2L antibody resulted in increased resistance to *Leishmania major* infection in BALB/c mice and exacerbated collagen-induced arthritis in DBA/1 mice by modulation of the Th1/Th2 balance. Thus, ST2L was postulated to be involved in Th2 cell effector functions.

An important unanswered question is the role of ST2 in immune responses. It might, like other soluble cytokine receptors, act as an antagonist to ST2L by binding to its putative ligand and so preventing ST2L-mediated signalling (Fig. 4.1 A, Fernandez-Botran *et al.*, 1996). Alternatively, since ST2 was found to bind directly to macrophages and trigger anti-inflammatory responses (Sweet *et al.*, 2001), ST2 has the potential to act as a ST2L agonist on cells expressing the putative receptor for ST2 and ST2L and to induce signalling through this receptor (Fig.4.1 B). In support of the first model, a ST2-Fc fusion protein, consisting of the extracellular part of ST2L and the Fc part of human IgG1, suppressed both differentiation to and activation of Th2, but not Th1 effector populations *in vitro,* and abrogated Th2 cytokine production *in vivo* (Coyle *et al.*, 1999). Additionally, the ST2-Fc protein attenuated eosinophilic inflammation of the airways, suggesting a crucial role for ST2L in Th2 effector functions (Coyle *et al.,* 1999; Loehning *et al.,* 1998). Coyle's findings that ST2-Fc also had a priming effect on unpolarised Th cells raised the question, as to how ST2 functions on ST2L(-) Th cells, where a competition between the two ST2 splice variants for the unknown ligand is unlikely (Coyle *et al.,* 1999). The effect of ST2-Fc on the cell viability and cytokine

A) Competition of ST2 and ST2L for their ligand



B) Binding of ST2 or ST2L to their receptor

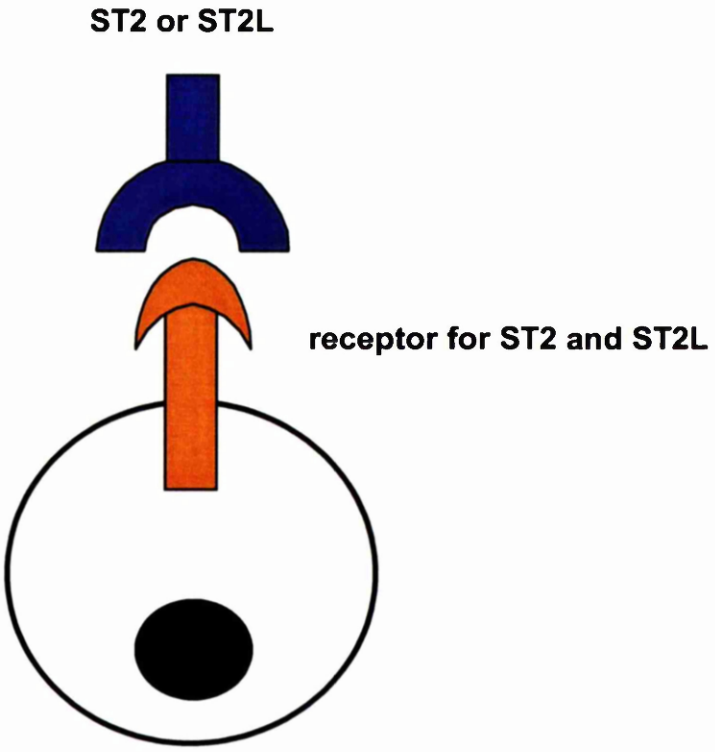


Fig.4.1 Models for ST2 function on T helper cells

A) ST2 competes with ST2L for their soluble ligand and therefore prevents signalling through ST2L.

B) ST2 or ST2L bind to their receptor on Th cells and thereby initiate signalling.

expression of naïve and committed CD4(+) T cells was therefore examined. The possibility of ST2-Fc mediating its effects by binding to the cell surface was also investigated by flow cytometry.

Results

4.1. The effect of ST2-Fc on T helper cells

4.1.1. The effect of ST2-Fc on naive CD4(+) T cells during an antigen-driven immune response

At the time of this study little was known about the *in vitro* effects of ST2-Fc on T cells. ST2-Fc or human IgG (100 pg/ml - 1 μg/ml) was added to spleen cells derived from DO11.10 mice. The cells were stimulated with antigen or plate-bound anti-CD3 to activate T cells, and LPS or LPS/IFN-γ to activate macrophages, which may produce ST2 ligand or receptor and therefore provoke a cytokine response by Th cells. ELISA analysis of Th1 and Th2 cytokines revealed no difference between ST2-Fc-treated and hIgG-treated control cells (data not shown). Next, CD4(+) T cells were enriched by negative selection as described in section 2.8 and polarised to Th1 and Th2 cells (section 2.9). ST2-Fc (100 pg/ml - 1 μg/ml) added at the time of stimulation did not influence the cytokine expression profiles of either Th1 or Th2 cells (data not shown). The effect of ST2-Fc on *in vitro* polarised Th1 and Th2 cells was then compared to an *in vivo* Th2 dominated disease model, *Leishmania major* in BALB/c mice. Spleen cells from infected animals were stimulated *in vitro*

with *L. major* antigen (section 2.16) and ST2-Fc or hIgG (100 pg/ml - 1 μg/ml). Th1 and Th2 cytokine expression profiles were not altered by the addition of ST2-Fc (data not shown). In summary, ST2-Fc at concentrations of 100 pg/ml - 1 μg/ml did not alter T helper effector functions in the applied models.

Coyle *et al.* (1999) described a shift from Th2 to Th1 cytokines in antigen restimulated Th cells, when the first antigenic stimulation was administered in the presence of 100 μg/ml ST2-Fc. Th2, but not Th1 cells were susceptible to ST2-Fc effects. To examine the effects of these doses of ST2-Fc on antigen-stimulated T helper cells, CD4(+) T spleen cells derived from DO11.10 mice and purified by negative selection as described in section 2.8, were stimulated with 300 nM OVA peptide antigen in the presence of irradiated APC in a ratio of 1:10 (T cells : APC). ST2-Fc or human IgG control (100 μg/ml) was added at the time of stimulation and the culture incubated for 3 days (Fig.4.2 A). Unexpectedly, the production of both Th1 and Th2 cytokines was significantly suppressed by the ST2-Fc protein. IL-2 expression was almost completely abolished and IFN-γ production was decreased by 70%; IL-5 expression levels were low, but a 75% decrease was observed. IL-6 expression was decreased by 85% in comparison to hIgG-treated cells and IL-4 expression was below the limit of detection of the ELISA (Fig. 4.3). ST2 did not significantly decrease cell proliferation, suggesting that toxicity due to the high concentration of ST2-Fc was not the reason for the observed suppression of cytokine release. Only ST2-Fc concentrations above 10 μg/ml proved to be effective in decreasing Th1 and Th2 cytokine production by antigen-stimulated Th cells (Fig. 4.4).

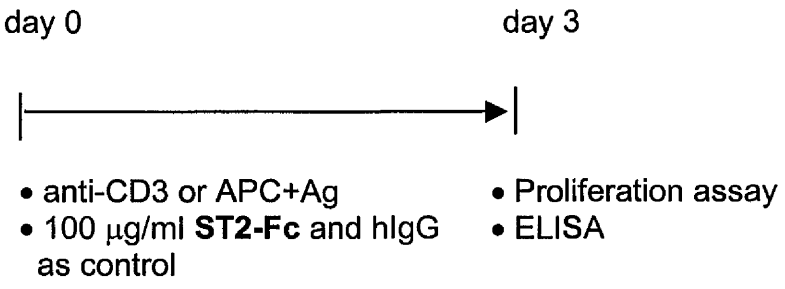
Fig. 4.2 Experimental protocols to investigate the effect of ST2-Fc on T helper cells

A) CD4(+) T cells, purified from DO11.10 mice as stated in section 2.4 and 2.8, were stimulated with either irradiated APC (1:10) and 300 nM OVA antigen or 4 μg/ml plate-bound anti-CD3. ST2-Fc (100 μg/ml) or human IgG (hIgG) were added for 3 days. Afterwards cell viability and cytokine expression were assessed by ^{3}(H)-Thymidine incorporation and ELISA.

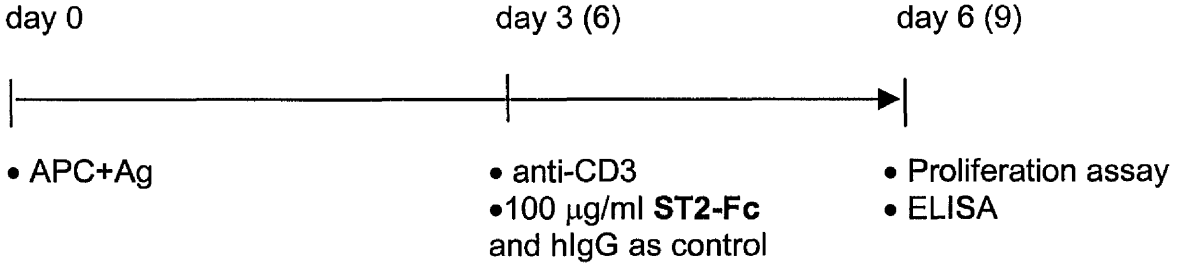
B) Purified CD4(+) T cells, purified from DO11.10 mice, were stimulated with irradiated APC (1:10) and 300 nM OVA antigen for 3 and 6 days before the cells were restimulated with 4 μg/ml anti-CD3 and the addition of 100 μg/ml ST2-Fc or hIgG. Three days later, cell viability and cytokine expression were assessed.

C) Purified CD4(+) T cells, purified from DO11.10 mice, were stimulated with 4 μg/ml anti-CD3 for 3 days. The cells were then incubated in 10 ng/ml IL-2-containing medium for 48h, before they were restimulated with 4 μg/ml anti-CD3 and 100 μg/ml ST2-Fc or hIgG. Three days later, cell viability and cytokine expression were assessed.

A)


day 0
day 3
• anti-CD3 or APC+Ag
• 100 μg/ml **ST2-Fc** and hIgG as control
• Proliferation assay
• ELISA

B)


day 0
day 3 (6)
day 6 (9)
• APC+Ag
• anti-CD3
•100 μg/ml **ST2-Fc** and hIgG as control
• Proliferation assay
• ELISA

C)

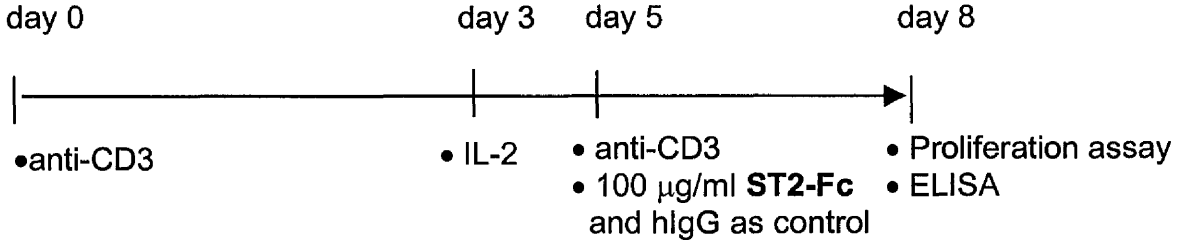
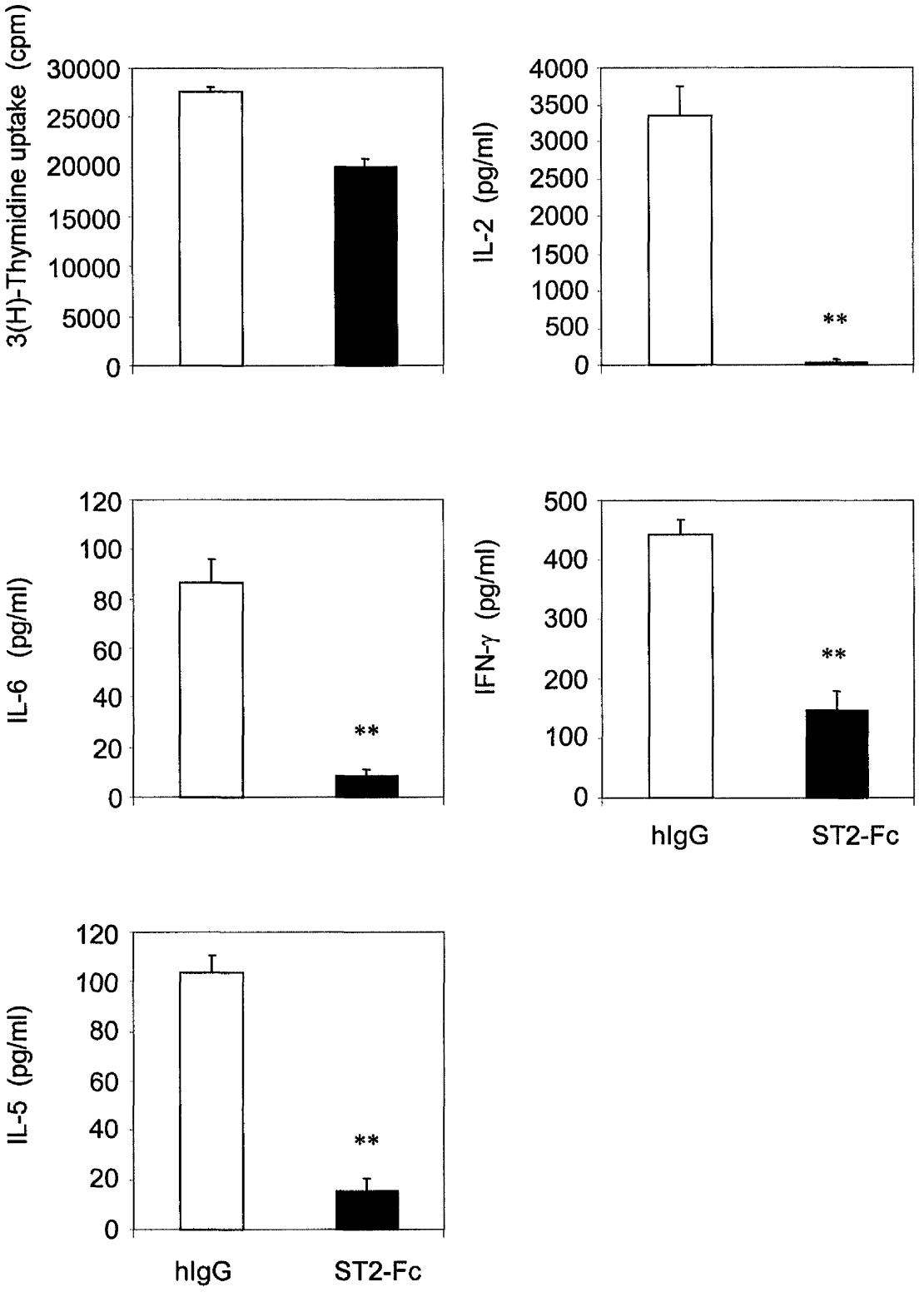

day 0
day 3
day 5
day 8
•anti-CD3
• IL-2
• anti-CD3
• 100 μg/ml **ST2-Fc** and hIgG as control
• Proliferation assay
• ELISA

Fig 4.3 The effect of ST2-Fc on antigen-stimulated Th cells

CD4(+) T cells were obtained from DO11.10 mice (section 2.4 and 2.8) and $1x10^6$ cells/well were stimulated with APC ($5x10^6$) and 300 nM OVA antigen for 3 days together with 100 µg/ml ST2-Fc and hIgG as control. Cytokine expression was assessed by ELISA. The detection level of the IL-5 was 20 pg/ml, the detection level of IL-6 was 10 pg/ml. IL-4 levels were below detection. Proliferation assays were performed to estimate cell viability. hIgG-treated samples were compared with ST2-Fc-treated samples: * $p<0.05$, ** $p<0.005$. These results (n=3, ELISA triplicates) are shown as the mean ± 1SD and are representative of 6 experiments.


3(H)-Thymidine uptake (cpm)
30000
25000
20000
15000
10000
5000
0
IL-2 (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
**
IL-6 (pg/ml)
120
100
80
60
40
20
0
**
IFN-γ (pg/ml)
500
400
300
200
100
0
**
hIgG
ST2-Fc
IL-5 (pg/ml)
120
100
80
60
40
20
0
**
hIgG
ST2-Fc

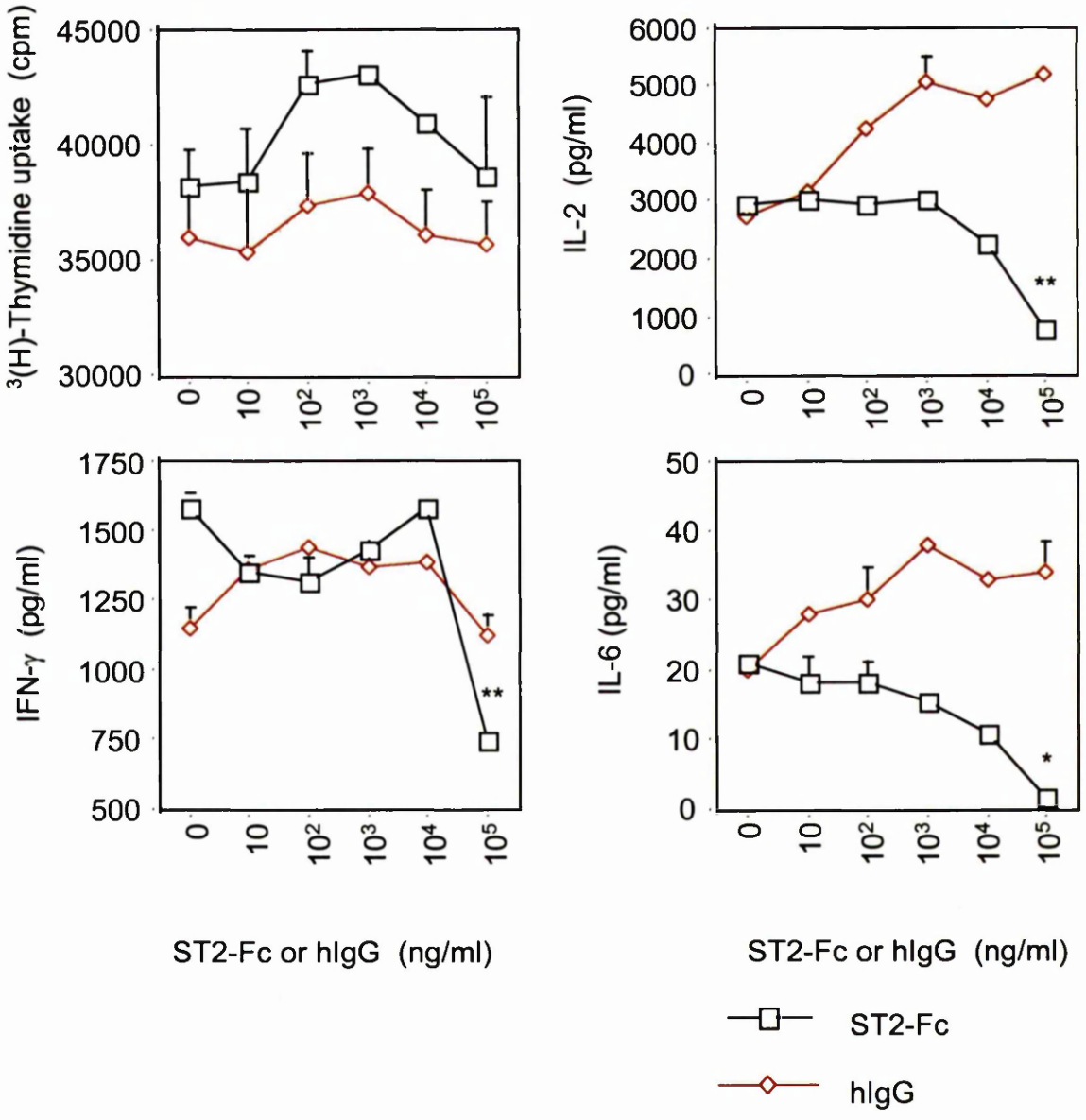


Fig 4.4 Dose dependent effect of ST2-Fc on antigen-stimulated Th cells polarised under neutral conditions

Purified CD4(+) T cells ($1x10^6$/well) were cultured for 3 days with APC ($5x10^6$) and 300 nM OVA antigen and ST2-Fc or hIgG in graded concentrations. Cytokines and proliferation profiles were assessed by ELISA and proliferation assay respectively. The detection level of the IL-6 was 5 pg/ml. hIgG-treated samples were compared with ST2-Fc-treated samples:

* $p<0.05$, ** $p<0.005$. Results (n=3) are shown as the mean ± 1 SD and are representative of 2 experiments.

4.1.2 The effect of ST2-Fc on naïve CD4(+) T cells primed with plate-bound anti-CD3

Next, the question of whether ST2-Fc-mediated suppression requires the presence of antigen presenting cells (APC) or whether it acts directly on the CD4(+) T cells was addressed. ST2-Fc might interact directly with the APC and thereby alter costimulatory signals for CD4(+) T cells or induce the expression of soluble factors, which mediate the cytokine suppression on CD4(+) T cells. CD4(+) T cells were stimulated with 4 μg/ml plate-bound anti-CD3 for 3 days and incubated from the time of stimulation with 100 μg/ml ST2-Fc protein or hIgG (Fig. 4.2 A and Fig. 4.5). ST2-Fc significantly decreased Th1 and Th2 cytokine release. IL-2, IL-4 and IL-5 production was almost completely suppressed by ST2-Fc treatment; IFN-γ expression was decreased to 45% of the control level, while cell viability, as measured by ^{3}(H)-Thymidine incorporation, was not affected. ST2-Fc concentrations lower than 10 μg/ml had no effect (Fig. 4.6). Thus, ST2-Fc has a suppressive effect on Th1 and Th2 cytokine release by T cell receptor stimulated CD4(+) T cells, irrespective of whether APC are present.

4.1.3 The effect of ST2-Fc on primed CD4(+) T cells during an anti-CD3-driven secondary response

Naïve and primed CD4(+) T cells might have a very different susceptibility to the suppressive effect of ST2-Fc. For example, another molecule with a suppressive effect on Th cells, TGF-β1, has been found to inhibit the proliferation and cytokine

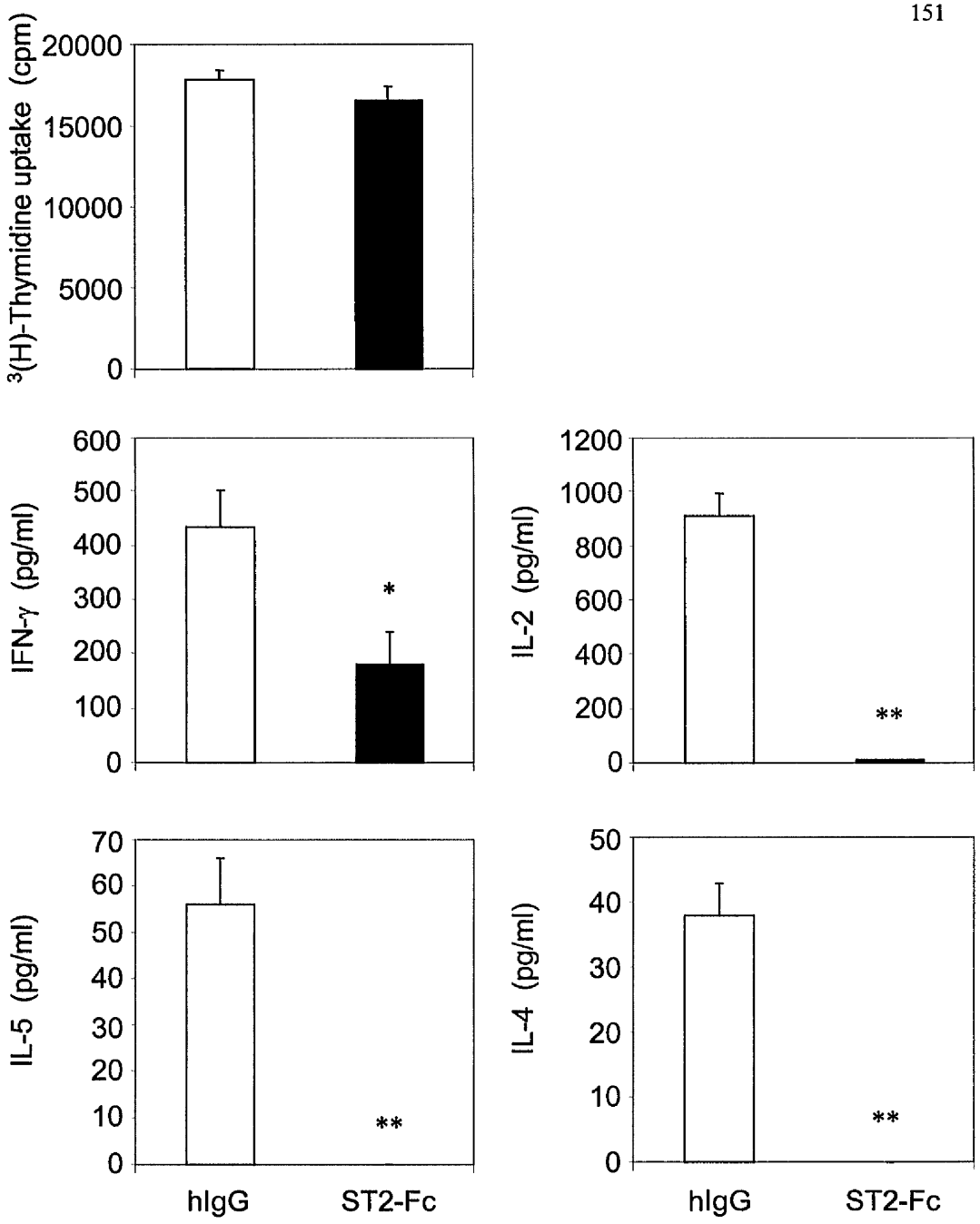


Fig. 4.5 The effect of ST2-Fc on anti-CD3-stimulated CD4(+) T cells

CD4(+) T cells (1x10^6/well), purified from DO11.10 mouse spleen cells by negative selection, were stimulated with 4 μg/ml anti-CD3 in the presence of 100 μg/ml ST2-Fc or hIgG for 3 days. Proliferation was assessed by ^{3}(H)-Thymidine incorporation and cytokine production was assessed by sandwich ELISA. hIgG-treated samples were compared with ST2-Fc-treated samples: * $p<0.05$, ** $p<0.005$. Results (n=3) are shown as mean ± 1 SD and representative of 4 experiments.

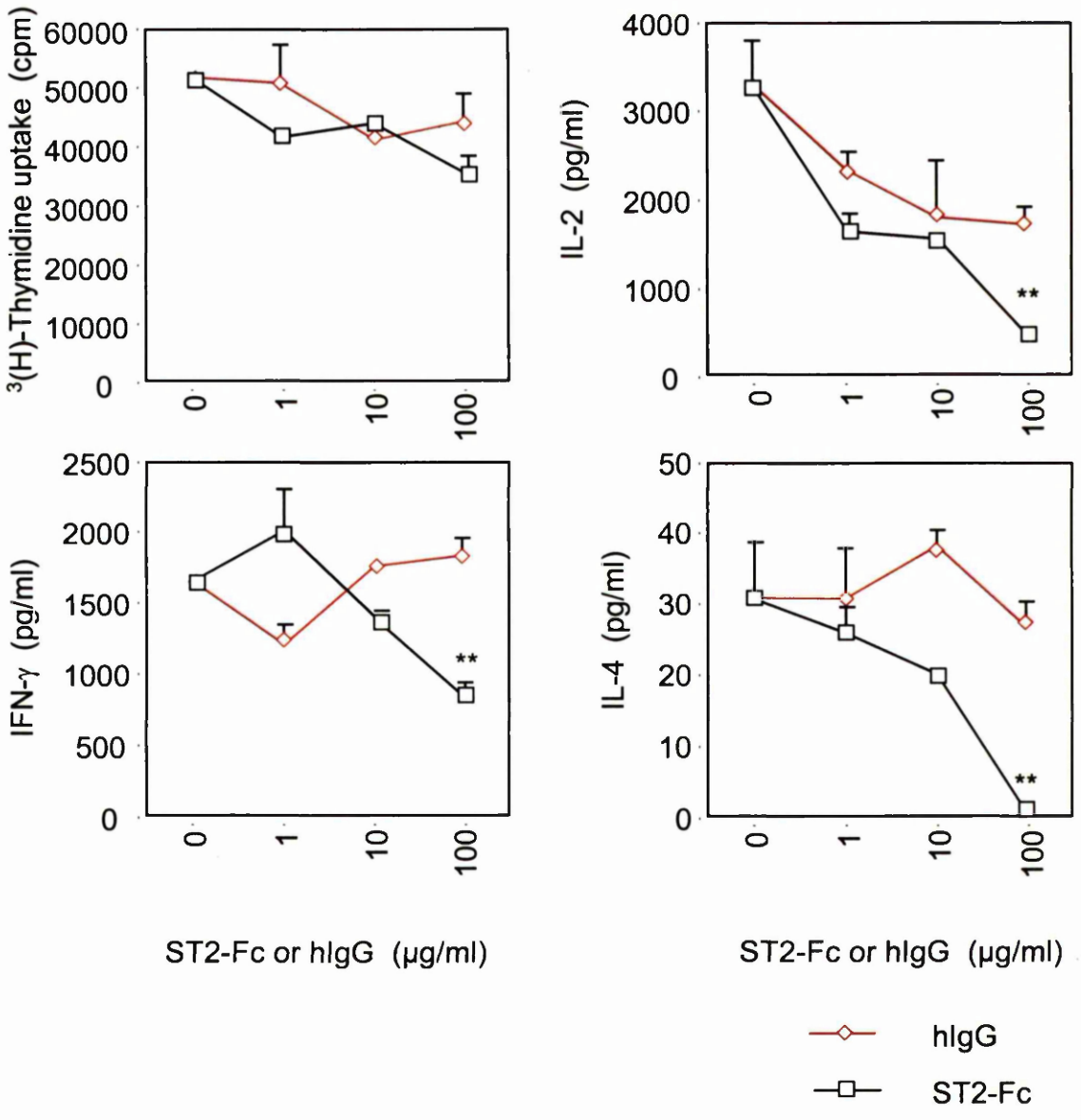


Fig. 4.6 Dose-dependent effect of ST2-Fc on anti-CD3-stimulated Th cells

Purified CD4(+) T cells (1x10^6/well) were stimulated with 4 μg/ml anti-CD3 and treated for 3 days with graded concentrations of ST2-Fc or hIgG. Cell proliferation and cytokine expression were determined by ^{3}(H)-Thymidine incorporation and ELISA. IL-5 levels were below detection. hIgG- treated samples were compared with ST2-Fc-treated samples: * $p<0.05$, ** $p<0.005$. Results (n=3) are shown as mean ± 1 SD.

expression of antigen-stimulated naïve Th cells, but not of antigen-stimulated memory Th2 cells (Ludviksson *et al.*, 2000).

To examine whether primed CD4(+) T cells are susceptible to ST2-Fc, CD4(+) T cells were derived from DO11.10 mouse spleen and purified by negative selection. They were primed with 300 nM OVA antigen in the presence of irradiated APC (1:10 ratio) for 3 or 6 days, and then restimulated with plate-bound anti-CD3 (4 μg/ml) for a further 3 days in the presence of 100 μg/ml ST2-Fc or hIgG (Fig. 4.2 B). ST2-Fc had no effect on either the proliferation or Th1 and Th2 cytokine production of restimulated T helper cells, indicating that restimulated T helper cells are resistant to ST2-Fc (Fig. 4.7 and 4.8). To assess whether the strong response of primed Th cells to the secondary anti-CD3 stimulation could have masked the suppressive effect of ST2-Fc, CD4(+) T cells were stimulated for 3 days with antigen in the presence of APC and subsequently incubated with 100 μg/ml ST2-Fc or hIgG for a further 3 days. Cell proliferation levels were low but similar between ST2-Fc- and hIgG-treated cells. Th1 and Th2 type cytokines could not be detected (data not shown).

4.1.4 The effect of ST2-Fc on CD4(+) T cells, twice stimulated with plate-bound anti-CD3

To investigate whether the engagement of the T cell receptor was responsible for this resistance to ST2-Fc, or whether the interaction with the APC or soluble factors expressed by APC was also essential, CD4(+) T cells were stimulated with plate-bound anti-CD3 (4 μg/ml) for 3 days, incubated with 10 ng/ml IL-2 for 48 h

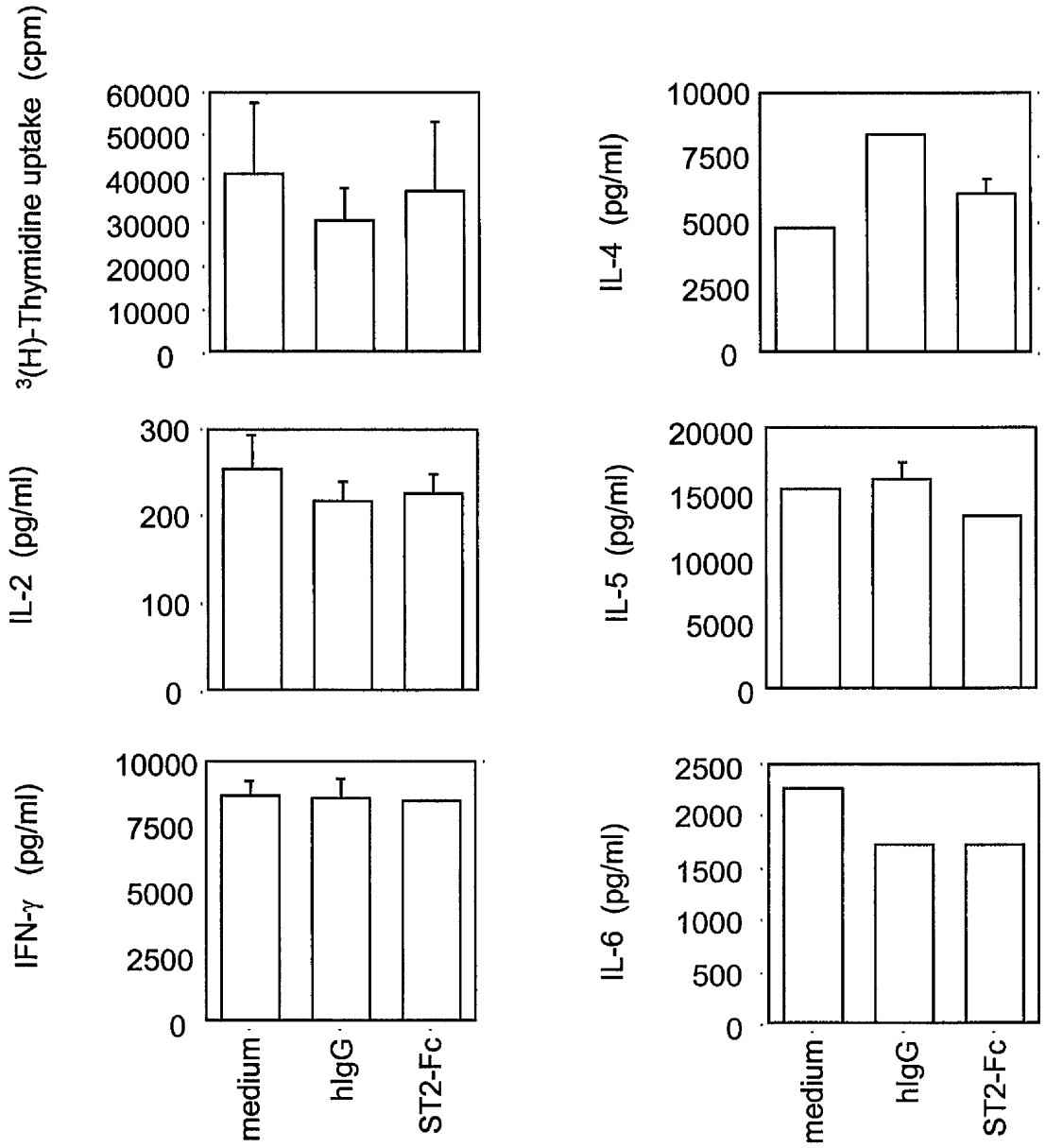


Fig. 4.7 The effect of ST2-Fc on restimulated Th cells (1)

Purified CD4(+) T cells ($1x10^6$/well) were stimulated with irradiated APC ($5x10^6$) and 300 nM OVA antigen for 3 days before being restimulated with 4 μg/ml anti-CD3 for a further 3 days. At the time of the restimulation with anti-CD3 100 μg/ml ST2-Fc or hIgG were added. Then proliferation and cytokine pattern were assessed. Results (n=3) are shown as mean ± 1 SD and representative of 3 experiments.

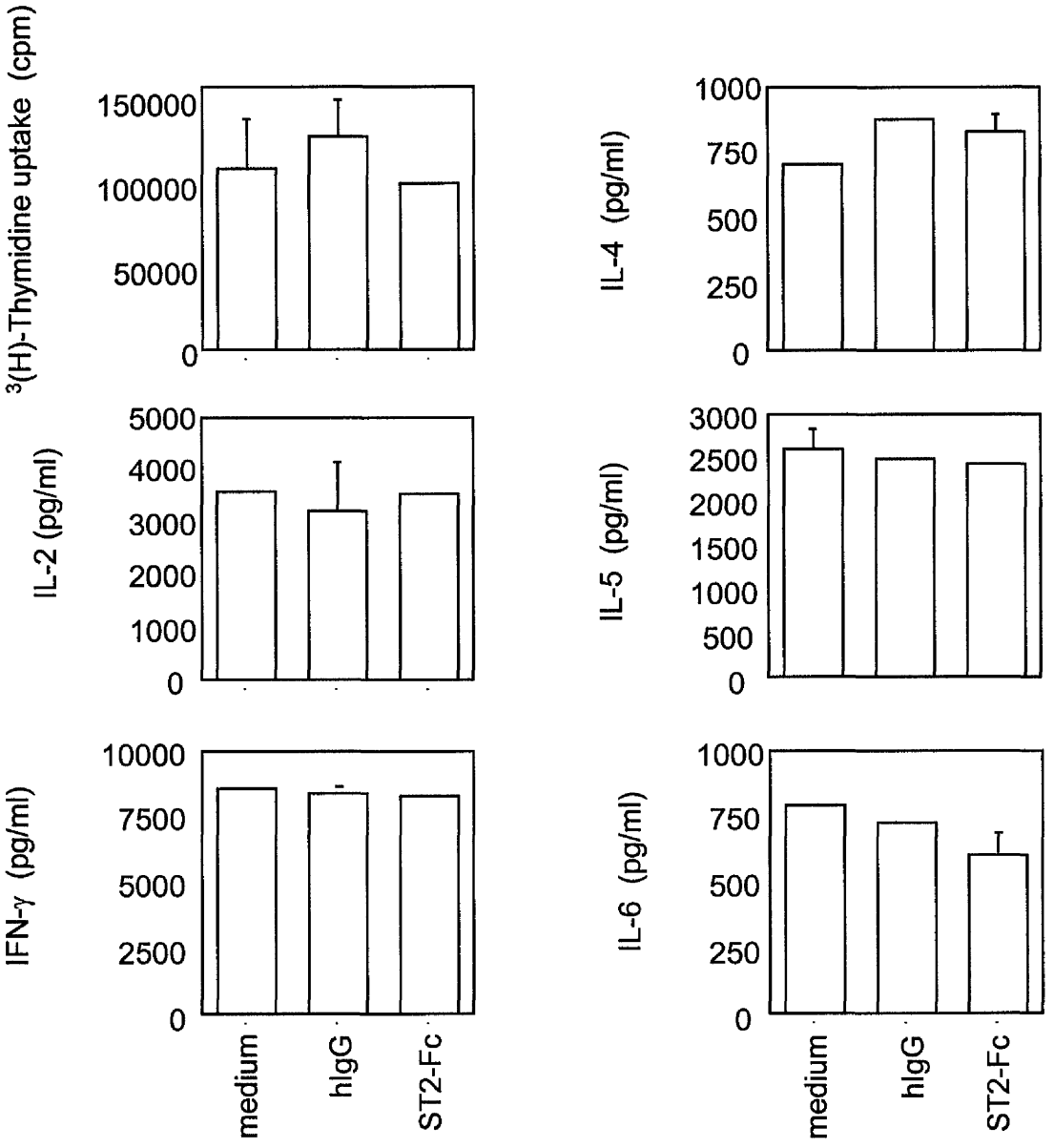


Fig. 4.8 Effect of ST2-Fc on restimulated Th cells (2)

Purified CD4(+) T cells (1x10^6/well) were stimulated with APC (5x10^6) and 300 nM OVA antigen for 6 days before being restimulated with 4 μg/ml anti-CD3 for 3 days. At the time of restimulation with anti-CD3 (day 6), 100 μg/ml ST2-Fc or hIgG were added. The cytokines released were measured by ELISA and the proliferation determined by ^{3}(H)-Thymidine uptake. Results (n=3) are shown as mean ± 1 SD and represent 2 experiments.

and then restimulated with anti-CD3 in the presence of 100 μg/ml ST2-Fc or hIgG for a further 3 days (Fig. 4.2 C; Fig. 4.9). The fusion protein did not suppress cytokine production or proliferation of the Th cells, confirming that triggering of the T cell receptor sends a sufficient signal to the T cell to undergo changes that prevent ST2-Fc action.

4.1.5 The effect of ST2-Fc on cloned Th1 and Th2 cells

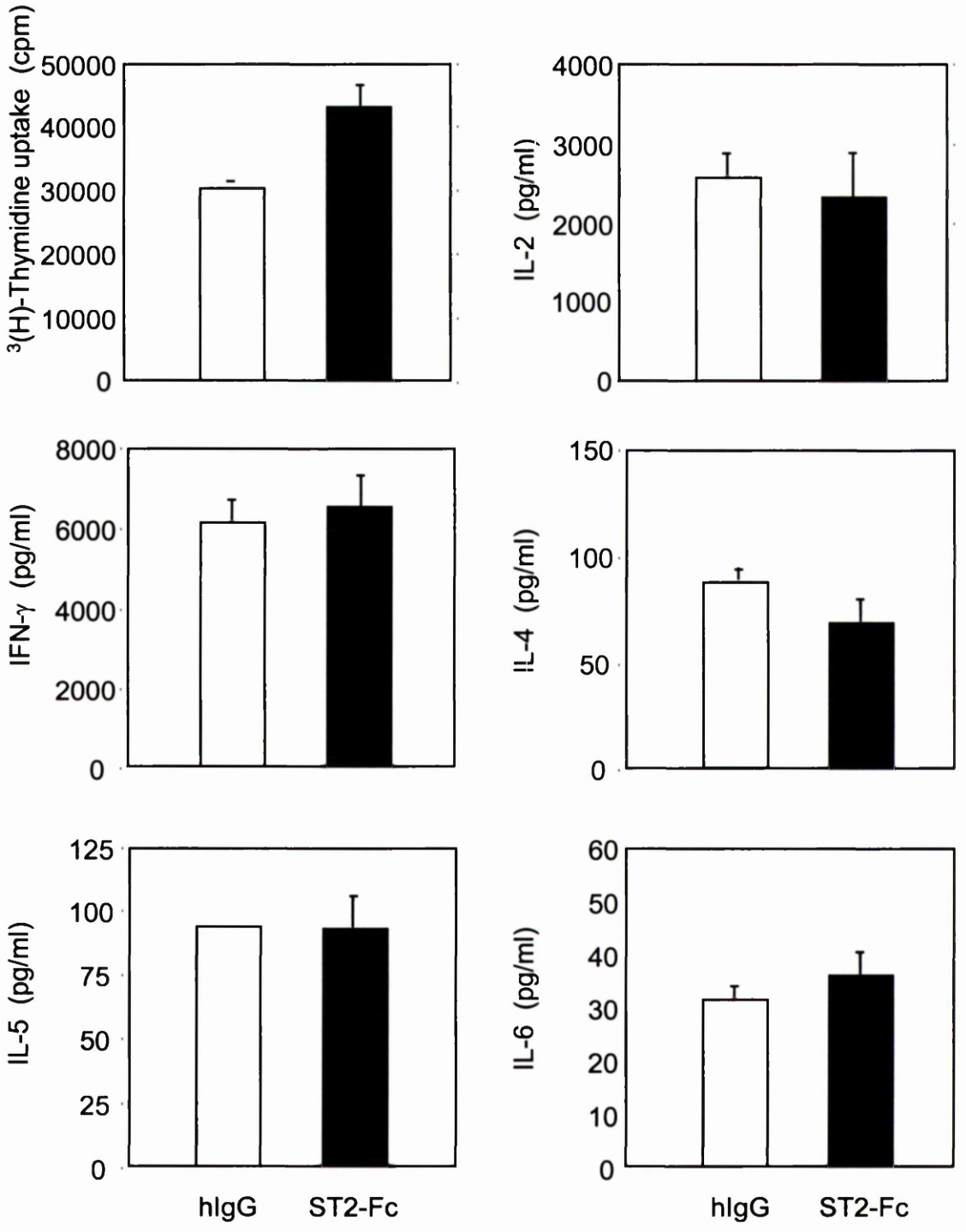
To further confirm that primed T helper cells are resistant to ST2-Fc, the effect of the fusion protein on the responses of fully committed Th1 and Th2 clones was examined. D10 (Th2) cells produced IL-4 and IL-5 but not IFN-γ when stimulated with antigen in the presence of APC, whilst Dorris (Th1) cells produced IFN-γ but not IL-4 and IL-5 (Fig.4.10). The presence of ST2-Fc did not affect cytokine production or proliferation of antigen-stimulated Th clones (Fig. 4.11). Interestingly, although ST2L is expressed on Th2 cells but not on Th1 cells (see Fig. 5.1), Th2 clones did not exhibit differential susceptibility to ST2-Fc.

4.1.6 Analysis of ST2-Fc binding to CD4(+) T cells and cloned T helper cells

The results presented above demonstrate that ST2-Fc has a suppressive effect on freshly stimulated CD4(+) T cells, but not restimulated cells. To investigate whether this phenomenon is due to the differential expression of a ST2 binding protein on the Th cells, ST2-Fc binding to naïve, freshly stimulated and restimulated CD4(+) T cells was analysed by flow cytometry. CD4(+) T cells were separated by negative selection and cultured with OVA antigen or anti-CD3 (as

Fig 4.9 The effect of ST2-Fc on anti-CD3-restimulated Th cells

Purified CD4(+) T cells ($1x10^6$/well) were stimulated with 4 μg/ml anti-CD3 for 3 days, then incubated for 2 days with 10 ng/ml IL-2 and afterwards restimulated with anti-CD3 for a further 3 days. At the time of the restimulation with anti-CD3, 100 μg/ml ST2-Fc or hIgG were added. The proliferation and cytokine pattern were determined by ^{3}(H)-thymidine uptake and ELISA respectively. Results (n=3) are shown as mean ± 1 SD.


^{3}(H)-Thymidine uptake (cpm)
50000
40000
30000
20000
10000
0
IL-2 (pg/ml)
4000
3000
2000
1000
0
IFN-γ (pg/ml)
8000
6000
4000
2000
0
IL-4 (pg/ml)
150
100
50
0
IL-5 (pg/ml)
125
100
75
50
25
0
IL-6 (pg/ml)
60
50
40
30
20
10
0
hIgG
ST2-Fc
hIgG
ST2-Fc

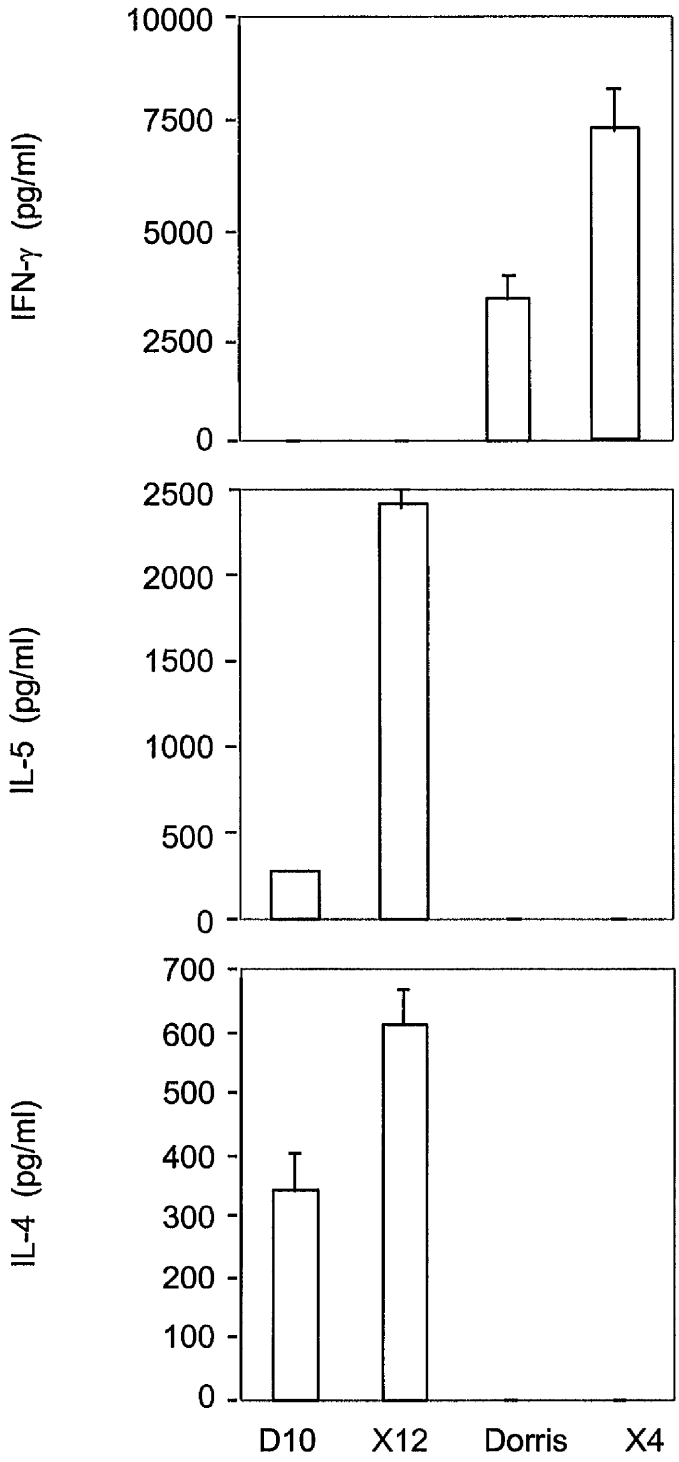


Fig. 4.10 Typical cytokine profile of T helper clones

Th1 clones (Dorris, X4) and Th2 clones (D10, X12) were stimulated with their respective antigen in the presence of APC (1:10) for 3 days as described in section 2.7. The supernatants were analysed for IFN-γ, IL-4 and IL-5 by ELISA. Results (n=3) are shown as mean ± 1 SD.

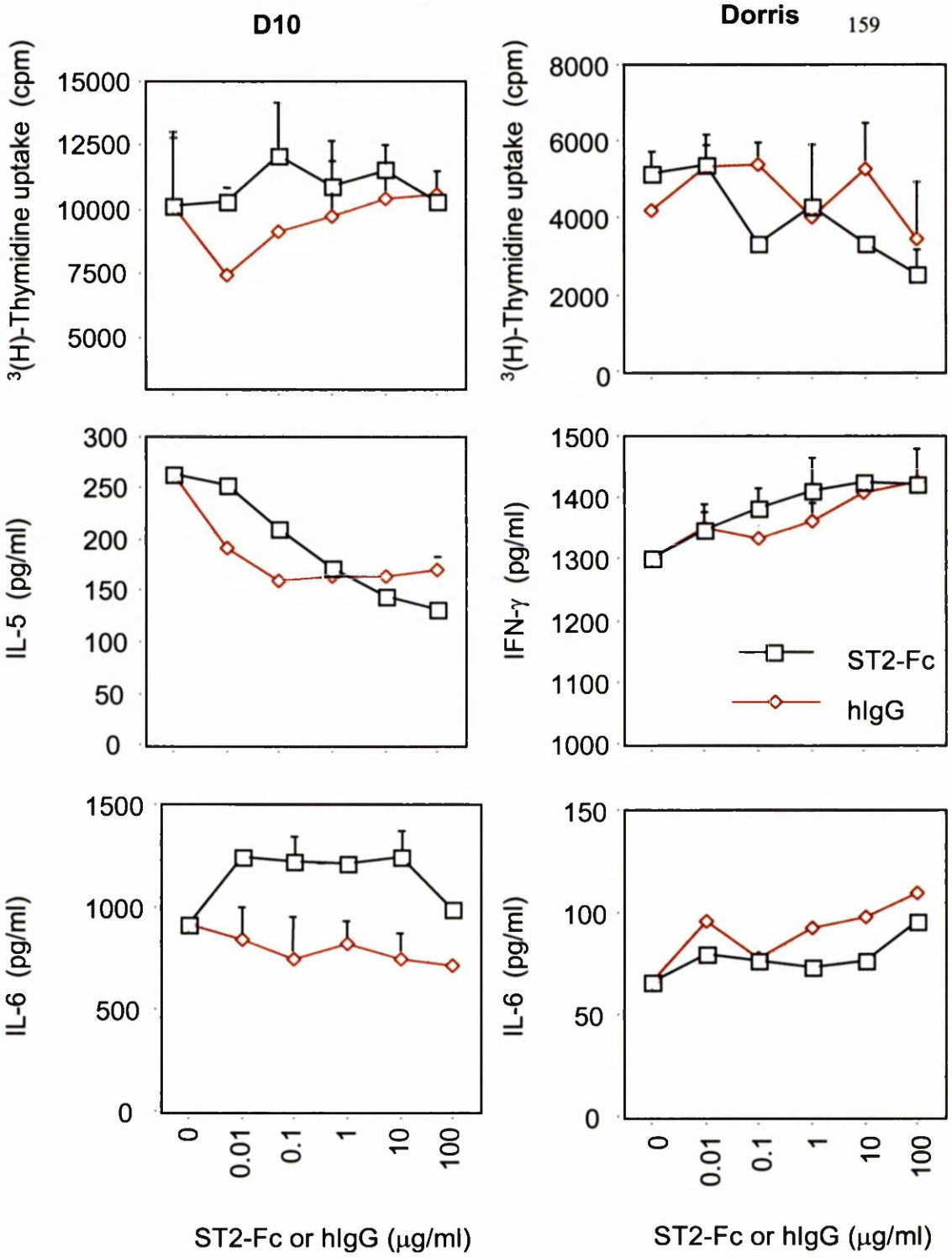


Fig. 4.11 Dose-dependent effect of ST2-Fc on Th cell clones

D10 (Th2) and Dorris (Th1) T cell clones (1x10^6/well) were stimulated with APC (1:10 ratio) and antigen for 3 days, ST2-Fc or hIgG at indicated concentrations were added. Proliferation and cytokine release was determined. Results (n=3) are shown as mean ± 1 SD.

described in Fig. 4.12) prior to incubation for 30 min with ST2-Fc fusion protein or hIgG (100 μg/ml) at 4°C to allow binding but prevent receptor-mediated endocytosis (Huang *et al.*, 1999; Ikushima *et al.*, 2000). ST2-Fc was detected by a FITC-labelled anti-human IgG1 antibody. In two separate experiments, 10-15% of naïve CD4(+) T cells stained positive for anti-hIgG1, suggesting binding of ST2-Fc to these cells (Fig. 4.12 A). Freshly stimulated CD4(+) T cells exhibited a similar percentage of ST2-Fc binding (Fig. 4.12 B), while restimulated CD4(+) T cells showed no binding of ST2-Fc (Fig. 4.12 C and D). Th1 and Th2 cell clones did not exhibit binding of the ST2-Fc fusion protein (Fig. 4.13).

Next, naïve CD4(+) T cells were double stained for ST2-Fc binding and CD4 to exclude the possibility that the fusion protein bound to the 5-10% CD4(-) cells not excluded by negative selection. As demonstrated in Fig.4.14, ST2-Fc bound to approximately 9% of the CD4(+) T cell population and no ST2-Fc positive staining could be detected in the 9.5 % CD4(-) cells. Hence, there was a direct correlation between cell populations that bound ST2-Fc and cell populations in which ST2-Fc exerted a suppressive effect on cytokine production and proliferation.

Discussion

To investigate the effects of ST2 on CD4(+) T cells, a fusion protein expressed in CHO cells and consisting of the extracellular part of ST2L and the Fc part of IgG1, κ, was used. Its biological function had been tested on LPS-stimulated macrophages and 50 - 100 μg/ml ST2-Fc proved to suppress pro-inflammatory

Fig. 4.12 Binding studies of ST2-Fc versus hIgG on purifled CD4(+) T cells

Purified CD4(+) T cells, which were derived from DO11.10 mice, were treated as described below and incubated with 100 μg/ml ST2-Fc or hIgG and stained with monoclonal FITC-anti-human IgG1 antibody (1:100 dilution) as described in section 2.35. A gate was set to exclude dead cells.

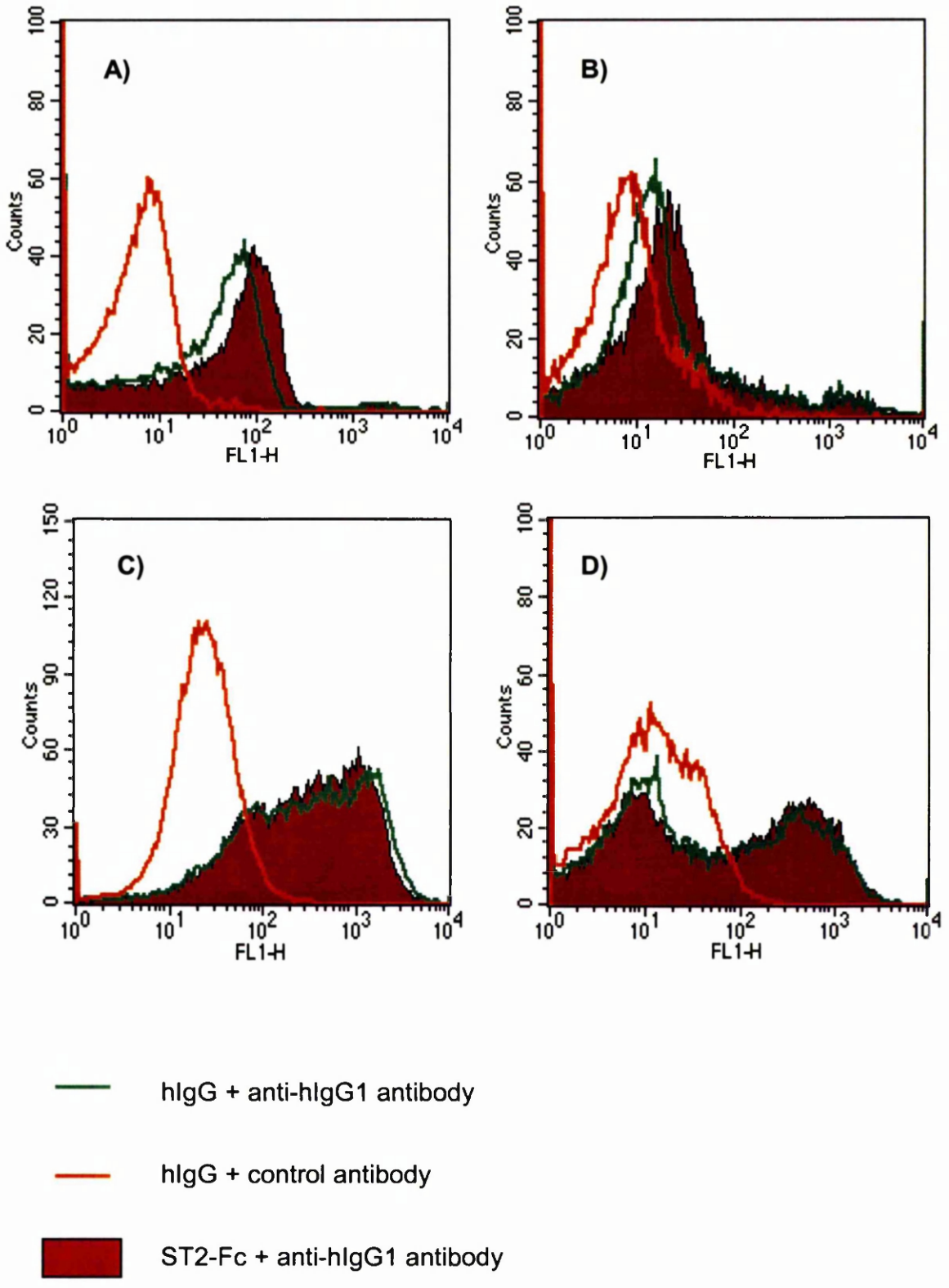
A) naïve CD4(+)T cells

B) CD4(+) T cells stimulated for 24 h with 4 μg/ml anti-CD3

C) CD4(+) T cells stimulated for 3 days with 4 μg/ml anti-CD3, then incubated for 48 h with 10 ng/ml IL-2 and then restimulated with anti-CD3 for 36 h

D) CD4(+) T cells stimulated for 6 days with (1:10) APC and 300 nM OVA Ag and then for 2 days with anti-CD3

These results are representative of 2 experiments.


A)
B)
C)
D)
Counts
FL1-H
hIgG + anti-hIgG1 antibody
hIgG + control antibody
ST2-Fc + anti-hIgG1 antibody

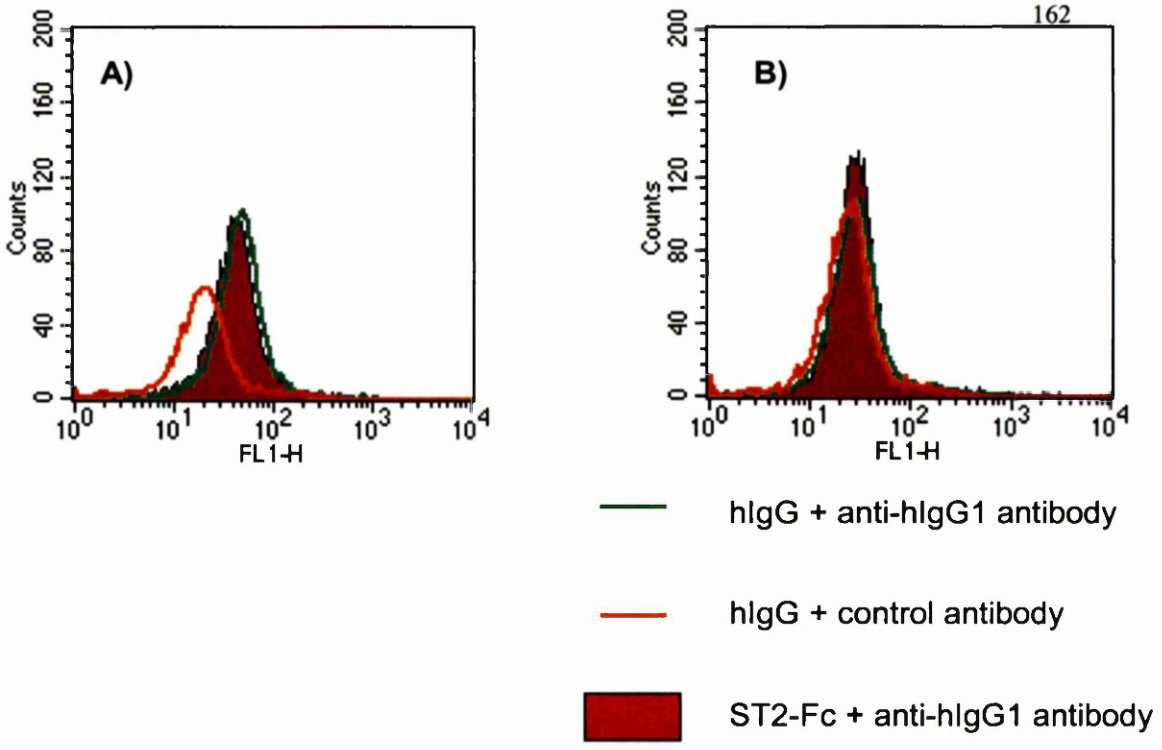
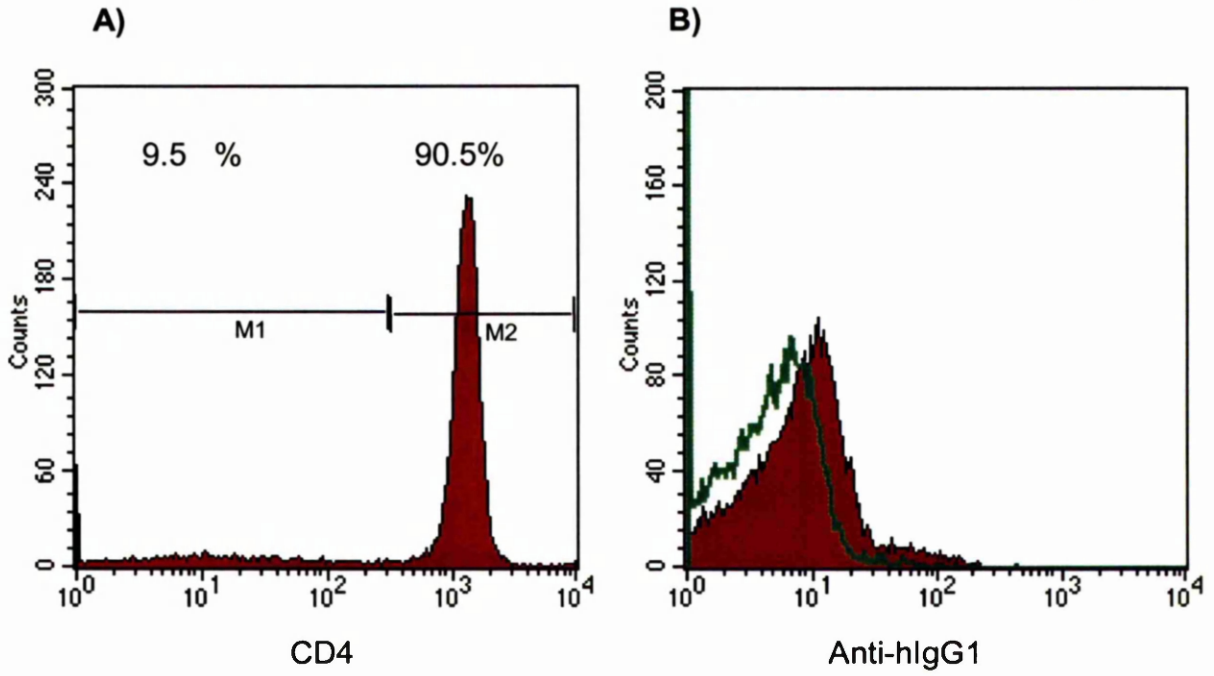


Fig. 4.13 ST2-Fc binding studies on Th cell clones

Th2 cell clone X12 **(A)** and Th1 cell clone X4 **(B)** were tested for ST2-Fc binding by incubating them with 100 µg/ml ST2-Fc or hIgG and staining with monoclonal anti-human IgG1 antibody (section 2.35). A gate was set to exclude dead cells These results are representative of 2 experiments.

	Treatment	D/s(n)	D	p
Statistics of Fig. 4.12, and 4.13	**Naïve CD4(+) T cells (A)**	15.42	0.29	<0.001
	CD4(+) T cells (B)	13.72	0.22	<0.001
	CD4(+) T cells (C)	4.2	0.04	<0.001
	CD4(+) T cells (D)	1.56	0.03	<0.001
	X4	6.32	0.08	<0.001
	X12	6.68	0.09	<0.001



Legend for Fig.B

hIgG + anti-hIgG1 antibody

ST2-Fc + anti-hIgG1 antibody

Treatment	positive for hIgG1
hIgG	0.14%
ST2-Fc	9.56%

Fig. 4.14 ST2-Fc binding to CD4(+) T cells

Naïve CD4(+) T cells from lymph nodes of D0.11.10 mice were purified by negative selection. They were incubated with 100 μg/ml ST2-Fc or hIgG for 30 min at 4°C and then washed before staining with monoclonal anti-human IgG1 antibody and anti-CD4 antibody as described in section 2.35. The cells were then analysed by flow cytometry. **(A)** shows CD4 expression among live cells and **(B)** shows CD4(+) cells (M2), which stained positive for anti-hIgG1.

cytokines such as IL-6 and IL-12. Kuroiwa *et al.* (2000) reported a serum concentration of 1.2 ng/ml ST2 in normal human blood and an enhanced ST2 concentration of up to 5 ng/ml in sera from patients with bronchial asthma. These results suggest a function for ST2 in Th2-mediated diseases, but do not indicate a physiologically relevant ST2 concentration, because local concentrations might be much higher than the measured serum levels. Suppressive ST2-Fc doses exceeded the serum ST2 levels dramatically, suggesting the need for a high local ST2 concentration or a differing function of ST2-Fc function from *in vivo* occurring ST2.

The results presented here showed that ST2-Fc suppressed both Th1 and Th2 cytokine production by naïve CD4(+) T cells in the absence of APC, e.g. T cell stimulation by anti-CD3, and such suppression was equal to, or even greater than, that observed in the presence of APC. This implies that ST2-Fc is not primarily acting via effects on APC, but instead can act directly on CD4(+) T cells. After restimulation through the TCR, CD4(+) T cells lost the ability to respond to ST2-Fc, which further confirms that the observed suppression of cytokines on freshly stimulated CD4(+) T cells was not due to a toxic effect of the high concentration of ST2-Fc on the cells. In addition, Coyle *et al.* (1999) reported that CD4(+) T cells treated with ST2-Fc during priming and then restimulated with antigen still exhibited a suppression of Th2 cytokines, while Th1 cytokines were upregulated. These findings suggest that the suppressive effects of ST2-Fc are maturation-and differentiation-dependent.

TGF-β1 has been shown to have similar suppressive effects, dependent on the maturation and differentiation state of the CD4(+) T cells (Ludviksson *et al.*, 2000). It has a profound inhibitory effect on the cytokine production and proliferation of antigen-stimulated, naive CD4(+) T cells undergoing differentiation under neutral (antigen stimulation only), Th1 and Th2 priming conditions. ST2-Fc also inhibited the cytokine production of antigen-stimulated, naive CD4(+) T cells undergoing differentiation under neutral, Th1 and Th2 priming conditions (Th1 and Th2 cell data not shown). Furthermore, memory Th1 cells remain susceptible to TGF-β1 suppression, while memory Th2 cells are not subject to ST2-Fc suppression (Ludviksson *et al.*, 2000). Only memory Th2 cells remain susceptible to ST2-Fc (Coyle et al., 1999). Both TGF-β and ST2 are upregulated upon UV irradiation (Kumar *et al.*, 1997, Dissanayake and Mason, 1998) and might limit immune responses at an initial differentiation stage, and later control the balance between Th1 and Th2 cells.

To investigate how ST2-Fc modulated the effector function of T cells, ST2-Fc binding studies by flow cytometry were performed. The results revealed that ST2-Fc bound only to naïve and freshly stimulated CD4(+) T cells, but not to restimulated CD4(+) T cells. This is consistent with the fact that naïve but not committed CD4(+) T cells responded to ST2-Fc. This may be the result of downregulation of the putative ST2 binding protein. There are several candidate ST2 binding molecules on CD4(+) T cells. Binding of ST2 to the myeloma-derived RPMI8226 cell line (Yanagisawa *et al.*, 1997), BALB/c 3T3 cell line and human vein endothelial cells (Kumar *et al.*, 1995), macrophages, activated B cells, DCs

and naïve Th cells (Sweet *et al.*, 2001; Lambrecht *et al.,* 2000, Coyle *et al.,* unpublished data; own data) has been reported. A putative ligand called ST2 binding protein (SBP) was cloned by Gayle *et al.* (1996). However, SBP was not able to induce signal transduction by ST2L in murine fibroblasts. The expression of SBP on T helper cells was investigated by RT-PCR. Naïve CD4(+) T cells expressed the short form of SBP (670 bp), which was previously demonstrated to bind ST2-Fc. Whether TCR activation of naïve CD4(+) T cells causes downregulation or loss of SBP expression has still to be investigated. However, strong evidence from polarised Th1 cells suggest that this might be the case. Th cells polarised for 7 days towards the Th1 phenotype lost the expression of the 670 bp fragment but expressed a larger transcript of 1.2 kb. SBP, which contains only a short intracellular sequence, is unlikely to induce a signalling response on its own. Addition of other components forming a receptor complex similar to the IL-1R complex might be necessary for a response to ST2, if indeed SBP really is involved in signalling in response to ST2.

Chapter 5 Regulation of ST2 and ST2L expression by Th1 and Th2 type cytokines

This chapter addresses the question of whether the cytokine environment that determines the pathway of differentiation for the Th cell subsets also affects the ST2L expression pattern. Further, the expression of both ST2 and ST2L gene transcripts were monitored under the influence of IL-4 and IFN-γ to investigate whether ST2 and ST2L are differentially regulated in Th cells. The Th2 cytokine IL-4 upregulated ST2L expression at the promoter activity, gene transcript and protein levels. Furthermore, ST2 message was also upregulated by IL-4 but peaked earlier than ST2L mRNA. IFN-γ on the other hand, decreased the level of ST2L expression, indicating a potential mechanism for cross-regulation of Th2 cell function by Th1 cells. These results suggest differential regulation of ST2 and ST2L in CD4(+) T cells and may offer an explanation for the selective expression of ST2L on Th2 but not Th1 cells.

Introduction

ST2L is selectively expressed on Th2 cells but not naïve CD4(+) T cells or Th1 cells (Xu *et al.*, 1998; Coyle *et al.*, 1999; Meisel *et al.*, 2001). The expression pattern for its shorter splice variant ST2 on CD4(+) T cells has not been analysed. Treatment with antibodies against ST2L decreased Th2 effector functions *in vivo* (Xu *et al.*, 1998; Loehning *et al.*, 1998; Coyle *et al.*, 1999). This raised the question of whether ST2L expression is influenced by Type 2 (IL-4, IL-5, IL-10) and Type 1 cell cytokines (IFN-γ, IL-2). So far, the regulation of murine ST2L and ST2 expression in T helper cells is largely unknown apart from the observation that expression of ST2L was upregulated by serum stimulation (Xu *et al.*, 1998).

Very recently, Meisel *et al.* (2001) reported that exogenous IL-6, IL-5 and TNF-α enhanced the expression of ST2L on Th2 cells. Monitoring the expression of ST2 and ST2L in response to such stimuli could provide further information as to whether ST2 and ST2L are differentially regulated, as suggested by observations in fibroblasts and mast cells (Kumar *et al.*, 1997; Gaechter *et al.,* 1998). This may help answer the question of whether ST2 acts as an agonist and/or antagonist in T cells. Therefore, in this study the expression patterns of ST2 and ST2L in naïve CD4(+) T cells as well as polarised Th1 and Th2 cells were determined, and the effects of Th1 and Th2 type cytokines on ST2/ST2L promoter activity, gene and protein expression were examined.

Results

5.1. Alteration of the cell surface expression of ST2L by IL-4 and IFN-γ

Although ST2L is clearly an important marker and functional receptor on Th2 cells, an analysis of factors influencing ST2L/ST2 expression in this cell type has not previously been performed. To address this question initially, regulation of protein levels in response to Th1 and Th2 cytokines was analysed.

5.1.1 Surface expression of ST2L

ST2L protein expression was detected by flow cytometry using a biotinylated monoclonal anti-ST2L antibody as described in section 2.34. As shown in Fig. 5.1 and 5.2, the antibody detected ST2L on Th2 cell clones and three times polarised Th2 cells, but not on Th1 cell subsets as expected.

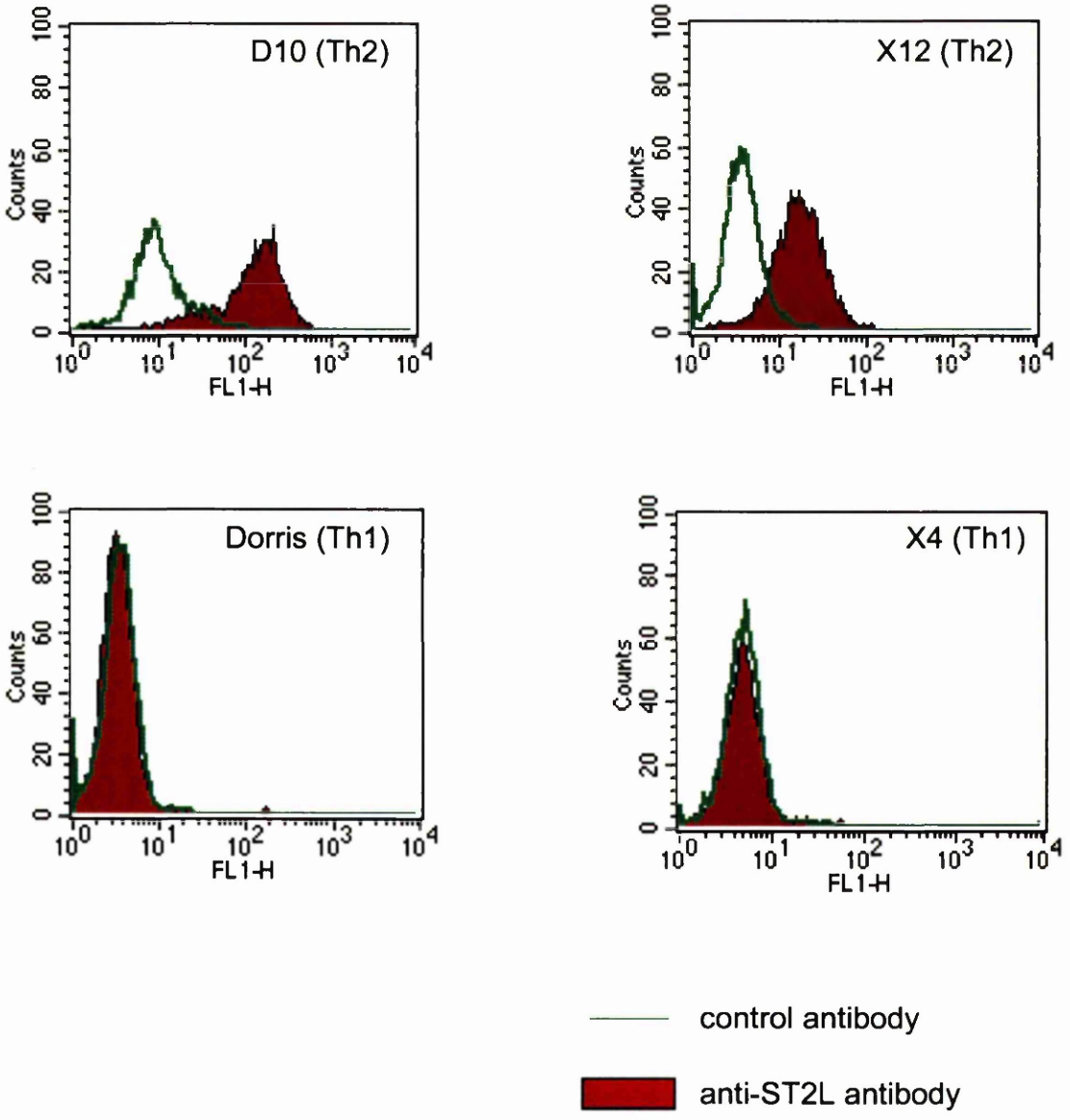


Fig. 5.1 Expression of ST2L on Th2 but not Th1 cell clones

Th1 and Th2 clones ($1x10^6$/well) were stained as described in section 2.34 with 250 ng/ml biotinylated anti-ST2L antibody or biotinylated control-antibody (rat IgG1, κ) and afterwards labelled with FITC-labelled streptavidin (1:1000) before being analysed by flow cytometry. A gate was set to exclude dead cells.

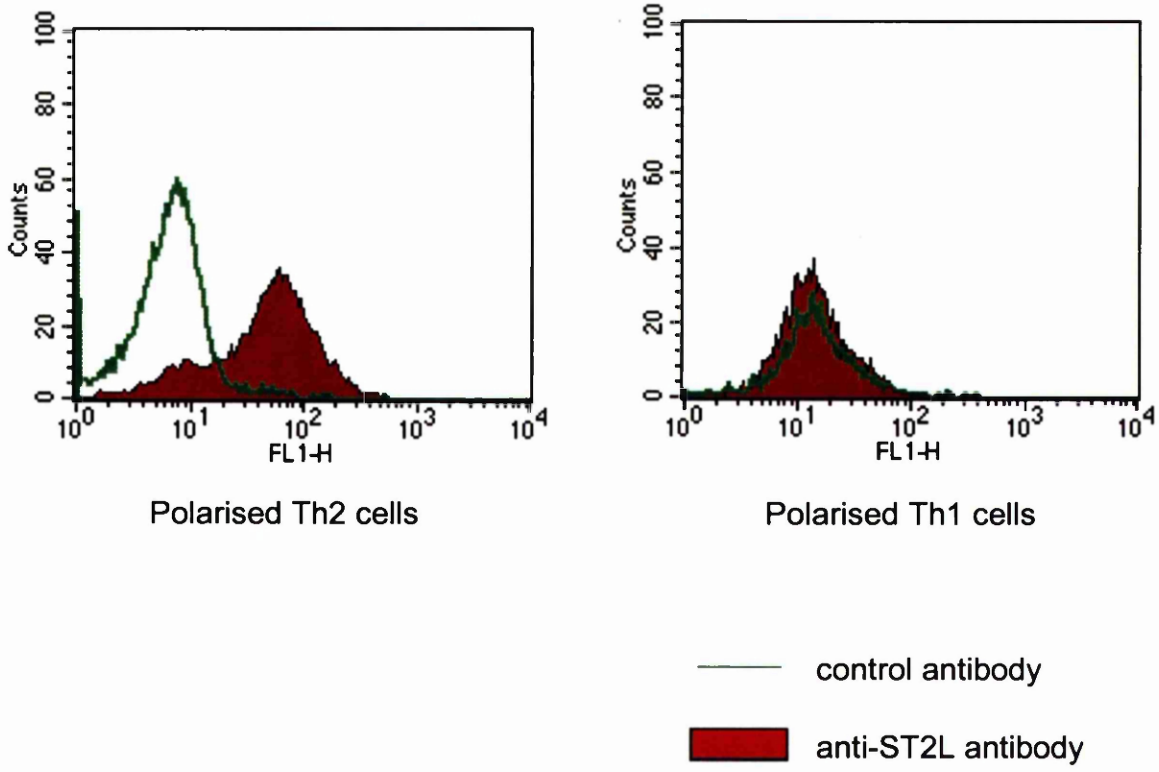


Fig. 5.2 Expression of ST2L on fully polarised Th2 but not on Th1 cells

CD4(+) T cells were purified by negative selection and polarised three times towards the Th1 or Th2 phenotype as described in section 2.8 and 2.9. Five days after the last stimulation, the Th cells were stained with 250 ng/ml biotinylated anti-ST2L antibody or the biotinylated control-antibody (rat IgG1, κ) and FITC-streptavidin (1:1000 dilution) as secondary antibody. Samples were analysed by flow cytometry. A gate was set to exclude dead cells.

5.1.2 Regulation of ST2L expression on cloned Th2 cells by IL-4 and IFN-γ

When Th2 clone cells (X12) were treated with 10 ng/ml IL-4 overnight, these cells showed a 50% increase in the proportion of ST2L positive cells compared to control cells treated with medium alone. Among the Th2 cells treated with 100 U/ml IFN-γ the proportion of ST2L positive cells was decreased by 30-50% (Fig. 5.3).

5.1.3 Regulation of ST2L expression on polarised Th2 cells by IL-4 and IFN-γ

In vitro polarised Th2 cells exhibit, like Th2 cell clones, all effector functions specific for this cell subset, but do not have the limitation of immortalised cloned cells. Hence, the results obtained in T cell clones were confirmed using long-term polarised resting Th cells. Overnight IL-4 treatment (10 ng/ml) doubled the proportion of ST2L positively stained cells in twice polarised Th2 cells. However, 100 U/ml (10 ng/ml) IFN-γ did not alter the cell surface expression of ST2L on these cells (Fig. 5.4). It is possible that Th cell clones and polarised Th cells might have different response thresholds to IFN-γ. This was not further investigated, but Carter *et al.* (2001) reported that IFN-γ (50 ng/ml) could suppress ST2L expression in antigen-stimulated, polarised Th2 cells. In addition, they reported an increase in ST2L expression on Th2 cell clones and semi-committed Th2 cells upon antigen and IL-4 (50 ng/ml) stimulation which is consistent with the results above (Carter *et al.*, in press EJI, 2001).

5.2 Regulation of ST2 and ST2L gene expression by IL-4 and IFN-γ

Since both IL-4 and IFN-γ regulated cell surface expression of ST2L, the quantitative technique of Real time PCR was employed to analyse ST2L gene

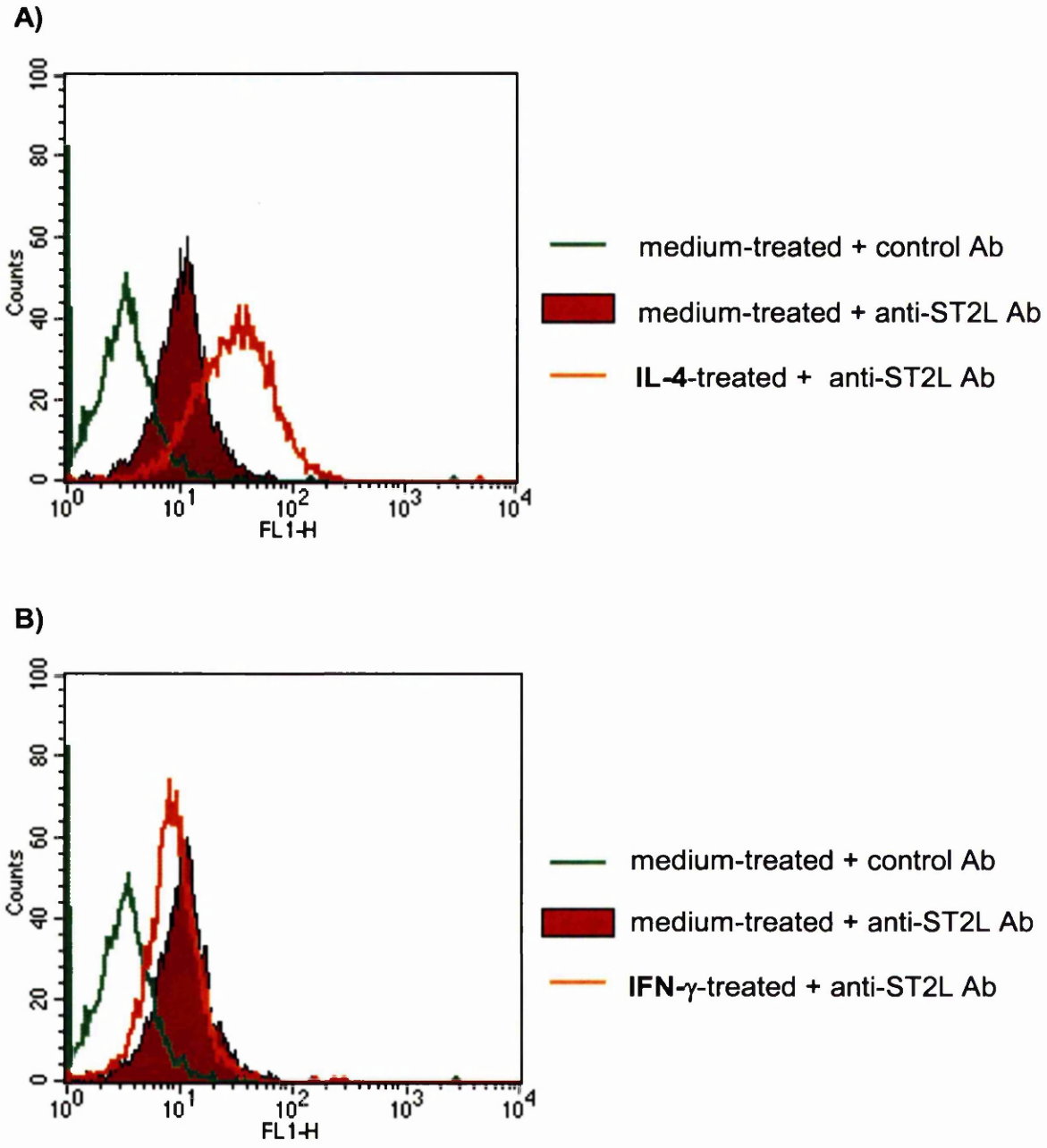


Fig.5.3 Regulation of ST2L expression on cloned Th2 cells by IL-4 and IFN-γ

Th2 clone (X12) ($1x10^6$/well) was treated overnight with 10 ng/ml IL-4 **(A)** or 100 U/ml IFN-γ **(B)** or medium alone and the expression of ST2L was assessed by flow cytometry staining with 250 ng/ml biotinylated anti-ST2L antibody or with biotinylated isotype control. A gate was set to exclude dead cells. IL-4 increases the ST2L expression by 50%, while IFN-γ drecreased ST2L expression by 30%.

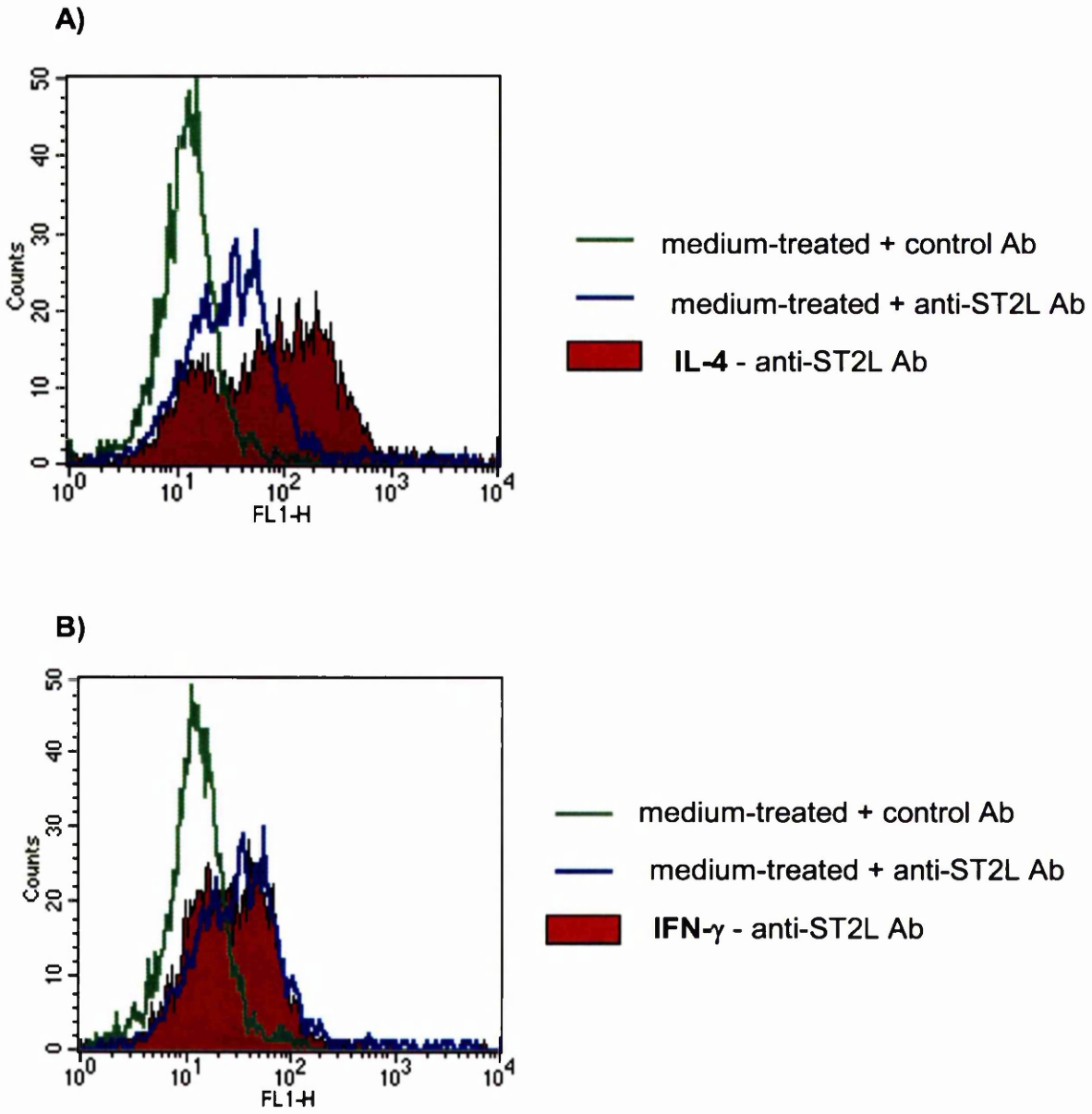


Fig.5.4 IL-4-increased ST2L expression on polarised Th2 cells

CD4(+) T cells were purified by negative selection and polarised twice towards Th2 cells as described in section 2.8 and 2.9. Three days after the last stimulation $1x10^6$/24 well Th2 cells were treated overnight with 10 ng/ml IL-4 **(A)** or 100 U/ml IFN-γ **(B)** or medium alone and the expression of ST2L was assessed by staining with 250 ng/ml biotinylated anti-ST2L antibody or the biotinylated isotype control and FITC-streptavidin as secondary antibody (1:1000). A gate was set to exclude dead cells.

expression in response to these stimuli. In addition, this method was also used to analyse the effects of IL-4 and IFN-γ on ST2 gene expression to investigate whether both transcripts are similarly regulated.

5.2.1 ST2 and ST2L mRNA expression patterns

ST2 and ST2L mRNA expression were assessed by TaqMan Real time PCR after total RNA had been extracted, DNAse-treated and reverse transcripted (section 2.29-2.32). ST2L mRNA was strongly expressed in twice polarised Th2 cells, three days after the last stimulation. Very low levels of ST2L mRNA were detected in naïve Th and twice polarised resting Th1 cells (Fig. 5.5 A). ST2 mRNA was found to be expressed at a low but detectable level in differentiated restingTh2 cells, was hardly detectable in Th1 cells and below the level of detection in naïve Th cells (Fig. 5.5 B). Hence, the expression pattern was as expected for ST2L. The expression pattern of ST2 in differentiated Th1 and Th2 cells and naïve Th cells has not previously been reported. The low expression levels of ST2 suggest that this soluble factor has no function in resting Th cells and is likely to react only to appropriate stimuli, e.g. IL-4.

5.2.2 Regulation of ST2 and ST2L mRNA expression in cloned Th2 cells by IL-4 and IFN-γ

When the Th2 clone (X12) was treated with 10 ng/ml IL-4, levels of ST2L mRNA increased after 4h to 170% of the control level, before decreasing to basal levels. IFN-γ (100 U/ml) reduced the amount of ST2L mRNA after 1h, and after 4-8h to 50-60% of its control level (Fig. 5.6 A). IL-4 enhanced ST2 mRNA levels after 1h of treatment, whilst IFN-γ had little effect on ST2 gene expression (Fig. 5.6 B).

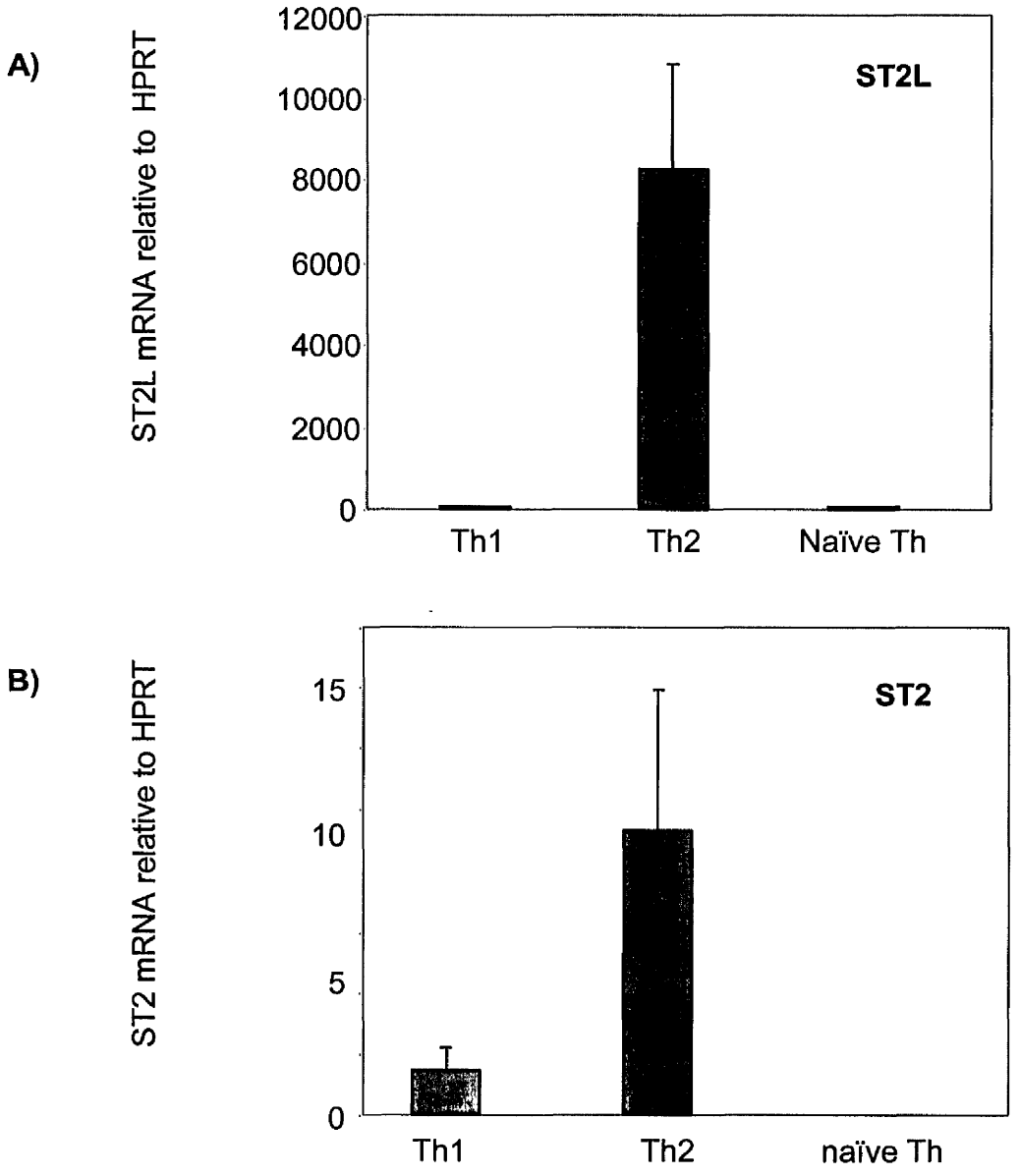


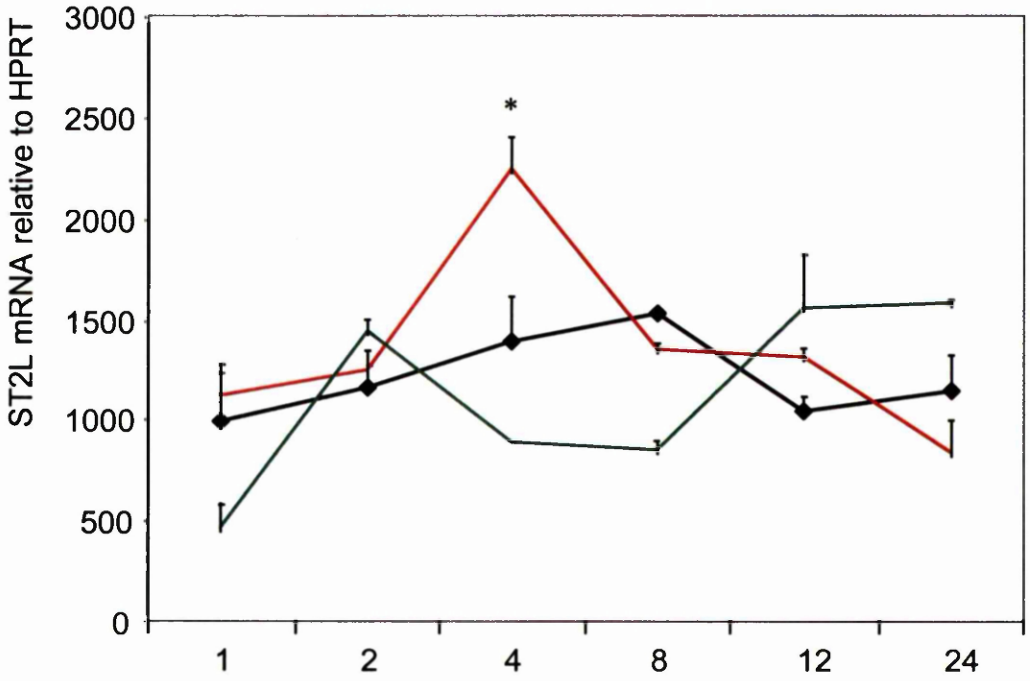
Fig. 5.5 Expression of ST2L and ST2 mRNA in naïve Th, Th1 and Th2 cells

CD4(+) T cells were purified from DO11.10 mice by negative selection (section 2.8) and twice polarised towards the Th1 or Th2 phenotype (section 2.9). Three days after the last stimulation, total RNA was extracted from $2x10^6$ naïve Th, Th1 and Th2 cells and reverse transcripted after DNAse treatment. Real time PCR was performed using specific primers for ST2L **(A)** and ST2 **(B)**. The error bars represent the standard deviation between triplicate results obtained from one cDNA sample.

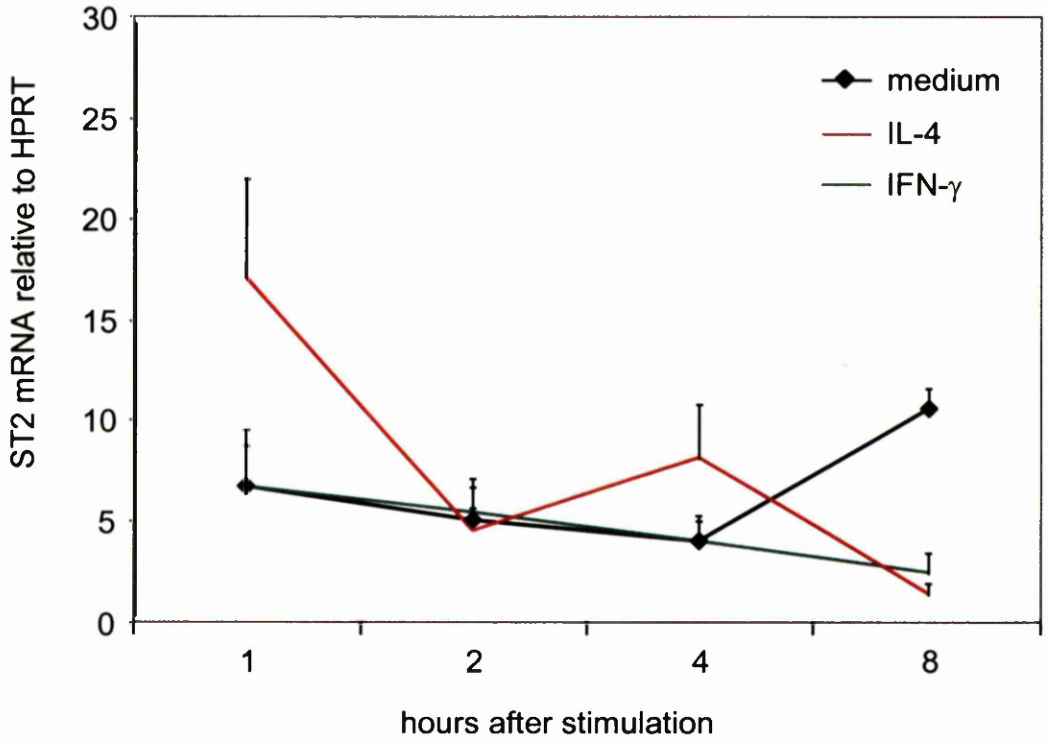
Fig. 5.6 IL-4 and IFN-γ alter ST2L and ST2 mRNA expression levels in cloned Th2 cells.

Th2 cell clone X12 ($2x10^6$/well) were treated with either 100 U/ml IFN-γ or 10 ng/ml IL-4 for 24 h, RNA was extracted and reverse transcripted after DNAse treatment and Real time PCR performed with specific primers for ST2L **(A)** and ST2 **(B)**. The results (n=3) are shown as mean ± 1 SD. The IFN-γ- and IL-4-treated sample was compared with the medium control: *$p<0.05$.

A) ST2L expression



B) ST2 expression



These findings corroborate the findings of cell surface expression of ST2L in response to IL-4 and IFN-γ. Furthermore, these data indicate that differences between ST2 and ST2L gene expression exist. For example, IFN-γ selectively suppressed ST2L but not ST2 mRNA.

5.2.3 Regulation of ST2 and ST2L mRNA expression in polarised Th2 cells by IL-4 and IFN-γ

The results obtained with cloned Th cells were confirmed with fully polarised Th2 cells, originally derived from DO11.10 mouse spleen cells.

Addition of IL-4 to twice polarised Th2 cells led to a 2.5 fold increase in ST2L mRNA expression 2 h - 8 h after stimulation (Fig. 5.7 A). ST2 mRNA in IL-4-treated cells increased 4 fold after 2 h and decreased to control level thereafter (Fig. 5.7 B). In agreement with the protein expression results, no significant changes of ST2 gene expression in response to IFN-γ were observed (data not shown).

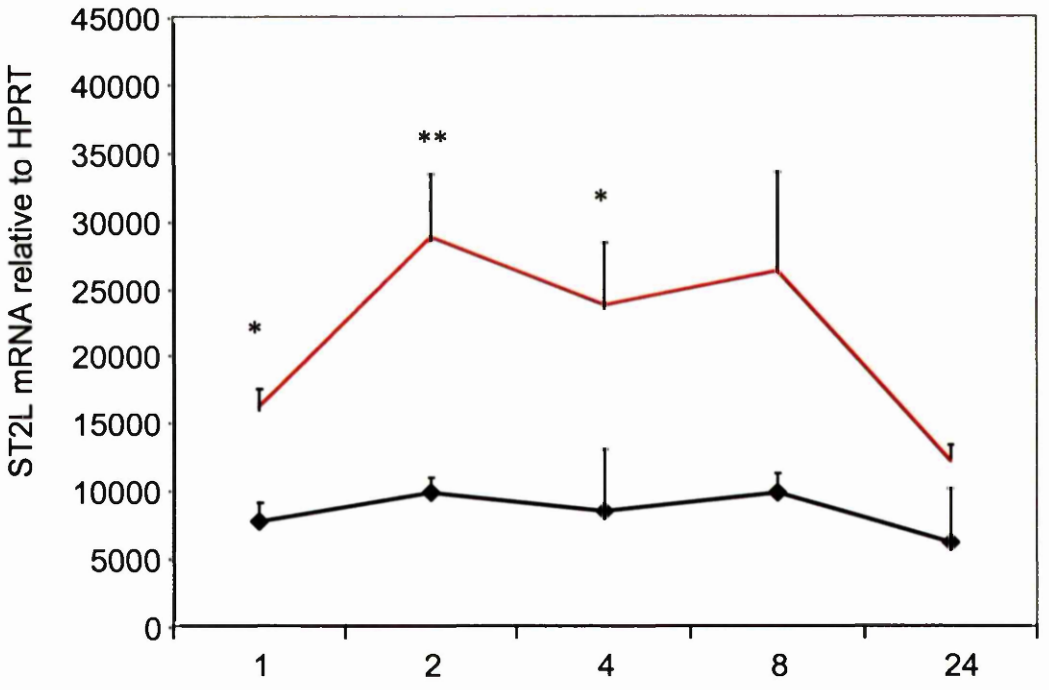
5.2.4 Regulation of ST2 and ST2L mRNA expression in naïve T helper cells by IL-4

Potential effects of IL-4 on the ST2L gene expression at the early stage of Th cell differentiation might explain the Th2 phenotype specific expression pattern. For this reason, the effects of IL-4 on ST2/ST2L gene expression in naïve Th cells were assessed. Low ST2L gene expression was found in unstimulated, naïve T helper cells (Fig. 5.8 A). IL-4 increased this amount after 4 h – 24 h by about 50%. Remarkably, IL-4 dramatically enhanced the expression of ST2 at 1 h post-stimulation; subsequently ST2 mRNA returned to basal levels (Fig. 5.8 B).

Fig.5.7 IL-4 alters ST2L and ST2 mRNA expression in polarised Th2 cells.

CD4(+) T cells were purified by negative selection (section 2.8) and polarised twice towards a Th2 phenotype (section 2.9). Three days after the last stimulation the cells were treated with 10 ng/ml IL-4 for 24 h. RNA was extracted and reverse transcribed after DNAse treatment and Real time PCR performed with specific primers for ST2L **(A)** and ST2 **(B)**. The results (n=3) are shown as mean ± 1 SD. The IL-4-treated sample was compared with the medium control: **p<0.005, *p<0.05.

A) ST2L expression



B) ST2 expression

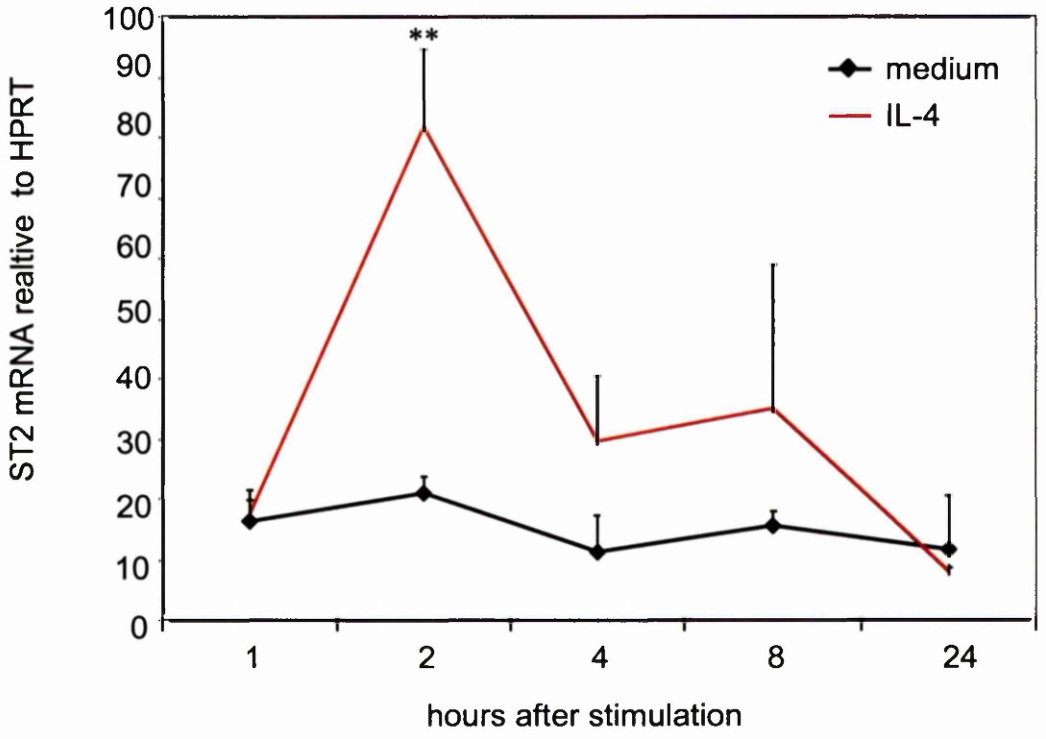
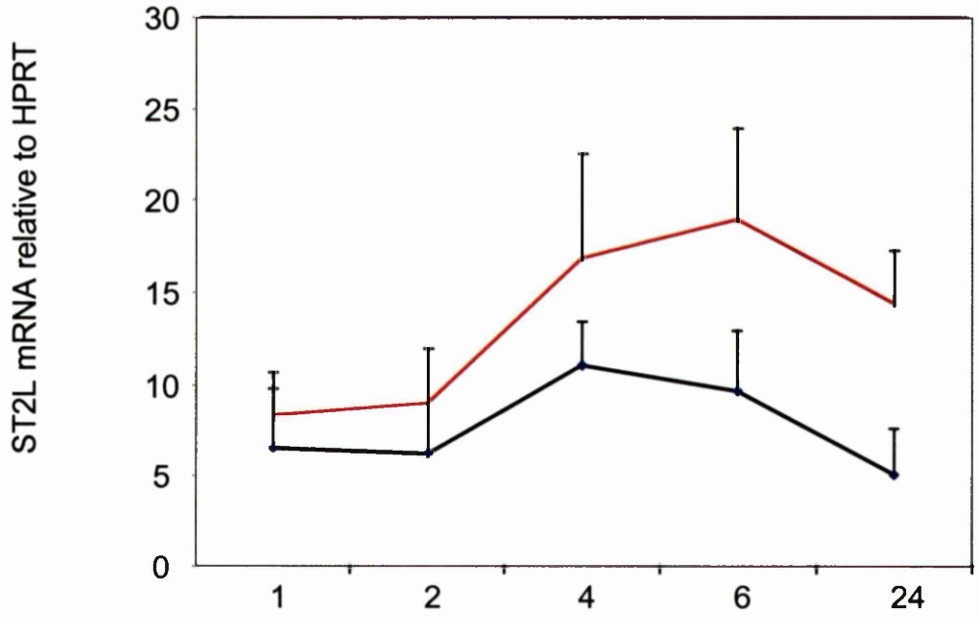


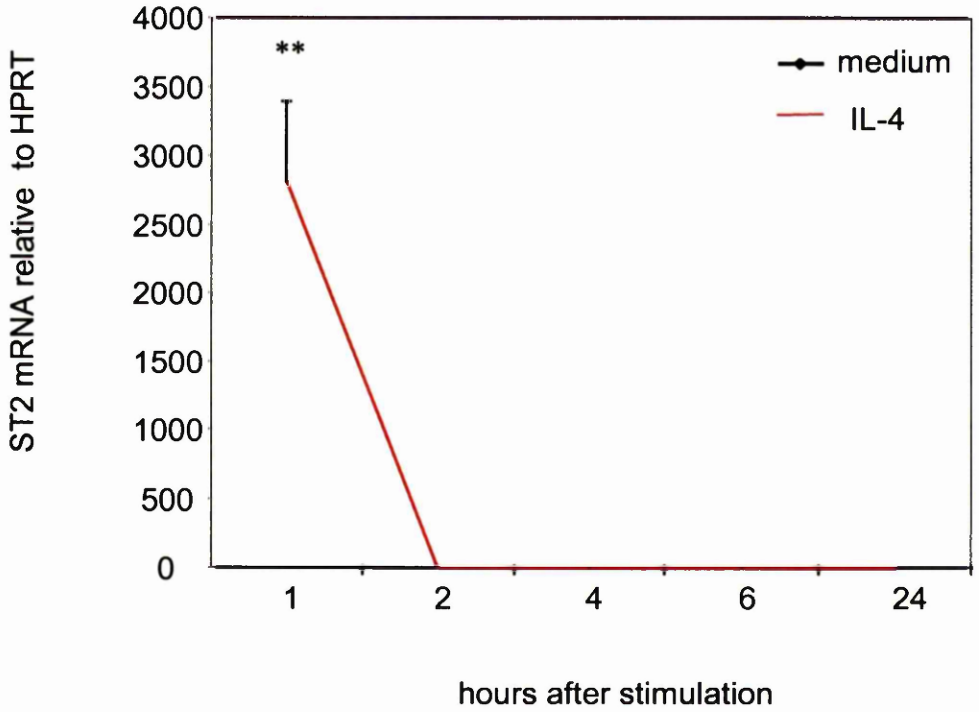
Fig. 5.8 IL-4 alters ST2L and ST2 mRNA expression in naïve Th cells.

CD4(+) T cells were purified by negative selection as described in section 2.8, treated with 10 ng/ml IL-4 for 24 h. RNA was extracted and reverse transcribed after DNAse treatment and Real time PCR was performed with specific primers for ST2L **(A)** and ST2 **(B)**. The results (n=3) are shown as mean ± 1 SD. The IL-4 treated sample was compared with the medium control: **p<0.005.

A) ST2L expression



B) ST2 expression



5.3 Influence of IL-4 and IFN-γ on the ST2/ST2L promoter

After establishing that IL-4 and IFN-γ regulate ST2L expression in an opposing manner, the mechanism of this phenomenon was investigated. This work was done primarily by Dr. Matthew Sweet but was in collaboration with the work described above on ST2 gene regulation.

There are various mechanisms (e.g. different promoter usage, mRNA stability, alternative splicing, alternate poly A site etc.) by which cells can regulate ST2/ST2L expression. Alternate usage of distal and proximal promoters, which are 10.5 kb apart, is thought to control haematopoietic and non-haematopoietic specific ST2/ST2L expression respectively. For example, while in murine mast cells ST2 and ST2L gene transcription is initiated from the distal promoter, in murine fibroblasts both variants are transcribed from the proximal promoter (Gaechter *et al.,* 1998, *Gaechter et al.*, 1996). However, in rat fibroblasts ST2L is transcribed from the distal promoter, while ST2 is transcribed from the proximal promoter (Bergers *et al.,* 1994), which suggests that these cells differentiate between the two forms through alternate promoter usage. In initial studies the question about promoter usage of ST2L in murine Th2 cell clones was addressed using primer extension analysis. It was shown that ST2L transcription was initiated from distal exon 1 (corresponding to upstream promoter usage) and not from proximal exon 1 (corresponding to downstream promoter usage, data not shown). To confirm these findings and to assess the relative importance of different transcription factors and signalling pathways in ST2/ST2L gene expression, four separate ST2/ST2L promoter/reporter gene constructs as well as a negative control plasmid were generated (Fig. 5.9). These constructs encompassed both the upstream and downstream promoters. Constructs were transiently transfected

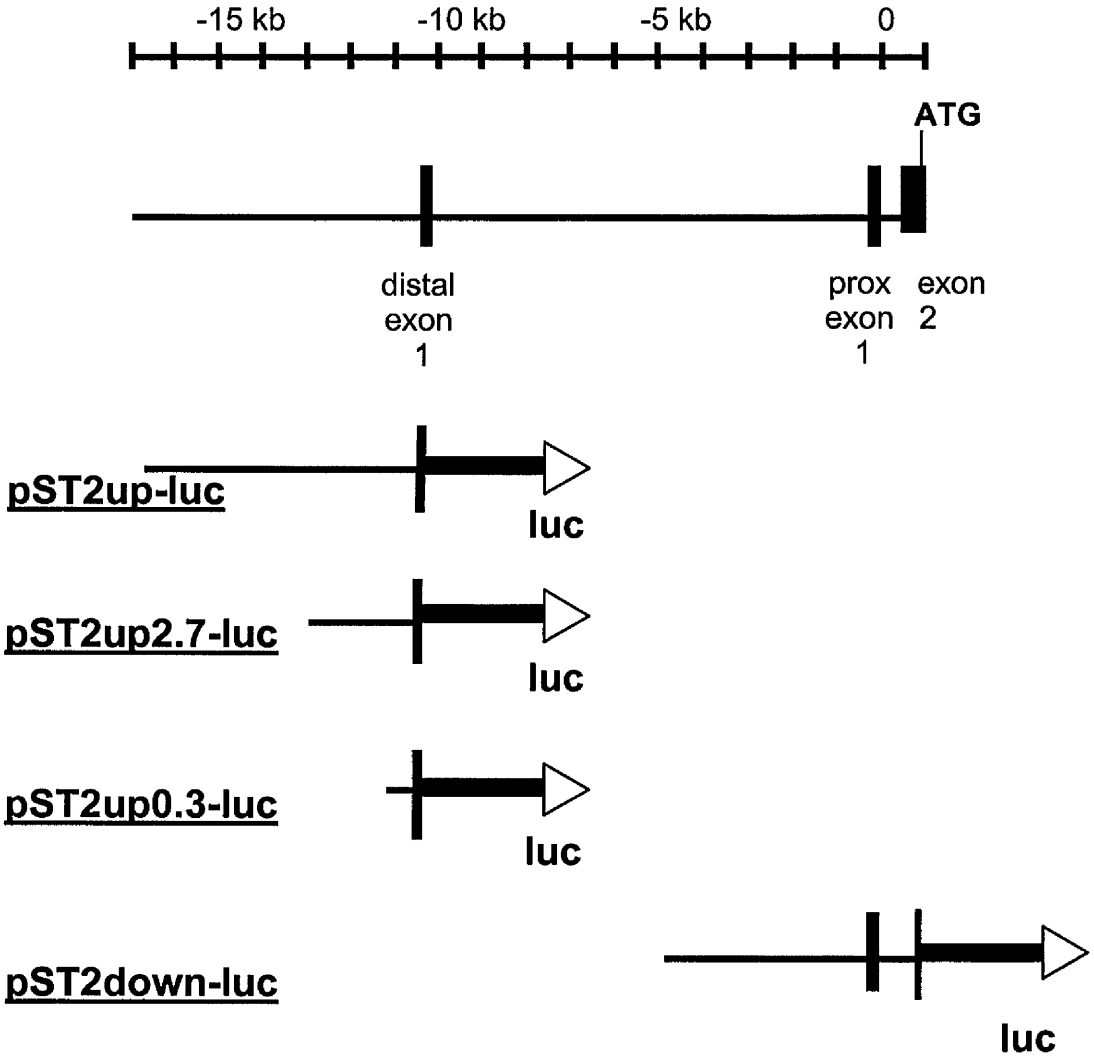


Fig.5.9 ST2/ST2L promoter-luciferase contructs (Sweet *et al.*, 2001)

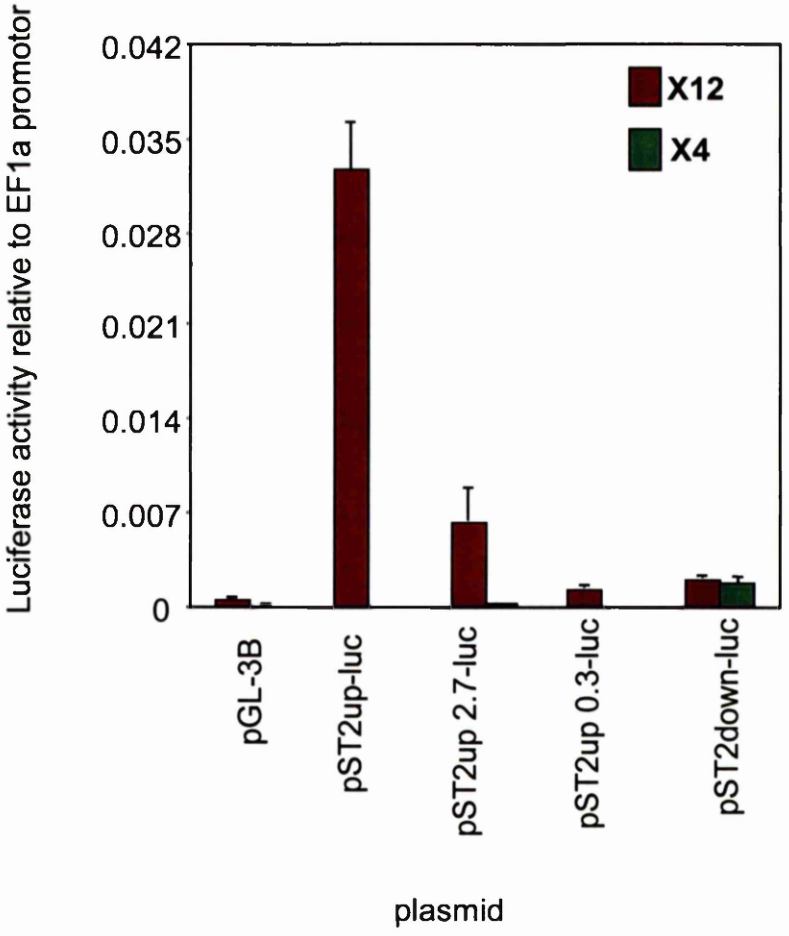


Fig.5.10 Luciferase assay to test activity of ST2/ST2L promoter contructs in Th1 and Th2 clones

Different length promoter contructs were cloned and transiently transfected into X4 (Th1) and X12 (Th2) cells. The luciferase activity was assayed to determine the active promoter. The error bars represent the standard deviation between triplicate samples.

into Th1 (X4) and Th2 (X12) cell clones and reporter gene expression (luciferase activity) was analysed (Fig. 5.10). These experiments revealed that the downstream (pST2down-luc) promoter was very weakly activated in both Th1 and Th2 cell clones, while the upstream (pST2up-luc) promoter was 60 fold more active inTh2 cells than in Th1 cells. Hence, these results corroborate the findings from primer extension experiments and suggest that the upstream (distal) promoter is responsible for Th2-specific ST2L expression. Deletion of the 6.3 kb upstream promoter to a 2.7 kb fragment (pST2up2.7-luc) reduced the promoter activity approximately 5-fold, indicating that the elements within the depleted regions are important for the expression of ST2L in Th2 cells. A further depletion to 0.3 kb (pST2-0.3-luc) reduced promoter activity still further, although this minimal promoter still displayed Th2 cell-specific activity. The activity of the upstream ST2/ST2L promoter was modulated by IL-4 and IFN-γ, which confirmed the findings made at the mRNA level (Fig. 5.11). IFN-γ suppressed luciferase expression 2.2-fold and treatment with IL-4 upregulated expression 2.3-fold. These data suggest that the IL-4 and IFN-γ regulate ST2L expression by modulation of the upstream promoter activity. The downstream promoter was not regulated in Th2 cells by the addition of these cytokines, providing further evidence that the upstream promoter and not the downstream promoter is the functional promoter in Th2 cells.

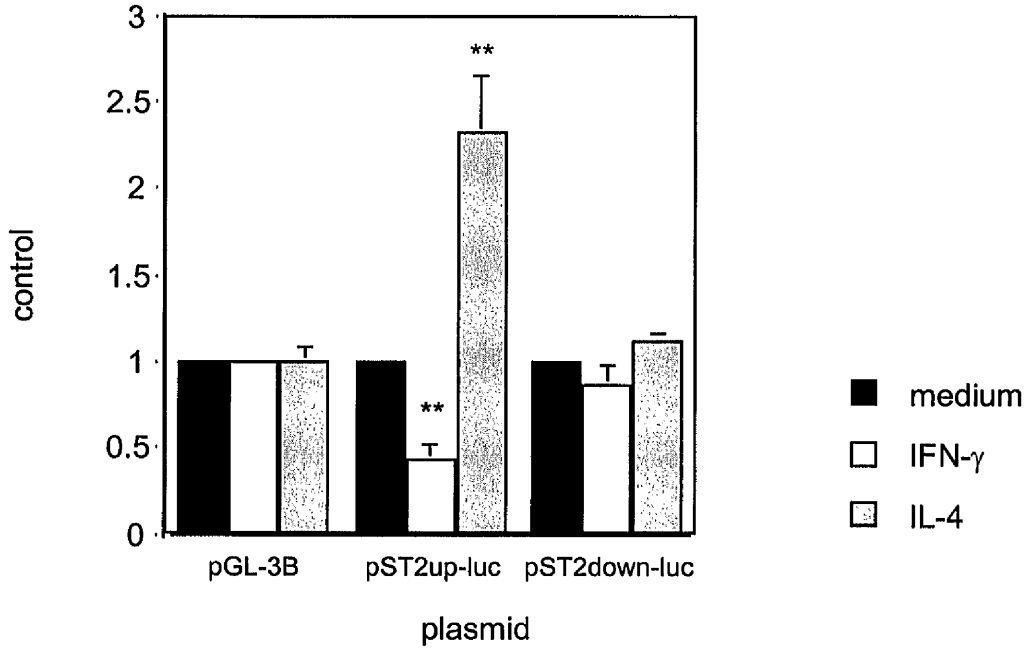


Fig.5.11 Influence of IL-4 and IFN-γ on upstream ST2/ST2L promoter in cloned Th2 cells

Th2 clone (X12) was transiently transfected with a promoter contruct containing the full length upstream ST2/ST2L promoter fused to the luciferase gene. Cells were treated overnight with either 100 U/ml IFN-γ or 10 ng/ml IL-4 and analysed for luciferase activity. These results represent two pooled experiments and are expressed as mean ± 1 SD. The IFN-γ- or IL-4-treated sample were compared to the medium control: ** $p<0.005$.

Discussion

Regulation of ST2L expression in T helper cells by IL-4 and IFN-γ

The cytokine environment in which naïve CD4(+) T cells are primed determines whether the cell differentiates towards a Th1 or Th2 phenotype. Th2 cells differentiate and proliferate through IL-4 stimulation, while IL-12 is essential for Th1 development and IFN-γ for Th1 cell effector functions (O'Garra and Arai, 2000).

ST2L was found to be expressed on Th2, but not on Th1 cells and its inhibition led to an attenuation of essential Th2 effector functions (Loehning *et al*, 1998; Coyle *et al.*, 1999). Furthermore, ST2L expression is closely linked to Th2 type cytokine expression as analysed by four-colour single-cell cytometry (Loehning *et al.*, 1999). These results indicate a strong link between ST2L expression and Th2 effector functions; however, the molecular mechanisms are still unknown.

IL-4 increased the ST2L expression on naive and polarised Th2 cells in a similar manner, suggesting either a conserved mechanism of ST2L gene regulation to which naïve and later, polarised Th2 cells respond due to their cytokine environment (Fig. 5.12 A), or an IL-4-induced increase in proliferation of the small number of ST2L-expressing Th2 cells among naïve Th cells (Fig. 5.12 B). CD4(+) T cells purified from DO11.10 TCR transgenic mice contain cells able to react to antigens other than OVA peptide and therefore contain activated, differentiated Th cells (Saparov *et al.*, 1999). The former hypothesis was supported by findings that IL-4 expression preceded the surface expression of ST2L and therefore ST2L expression is indicative of advanced Th2 commitment (Meisel *et al.,* 2001).

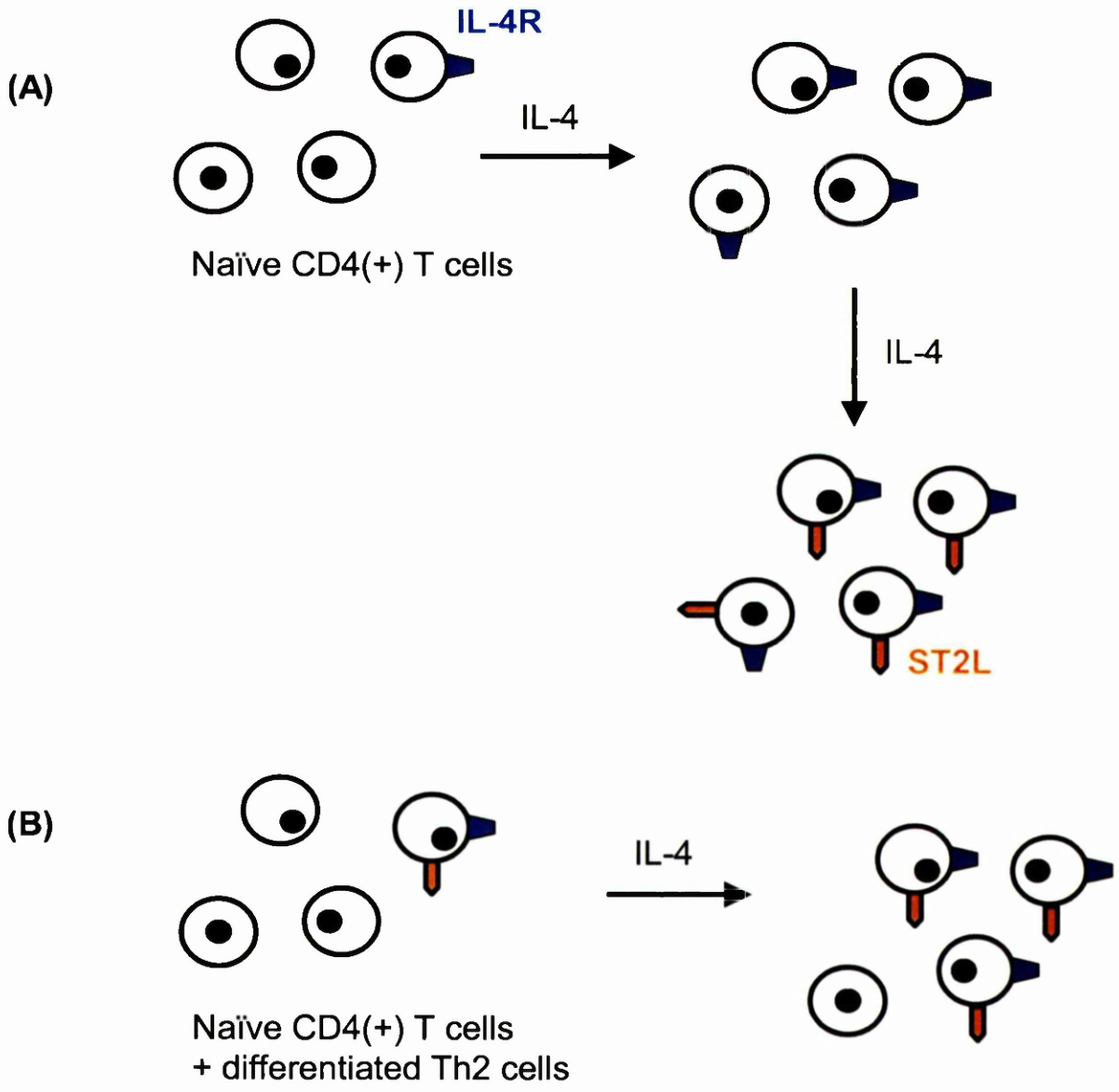


Fig. 5.12 Models of IL-4 effect on ST2L expression in Th2 cells

(A) Naïve Th cells are driven towards a Th2 phenotype by IL-4. The expression of IL-4 (and IL-4 R) precedes the expression of ST2L as a marker of advanced Th2 cell development.

(B) Among naïve Th cells there a small amount of already differentiated Th2 cells which are responsive to IL-4 and expressing ST2L. IL-4 addition enhances the proliferation of this subset.

Furthermore, only very low ST2L expression could be detected among naïve Th cells purified from TCR transgenic mice (Fig. 5.5).

IL-4 gene-targeted mice expressed similar ST2L levels as the wild type mice, whereas ST2L expression was reduced three fold in IL-5 knockout mice (Loehning *et al.*, 1998). These data suggest that ST2L is not sufficient to drive Th2 cell functions, assuming that IL-4 and IL-5 knockout mice develop impaired and normal Th2 responses respectively (Jankovic *et al.*, 2000, Pearce *et al.*, 1996; Noben-Trauth *et al.,* 1996; Xu *et al.,* 2000; Vajdy *et al.*, 1995; Burdach *et al.,* 1998).

Following priming of CD4(+) T cells under Th2-polarising conditions, the expression of ST2L was not dependent on further addition of exogenous IL-4 during subsequent rounds of stimulation (Meisel *et al.*, 2001). However, the neutralisation of endogenous IL-4 in these cells resulted in a delayed increase in ST2L expression (Meisel *et al.*, 2001). Hence, the ST2L expression does not seem to be absolutely dependent on Th2 cytokines such as IL-4 and IL-5, although ST2L expression can be upregulated by these cytokines as demonstrated in this chapter and by Meisel *et al.* (2001).

IFN-γ, which is released by Th1 cells and known to decrease Th2 cell proliferation (Fitch *et al.*, 1993), might suppress ST2L expression on Th2 cells, thereby potentially inhibiting ST2L-mediated Th2 effector functions, such as Th2 cell proliferation and IL-4 and IL-5 production (Meisel *et al.*, 2001).

In summary, ST2L expression correlates with the differentiation of naïve Th cells to Th2 cells and is positively regulated by Th2 type cytokines and negatively regulated by Th1 type cytokines. This level of regulation provides not only an

obvious mechanism for Th2-specific ST2 expression but also a mechanism by which Th1 cells can suppress Th2-type responses.

Differentially regulation of ST2 and ST2L by Th1 type and Th2 type cytokines

ST2 and ST2L are derived from the same gene, which raises the question of whether the two forms are similarly or differentially expressed and regulated. ST2 and ST2L expression were extensively studied in rat and mouse. ST2L was found in major haemopoietic organs such as spleen and liver, whilst ST2 was expressed in non-haemopoietic organs, e.g. embryonic bone and skin (Roessler *et al.*, 1995). Unstimulated murine fibroblasts predominantly express ST2, whilst unstimulated mast cells and Th2 cells express mainly ST2L (Gaechter *et al.*, 1998; Berger *et al.*, 1994). Gaechter and colleagues also reported that calcium ionophore stimulation of mast cells resulted in upregulation of ST2/ST2L gene expression and a switch from the long to the short transcript. These findings suggest that ST2 and ST2L are differentially expressed and regulated depending on the cell type and species.

In this chapter, IL-4 was shown to increase the expression of both transcripts. Timecourse experiments indicated that ST2L and ST2 mRNA were not co-ordinately regulated by IL-4. That is, IL-4 induced an early peak of ST2, which rapidly declined, whilst ST2L was induced more slowly over a prolonged period, demonstrating that the two isoforms are independently regulated by this cytokine. It is not inconceivable that ST2 and ST2L could also regulate the expression of each other in response to IL-4. For example, the rapid increase in ST2 expression in response to IL-4 might enhance ST2L expression or alternatively ST2L expression might suppress ST2 expression. These possibilities were not

investigated during the course of these studies. The hypothesis of differential regulation of ST2 and ST2L was supported by the findings in murine fibroblasts treated with pro-inflammatory cytokines such as IL-1 and TNF-α (Kumar *et al.*, 1997). The short form of the ST2 gene was strongly upregulated by these treatments, while ST2L gene expression was not affected.

Alternate promoter usage has been suggested as a mechanism for the differential expression of ST2 and ST2L in rat fibroblasts (Berger *et al.*, 1994). In order to investigate the ST2/ST2L promoter usage in Th2 cells primer extension experiments were performed (data not shown) and transient transfection analysed (Fig. 5.10). Both confirmed the distal promoter as the one being responsible for Th2 cell-specific ST2L expression as suggested in murine mast cells (Gaechter *et al.*, 1998). These data were further corroborated in promoter regulation studies where cytokines that regulated ST2L mRNA and protein (IL-4 and IFN-γ) acted in a similar manner on the upstream but not the downstream ST2/ST2L promoter.

In promoter analysis experiments, luciferase activity was only assayed after 24 h. Since luciferase is a relatively unstable enzyme (Sweet *et al.*, 1995), it is possible that the acute effect of IL-4 on ST2 expression was not observed in promoter analysis experiments. Timecourse experiments examining the effect of IL-4 on both the upstream and downstream promoters would therefore be warranted. However, in the rodent system, differential expression of ST2L and ST2 has been previously reported as a consequence of alternative splicing at exon 8 (Berger *et al.,* 1994, Gaechter *et al.*, 1996). Hence, it is likely that the effects of IL-4 on production of ST2 would not be observed using this technique.

Chapter 6 Immunomodulating effects of G418 and gentamicin *in vitro* and *in vivo*

During transfection studies I discovered that Th1, but not Th2 cells were resistant or less susceptible to the toxic effects of G418, the selection marker used for these transfections. *In vivo* applications for G418 are limited due to its overall toxicity to eukaryotic cells. Therefore, its structural analog and commonly used antibiotic, gentamicin, was tested *in vitro* for its ability to differentially inhibit Th cells and was found to behave in a similar manner to G418. According to these findings, the *in vivo* effects of gentamicin on *L. major* infections in susceptible BALB/c mice were assessed. Aminoglycosides such as paromomycin in combination with gentamicin have been linked to successful treatment of *L. major* infections in mice and humans, but the mechanism remained unclear. When susceptible BALB/c mice were treated with injections of 50 mg/kg of gentamicin for 12 days (twice per day) from the time of infection, the onset of the disease was completely prevented. This extensive treatment with gentamicin provoked a general suppression of the *L. major* antigen-specific responses among immune cells, e.g. Th and B lymphocytes, whilst shorter treatment, in agreement with *in vitro* results, showed that this antibiotic altered the Th1/Th2 type balance, which is known to determine the outcome of *L. major* infections. Further, gentamicin at estimated *in vivo* concentrations partially decreased the cell viability of macrophages and of parasites *in vitro*. The same gentamicin dose induced shape and activity changes in the parasite, which might influence the parasite - macrophage interaction. Parasites, short-term pretreated with high doses of gentamicin, did not loose their infectivity *in vivo*.

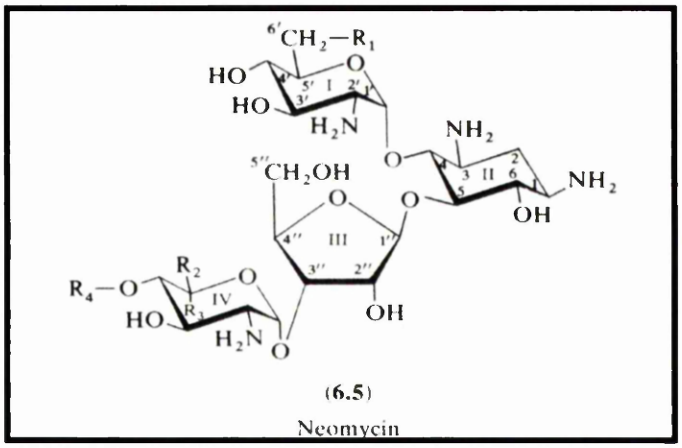
Taken together, these results suggest an immunomodulative ability of gentamicin given for a short period of time or at low doses and a immunosuppressive ability of the drug on parasites and immune cells when given for longer periods at high doses.

Introduction

In studies described in this chapter, Th2 cells were due to be transfected with a plasmid encoding antisense ST2 and a neomycin resistance gene. G418 is widely used as selection marker for transfection experiments since expression of neomycin resistance gene in mammalian cells confers resistance to the otherwise toxic antibiotic. G418 toxicity dose-response experiments on untransfected Th1 and Th2 cell clones were therefore performed to determine the concentration of G418 required to select stable transfectants. Surprisingly, Th1 but not Th2 cells were resistant to the toxicity of G418.

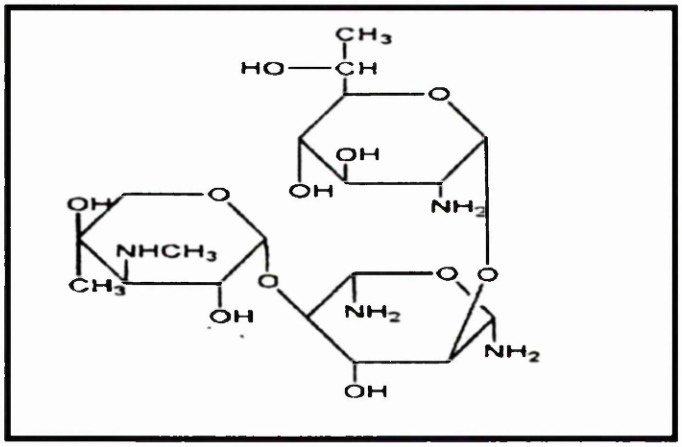
G418 (geneticin sulfate), exhibiting toxicity towards both eukaryotic and prokaryotic cells, belongs to the family of aminoglycoside antibiotics. Aminoglycosides, including streptomycin, neomycin, paromomycin, gentamicin and geneticin (G418) are characterized by the presence of an aminocyclitol ring linked to aminosugar (Fig. 6.1). They have established effects against prokaryotic cells, and some are widely used against aerobic and facultative aerobic Gram-negative bacilli and some Gram-positive bacteria (Mingeot-Leclercq *et al.*, 1999). Their bactericidal activity is attributed to the irreversible binding to the ribosomes,

Neomycin

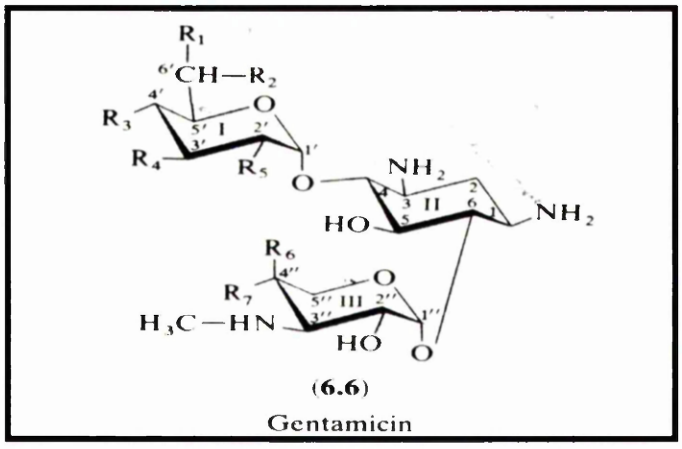


(6.5)
Neomycin

G418



Gentamicin



(6.6)
Gentamicin

Fig. 6.1 Structures of aminoglycosides

leading to mistranslation (Mingeot-Leclercq *et al.*, 1999). At higher concentrations these antibiotics have also known effects against eukaryotic, mammalian cells (Mingeot-Leclercq *et al.*, 1999). In eukaryotic cells G418 and its structural analogue gentamicin have been shown to reduce the efficiency of translation termination by a mechanism that alters the ribosomal proof-reading process. This leads to the misincorporation of an amino acid through the pairing of a near-cognate aminoacyl tRNA with the stop codon. Intact cells have to be exposed to 50-200 μg/ml aminoglycosides to facilitate a significant level of read-through of a translation termination codon. However, due to the poor permeability of these charged compounds across the plasma membrane, the actual intracellular concentration required to facilitate read-through during translation is unclear (Manuvakhova *et al.*, 2000). The interference of G418 with the ribosomes during eukaryotic protein synthesis causes the synthesis of stress proteins and other abnormal proteins as shown in fibroblasts (Eustice *et al.,* 1984; Wilhelm *et al.*, 1978; Buchanan *et al.*, 1987). This may lead to loss of fidelity and a shortening of cellular life span.

Apart from resistance to G418 by transfection with a plasmid encoding the neomycin resistance selectable marker, others have reported that mammalian cells can display inherent resistance to G418. Sweet *et al.* (1996) demonstrated that the murine macrophage cell line RAW264 became resistant to the toxic effects of G418 after treatment with bacterial lipopolysaccharide (LPS). It was suggested that LPS might induce expression of a membrane transport system such as multidrug resistance proteins (MDR) or an organic anion transporter.

G418 is toxic towards pro- and eukaryotic cells, excluding the possibility for clinical usage, but gentamicin, a structural analogue of G418, is widely used in humans as an antibiotic against diseases caused by aerobe bacteria such as septicaemia, meningitis, endocarditis (caused by viridans streptococci or *Enterococcus faecalis*) and pneumonia (British National Formulary, BNF, 1997).

Gentamicin is a multiple charged compound that interferes with prokaryotic protein synthesis by binding to the 16S rRNA and inhibiting several catalytic RNAs such as self-splicing group I introns, RNase P and small ribozymes (Schroeder *et al.*, 2000). Similar to G418, gentamicin is known to interfere also with eurkaryotic cells. In single eukaryotic cells like *Tetrathymena thermophila*, gentamicin inhibits protein synthesis by progressive depletion of polysomes (Eustice *et al.,* 1984). Gentamicin can suppress translation termination signals in mammalian cells similar to G418, but at a 2-14-fold lower level of read-through (Manuvakhova *et al.,* 2000). These findings may explain why long term treatments with gentamicin can produce dose-related adverse effects in humans, such as ototoxicity and nephrotoxicity (BNF, 1997; Wilhelm *et al.*, 1978, Buss *et al.*, 1985). Gentamicin as a polycation has been reported to induce the release of soluble intermembrane mitochondrial proteins and therefore support apoptosis (Mather and Rottenberg, 2000), offering another mechanism for gentamicin to interfere with eukaryotic cells. Negative effects of gentamicin on cytokine production of T cells, delayed-type hypersensitivity (DTH) response, NK cell activity, phagocytosis and antibody response have been reported (Van Vlem *et al.*, 1996; Ohya *et al.*, 1998). Due to

its ability to selectively kill or suppress Th2 cells, but not Th1 cells as shown in results, *L. major*-infected mice were treated with gentamicin.

Leishmaniasis is a widespread and debilitating protozoal disease of humans and animals. *Leishmania major* parasites cause cutaneous leishmaniasis, which can leave disfiguring scars upon healing or, alternatively, lead to long-term chronic infections. Hence, extensive research has been done on murine Leishmaniasis models such as BALB/c mice, that are highly susceptible to the disease. This led to the identification of a number of treatments that are now partly used in humans. Among aminoglycosides, paromomycin is the only one with clear antiprotozoal and antileishmanial activity (El-On and Greenblatt, 1983). However, early work by El-On *et al.* (1984) demonstrated that 12% gentamicin had a modest activity in *L. major* infections when used topically. Others reported gentamicin as part of a successful topical treatment (15% paromomycin, 0.5% gentamicin) for *L. major* infections (Grogl *et al.*, 1999). Nevertheless, the mechanism of action of gentamicin during *L. major* infections remains unclear.

In this chapter the effect of gentamicin on Th1 and Th2 cells activity was investigated and the question addressed whether the beneficial effect of gentamicin in *L. major* infection in mice is due to its ability to modulate the Th1/Th2 cell balance.

Results

6.1 Cloned Th1 cells but not Th2 cells are resistant to G418

The original experiment leading to the observations in this report involved the stable transfection of Th1 and Th2 cell clones with a plasmid containing antisense ST2 and a neomycin resistance gene, a selectable marker conferring G418 resistance, followed by selection in the presence of G418. The aim was to switch off ST2L and ST2 expression in Th2 cells. In a pilot experiment different concentrations of G418 were tested in Th1 and Th2 clone cells for their ability to select stable transfectants. Th1 and Th2 cells were cultured with 20-800 μg/ml G418 and 10 ng/ml IL-2 for 3 days. Th1 cells showed no changes in cell shape even at 800 μg/ml G418 (Fig. 6.2), whilst Th2 cells that did survive at a dose of 200-800 μg/ml G418 were severely affected as was evident by irregular cell shape that is indicative of cell death (Fig. 6.3).

To quantify the phenomenon, both proliferation assays and MTT cell viability assays were employed. Since absolute values for proliferation and succinate dehydrogenase activities varied between Th1 and Th2 cells, all results are displayed as a percentage of control cells, that did not receive G418, to allow a direct comparison between Th1 and Th2 cells. Th1 cells generally exhibited a lower proliferation rate than Th2 cells (Fig. 6.4, *basal levels*). The selective toxicity of G418 for two sets of Th2 but not Th1 cell clones is apparent in Fig. 6.4. The proliferation as well as the mitochondrial activity of Th2 cells decreased dramatically with 50-200 μg/ml G418, whereas Th1 cells were only slightly

Fig. 6.2 and 6.3 The effect of G418 on cloned Th1 and Th2 cells

Th1 cell clone X9 (Fig. 6.2) and Th2 cell clone X12 (Fig. 6.3) were stimulated with 10 ng/ml IL-2 and treated with 0, 400 and 800 μg/ml of G418 for 3 days. A wet preparation of the cells on slides was performed which was examined under a phase contrast microscope. Photographs were taken at x40 magnification.

Fig. 6.2

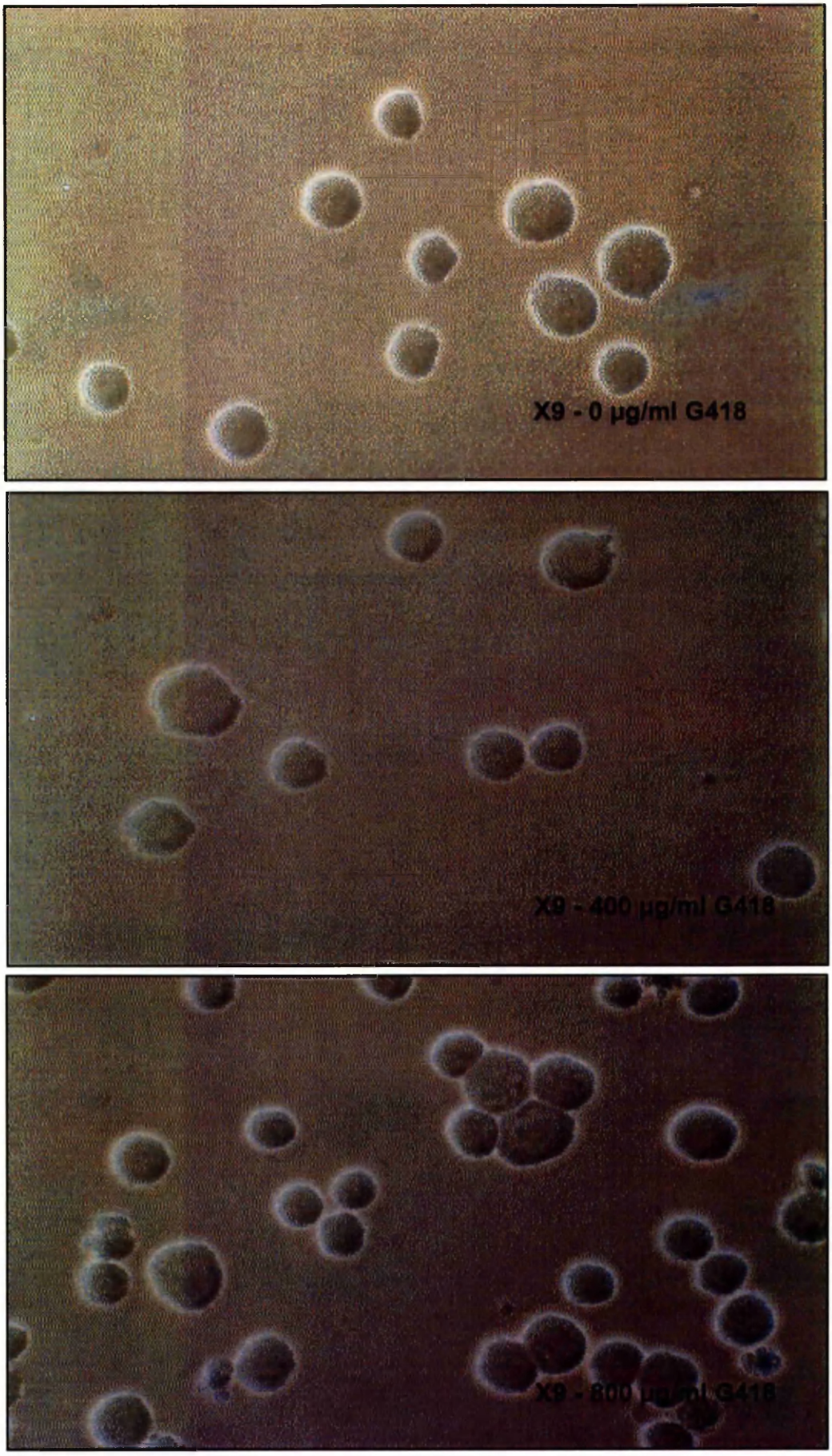
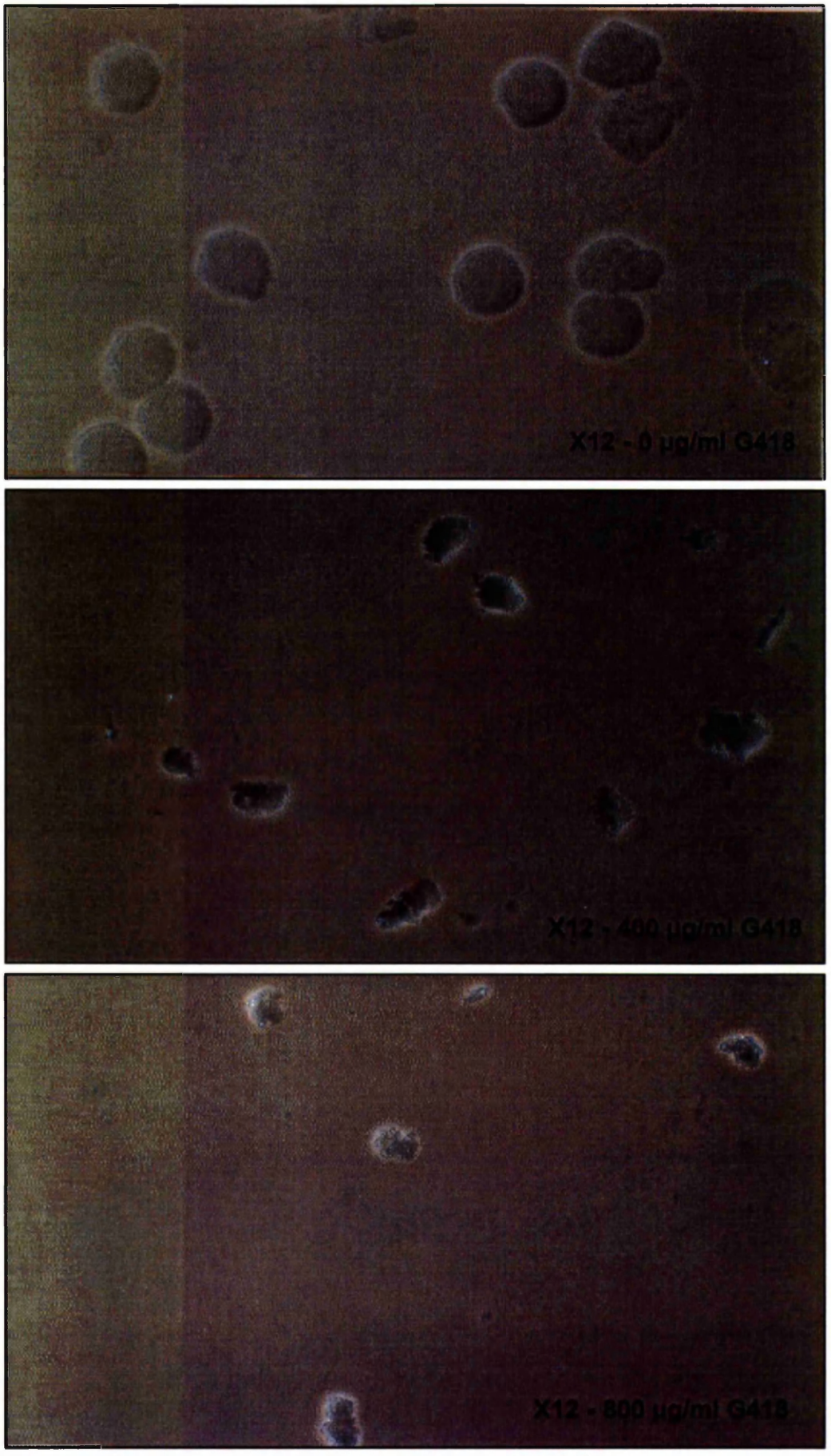


Fig. 6.3



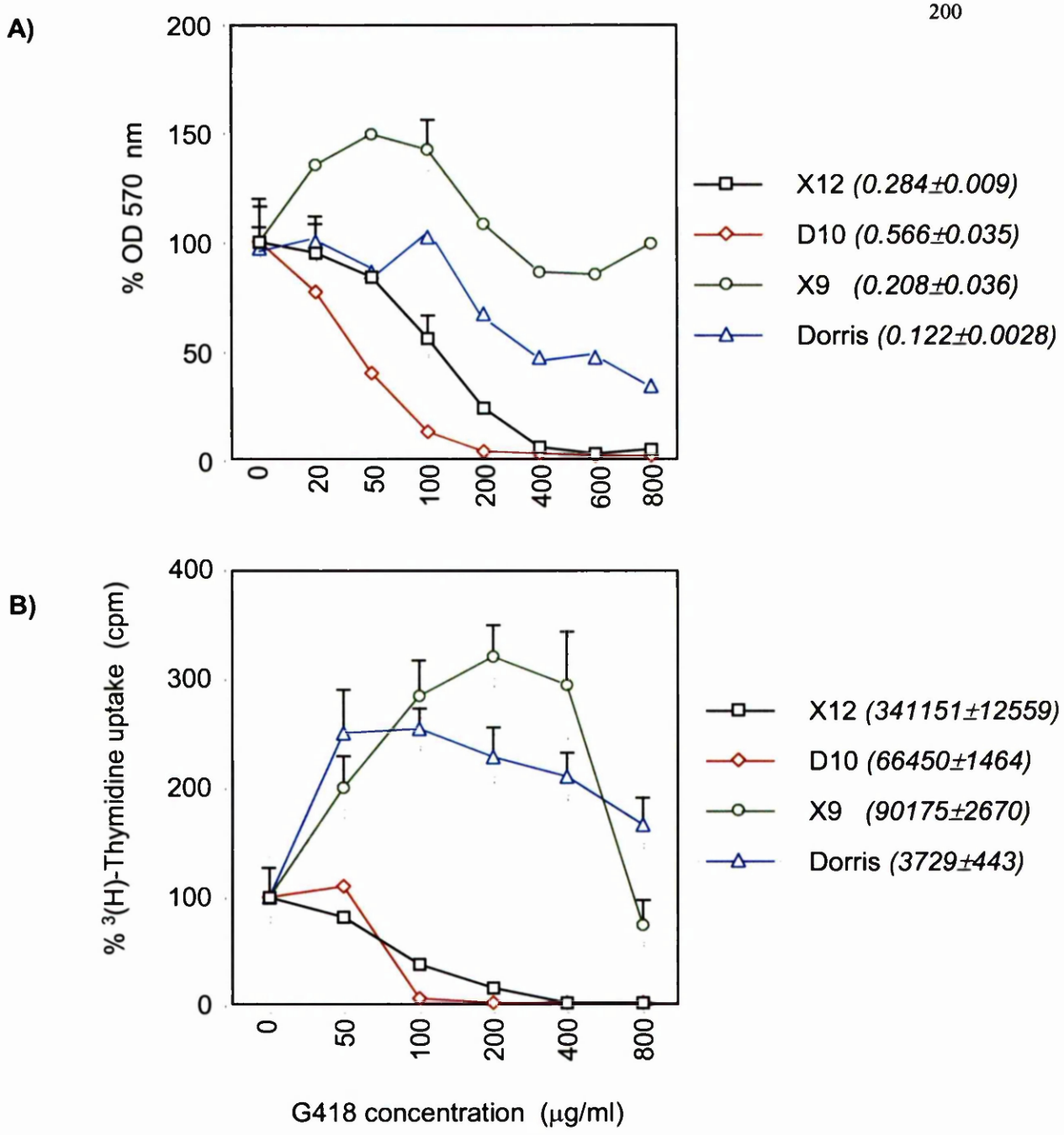


Fig. 6.4 Differential effect of G418 on cloned Th1 and Th2 cells

Th1 (X9, Dorris) and Th2 (X12, D10) cell clones ($1x10^5$/well) were treated with 10 ng/ml IL-2 and a concentration range of G418 for 3 days. MTT assays **(A)** and proliferation assays **(B)** were performed to assess the cell viability. The *basal levels* of proliferation (in cpm) and mitochondrial activity are given in brackets. The results are presented as percentage of untreated cells. Data (n=3) are shown as the mean ± 1 SD and are representative of 3 experiments.

affected by 600-800 μg/ml G418. In fact, treatment with 50-400 μg/ml G418 caused a two to three fold increase in the proliferation of Th1 cell clones. To determine whether the enhanced susceptibility of Th2 cell clones to G418 was associated with an increase in apoptosis, Th cell clones were treated with G418 for 2 days and then stained for propidium iodide and annexin V as described in section 2.36. After 2 days of G418 treatment, no early apoptotic cell population (annexin V(+)/PI(-)) could be detected (data not shown). Instead, G418 increased the number of annexin V(+)/PI(+) cells, which either undergo necrosis or are at the late stage of apoptosis (Fig. 6.5 A). At a concentration of 500 μg/ml, G418 induced 11% more annexin V(+)/PI(+) cells among Th2 than among Th1 cells. The PI(+)/annexin V(-) cell population, characteristic for necrotic cell death, was also more evident in Th2 cells (47%) in comparison to Th1 cells (38%) after treatment with 1000 μg/ml G418 (Fig. 6.5 B). Earlier timepoints have to be investigated to answer the question, whether G418 kills Th cells by apoptosis or necrosis.

6.2 G418 affects primary polarised Th2 cells but not Th1 cells

Since G418 appeared to have a selective toxicity for cloned Th2 cells over Th1 cells, the effect of G418 on primary Th1 and Th2 cells was next investigated.

The proliferation rate of Th1 cell clones was usually lower than the proliferation rate of Th2 cell clones (D10 versus Dorris, X12 versus X9) (Fig. 6.4), which might offer one explanation for the differential effect of G418 on Th cell clones. To assess whether other mechanisms apart from a differing proliferation rate might play a role in the selective killing process, G418 was added to polarised Th1 and

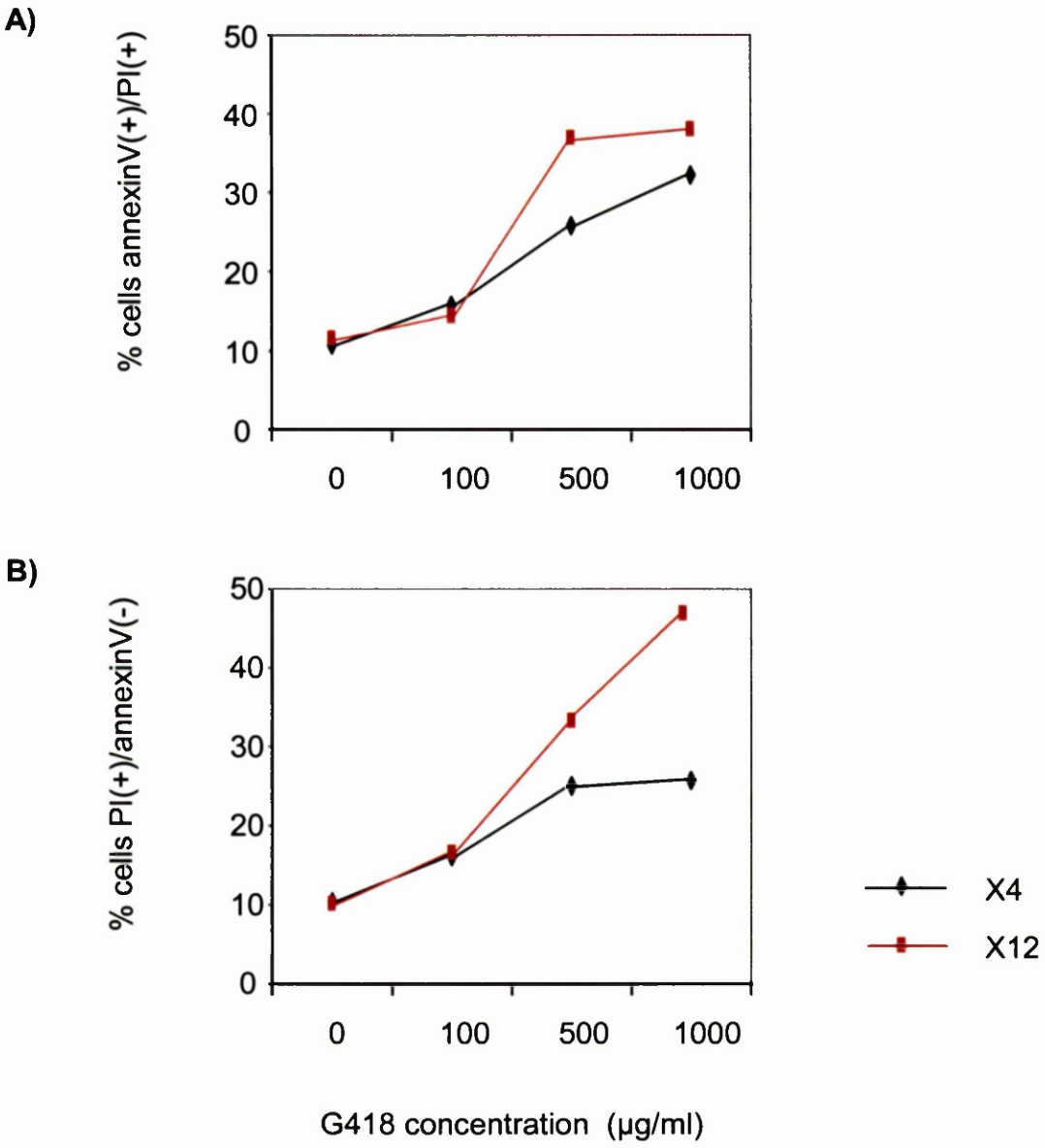


Fig. 6.5. G418 induces more cell death in Th2 cells than in Th1 cells

Th1 (X4) and Th2 (X12) clones ($1x10^6$/well) were treated for 2 days with a concentration range of G418 and were stained with PI and annexin V in order to detect apoptotic/necrotic cells.

(A) shows the percentage of PI(+)/annexinV(+) stained cells against G418 concentration.

(B) shows PI(+)/annexinV(-) stained cells.

Th2 cells at a timepoint (day 3 after antigen stimulation) when both cell types exhibited similar cell viability as assessed by proliferation and MTT assay (Fig. 6.6). The levels of proliferation and succinate dehydrogenase activities of polarised Th1 and Th2 cells, restimulated with anti-CD3, varied depending on length of time these cells had been stimulated with antigen presenting cells (APC) before. Th1 and Th2 cells, which were taken 3 days after antigenic stimulation and restimulated with anti-CD3, exhibited similar cell viability (Fig. 6.6). Cells taken after 5 days antigenic stimulation differed dramatically in the cell viability measures when restimulated with anti-CD3. The proliferation rate in Th1 cells decreased to 60% of its level on day 3 and the MTT assay showed a 70% decrease compared to day 3, whilst Th2 cells showed equal or higher cell viability measures. Hence, Th1 cells taken on day 5 after antigenic stimulation exhibited only 18% of the cell viability of Th2 cells. According to these findings, CD4(+) T cells were stimulated with plate-bound anti-CD3 (4 μg/ml) and incubated with 5-100 μg/ml G418 on day 3 after the second restimulation. The cell viability was then assessed by proliferation and MTT assays. Th1 cells, treated with 100 μg/ml G418 showed 80-100% higher proliferation and succinate dehydrogenase activities than Th2 cells, which showed severe signs of cell death (Fig. 6.7).

6.3 G418 reduces Th2 type cytokine production by BALB/c spleen cells

The results from both T cell clones and polarised T cells suggested that G418 was selectively toxic to Th2 cells. The effect of G418 on BALB/c spleen cells was next investigated to determine whether a similar phenomenon could be observed in a

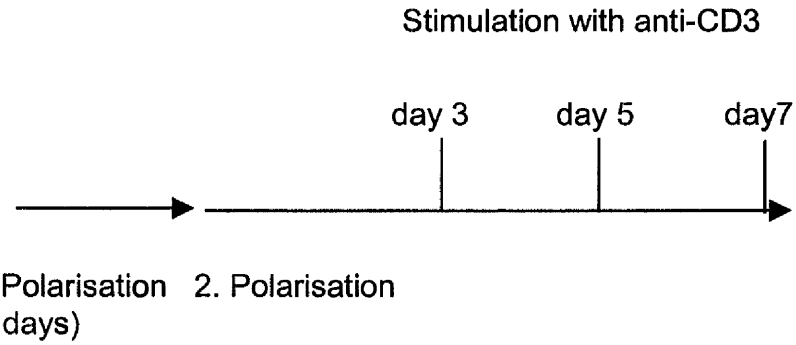
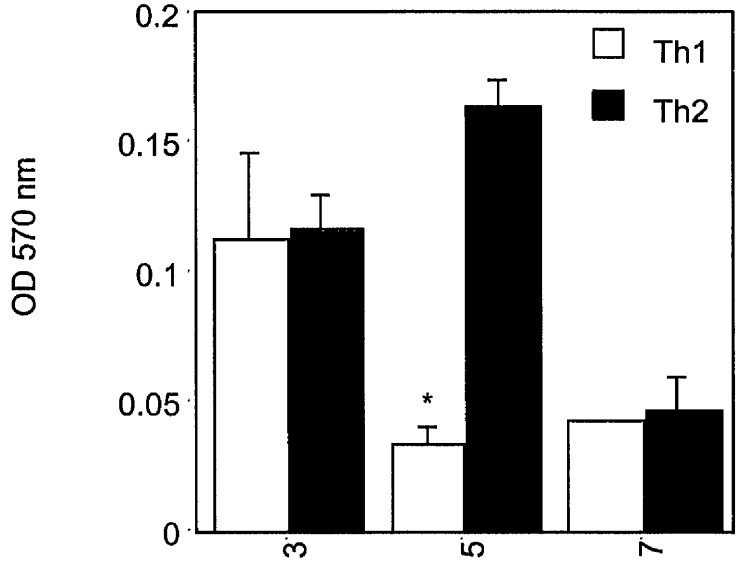


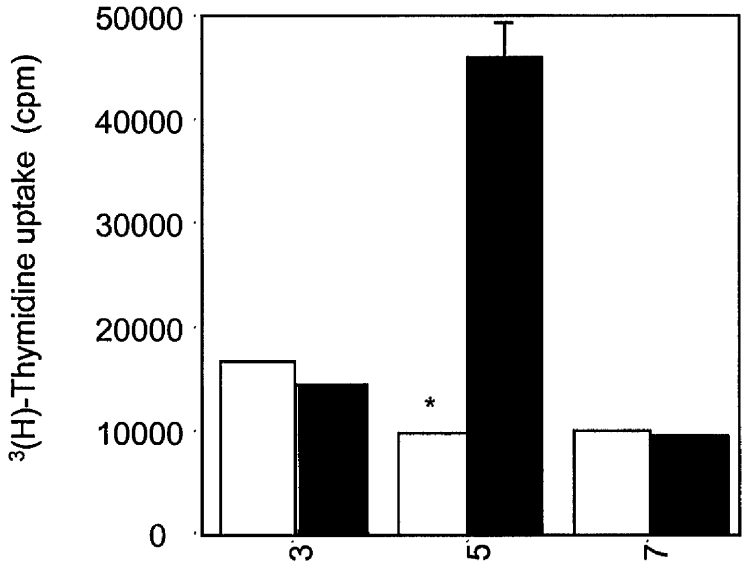
Fig. 6.6 The effect of anti-CD3 stimulation on cell viability of polarised Th cells

Purified CD4(+) T cells were polarised twice as described in section 2.9 and stimulated at 3, 5, 7 days after the second antigenic stimulation for 3 days with 4 μg/ml anti-CD3. The cell viability was then assessed by MTT **(A)** and proliferation assay **(B).** Results (n=3) are shown as mean ± 1 SD and and are representative of 4 experiments. (* $p<0.05$)

A)



B)



days after 2. polarisation

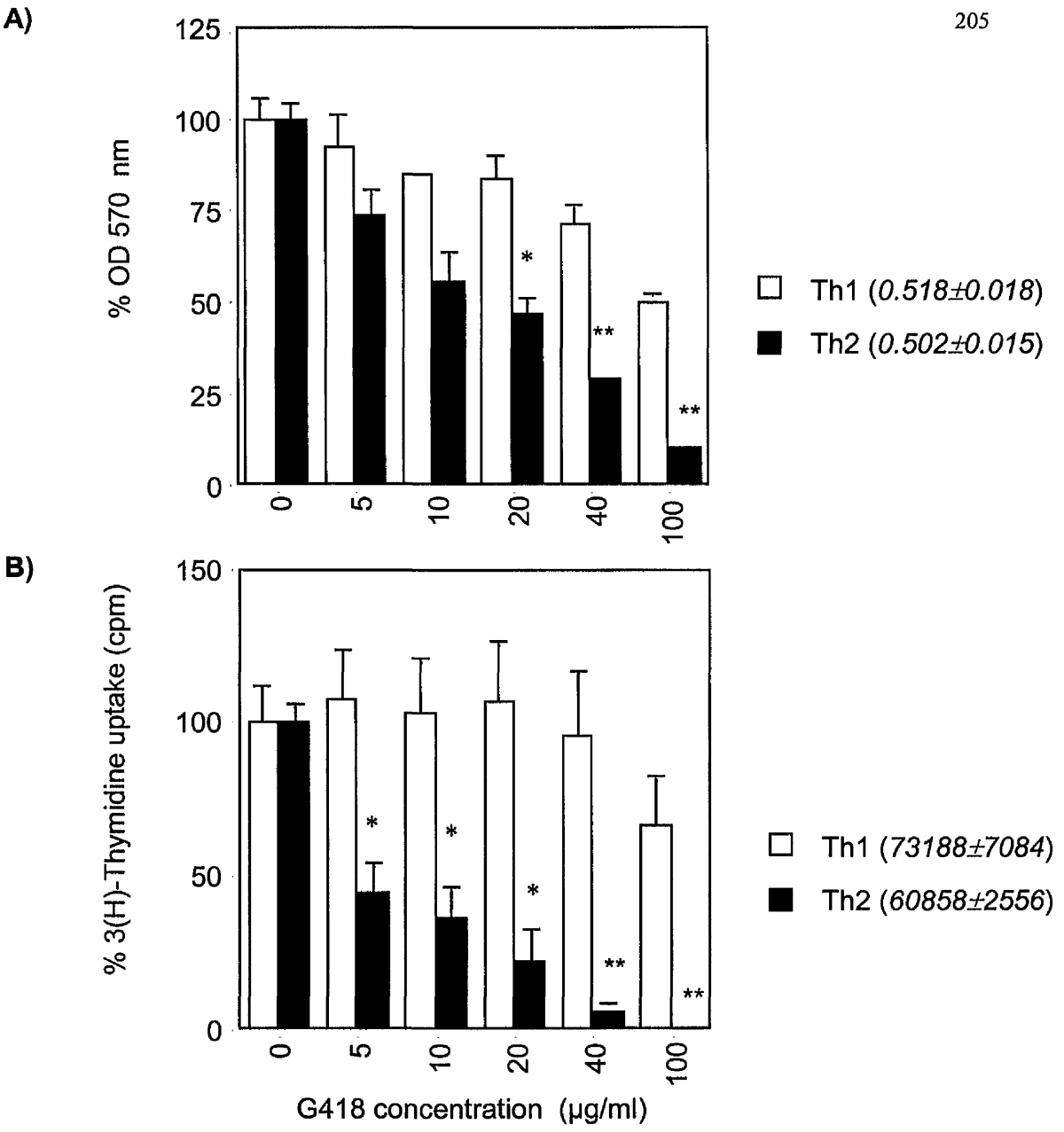


Fig. 6.7 The effect of G418 on twice polarised Th1 and Th2 cells

CD4(+) T cells were purified from DO11.10 mice spleen by negative selection and twice polarised as described in sections 2.8 and 2.9. The cells were collected three days after the second stimulation, treated with a concentration range of G418 and stimulated with 4 µg/ml anti-CD3 for 3 days. MTT assay **(A)** and proliferation assay **(B)** were then performed. The *basal levels* of proliferation (in cpm) and of mitochondrial activity are given in brackets. The results (n=3) are shown as mean ± 1 SD and as percentage of untreated cells (** $p < 0.005$, * $p < 0.05$). Data are representative of 5 experiments.

mixed cell population. Spleen cells, stimulated with anti-CD3 and incubated with 100-800 μg/ml G418, showed impaired proliferation and cytokine production (Fig. 6.8). 100 μg/ml G418 reduced the output of Th2 type cytokines IL-4 to 34% and IL-5 to 24%, but Th1 type cytokines IFN-γ and IL-2 were affected only marginally. These results suggest that among the spleen cell population G418 selectively inhibited or depleted cells secreting Th2 type cytokines and only higher G418 concentrations were able to reduce cells secreting Th1 type cytokines.

6.4 Gentamicin decreases the cell viability and cytokine production of Th2 cells over Th1 cells

Although these results obtained using G418 as a compound to favour survival of Th1 over Th2 cells, G418 can not be used *in vivo* because of its universal toxicity to mammalian cells. Therefore, the possibility that gentamicin, an antibiotic which is related to G418, might share this feature of Th2-specific toxicity was next explored. Th1 and Th2 cell clones were cultured with 10 ng/ml IL-2 and 200-1000 μg/ml gentamicin for 3 days, lower gentamicin concentrations did not affect cell viability (data not shown). As demonstrated by both proliferation and mitochondrial activity assays, 600-1000 μg/ml gentamicin reduced the cell viability of Th2 cells to 20-30% whilst the same doses of gentamicin had little effect on Th1 cells which remained at 55-95% of their untreated cell viability (Fig. 6.9). The selective effect of gentamicin was then tested on twice polarised Th1 and Th2 cells. Proliferation of Th2 cells was reduced by 70% in response to 1000 μg/ml gentamicin whilst Th1 cells had only a 15% impairment in proliferation (Fig. 6.10). These findings clearly

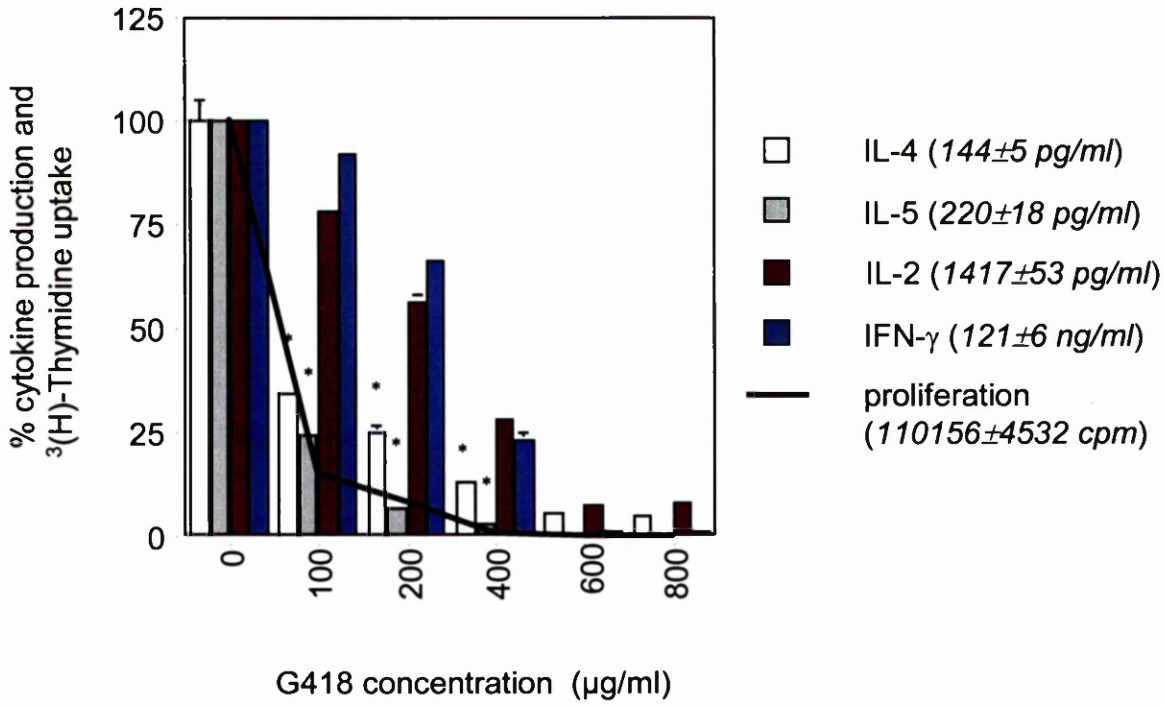


Fig. 6.8 The effect of G418 on BALB/c spleen cell cytokine production

BALB/c spleen cells ($1x10^6$/well) were stimulated with 4 µg/ml anti-CD3 and treated with a concentration range of G418 for 3 days. Cytokine production was measured by ELISA and cell viability was assessed by proliferation assay. The *basal cytokine* and *proliferation levels* are given in brackets. Results (n=3) are shown as percentage of untreated cells (100%) and presented as mean ± 1 SD (*$p<0.05$; **$p<0.005$). These data are representative of 5 experiments.

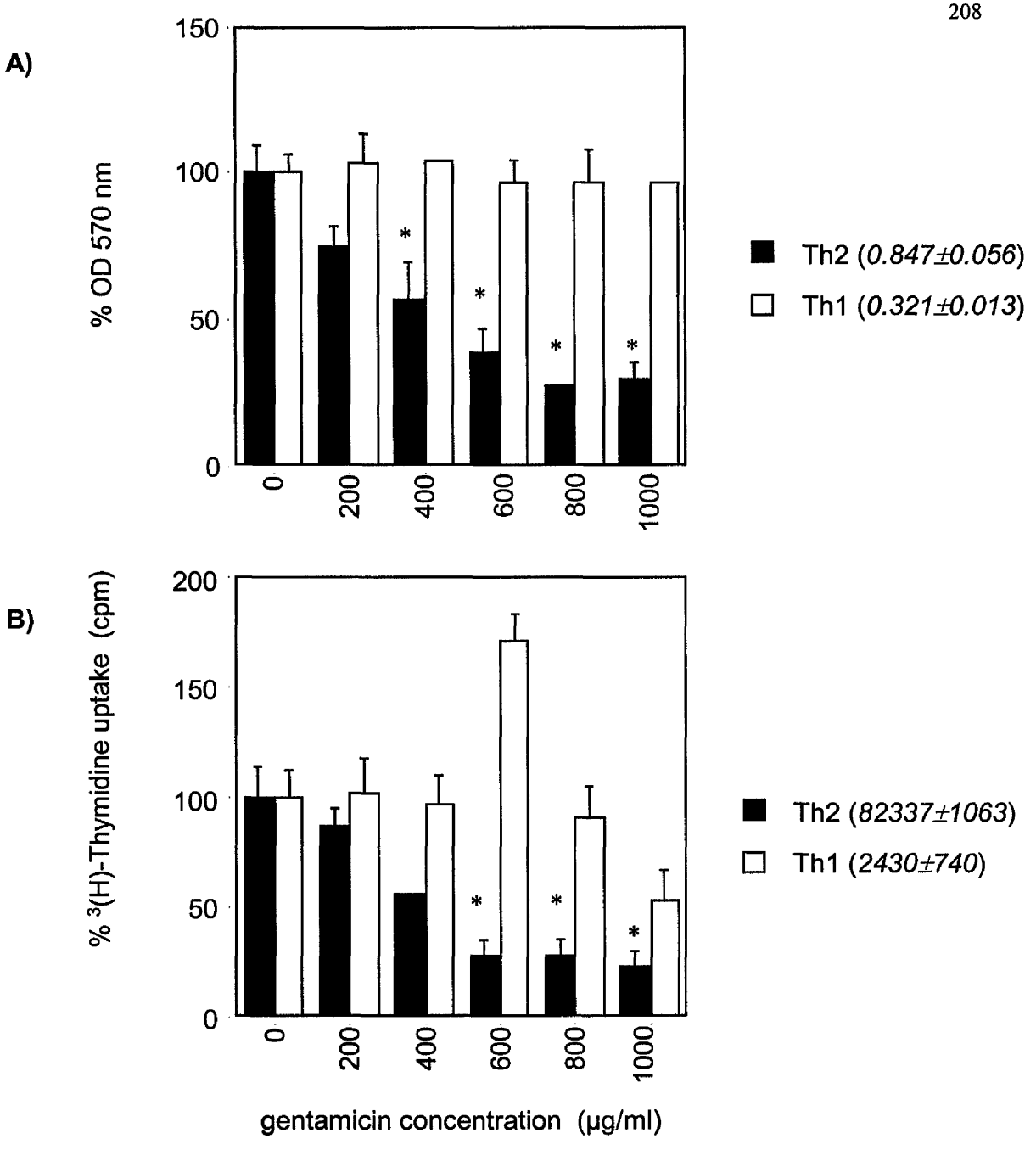


Fig. 6.9 The effect of gentamicin on cioned Th1 and Th2 cells

Th1 (Dorris) and Th2 cell (D10) clones ($1x10^5$/well) were treated for 3 days with a concentration range of gentamicin and 10 ng/ml IL-2. Cell viability was assessed by MTT assay **(A)** and proliferation assay **(B)**. The *basal levels* of proliferation and MTT are shown in brackets. The results (n=3) are shown as percentage of untreated cells (100%) and presented as mean ± 1 SD (* $p<0.05$). Data are representative of 2 experiments.

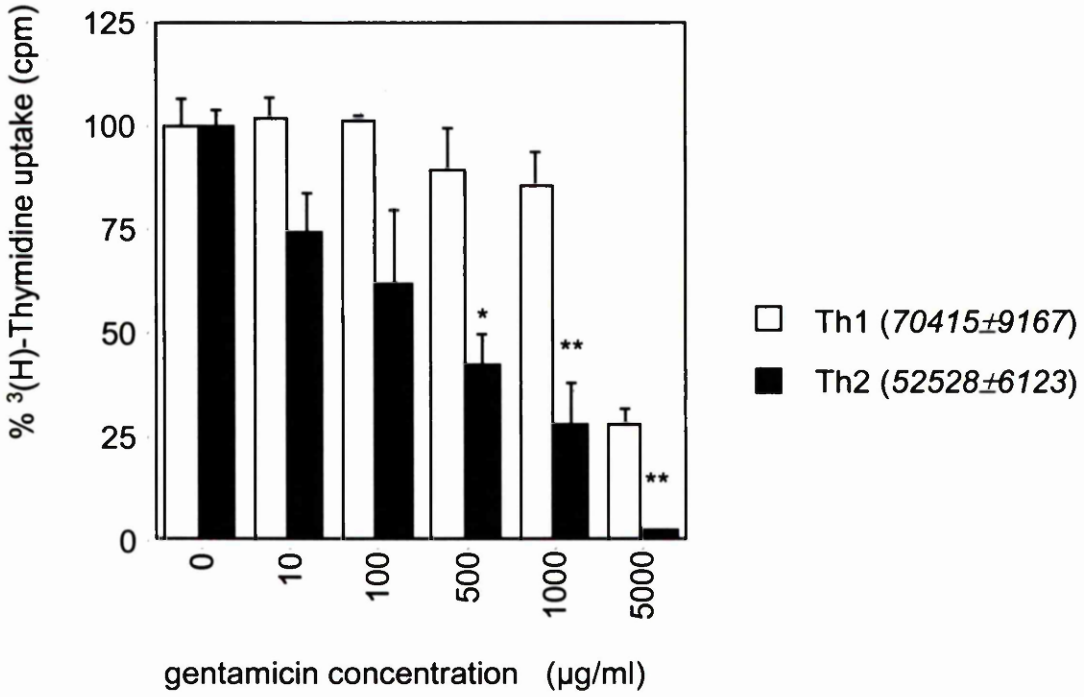


Fig. 6.10 The effect of gentamicin on polarised Th1 and Th2 cells

Purified CD4(+) T cells were polarised twice as described in section 2.9. Three days after the second stimulation they were stimulated with 4 µg/ml anti-CD3 and with a concentration range of gentamicin for 3 days. Cell viability was assessed by proliferation assay. *Basal proliferation levels* are given in brackets (in cpm). The results (n=3) are presented as percentage of untreated cells (100%) and shown as the mean ± 1 SD (** $p<0.005$; *$p<0.05$). Data are representative of 3 experiments.

indicate a similar killing specificity of gentamicin as seen with G418, suggesting that Th1 cells were less susceptible to the aminoglycoside than Th2 cells. In the mixed cell population of BALB/c spleen cells, a three-day gentamicin treatment (50-1500 μg/ml) decreased production of Th2 cytokines much more dramatically than Th1 cytokines (Fig. 6.11). For example, 1000 μg/ml gentamicin caused a 21% reduction of IL-2 and a 33% reduction of IFN-γ, whilst the Th2 cytokine IL-4 was reduced by 75% and IL-5 by 95%. Summarizing the findings, gentamicin was able to selectively kill or suppress Th2 cell clones, polarised Th2 cells and Th2 cytokine producing spleen cells compared with Th1 cells.

6.5 Gentamicin inhibits the progress of *Leishmania major* in susceptible BALB/c mice

Both G418 and gentamicin altered the balance between Th cells in favour of Th1 cell survival and cytokine production *in vitro*. Whether this observation could be applied to modulation of Th1/Th2 responses *in vivo* was assessed next. leishmaniasis is caused by the intracellular parasite *Leishmania,* which infects macrophages, multiplies in their vacuoles and thereby escapes immune detection. Disease progress is exacerbated by strong Th2 responses in infected organisms, while a strong Th1 response is protective (Scott *et al.*, 1988). Healing is mediated by the Th1 type cytokine IFN-γ, that activates macrophages to produce nitric oxide which has been shown to kill intracellular parasites *in vitro* (Liew *et al.*, 1991; Nacy *et al.*, 1985). Disease progression correlates with production of Th2 type cytokines such as IL-4, which decreases macrophage expression of NO synthase and NO

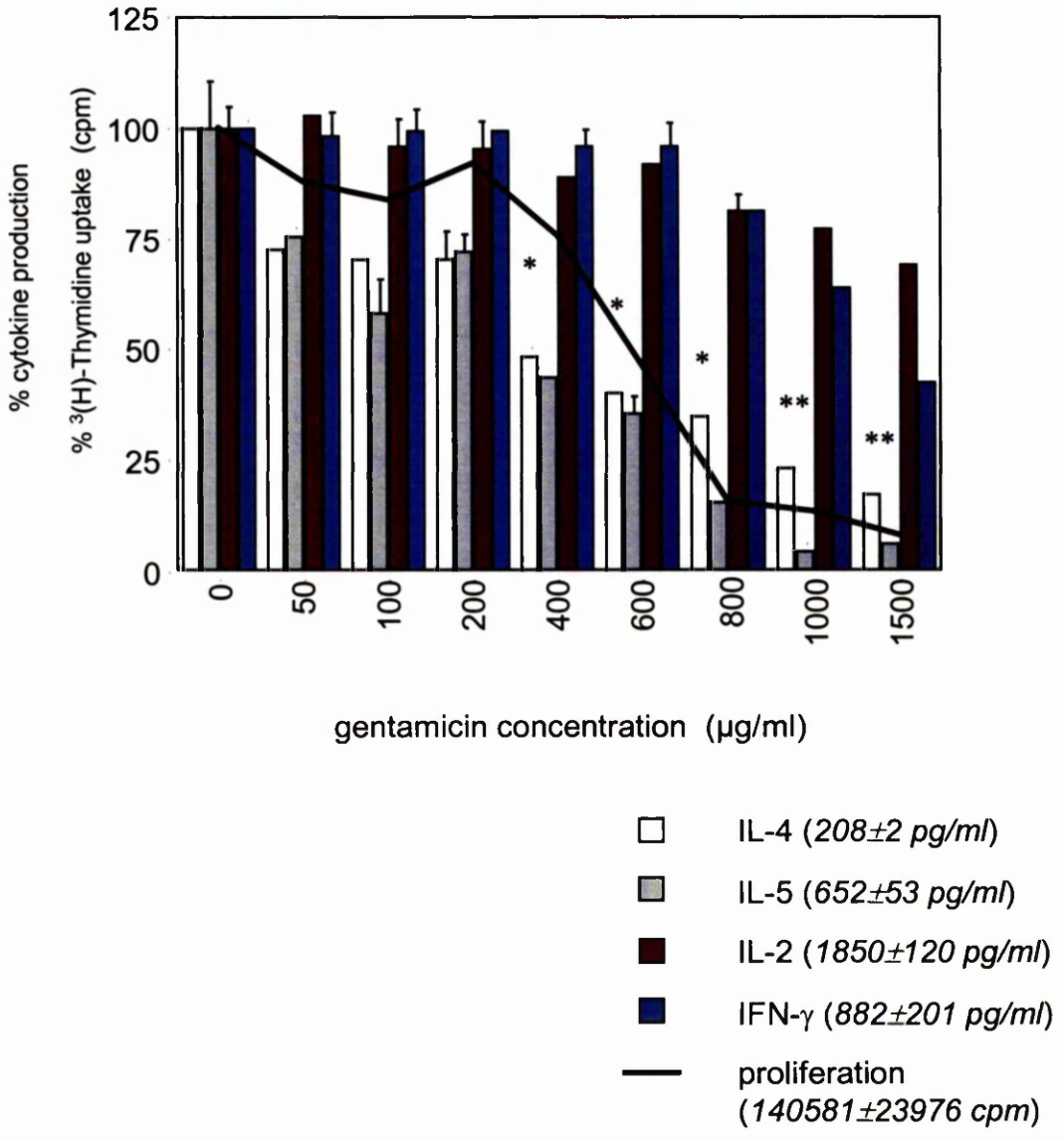


Fig. 6.11 The effect of gentamicin on BALB/c spleen cell cytokine production

BALB/c spleen cells (1x10^6/well) were stimulated with 4 µg/ml anti-CD3 and treated with a concentration range of gentamicin for 3 days. Cytokine release was assessed by ELISA and cell viability by proliferation assay. The *basal levels* are shown in brackets. The results (n=3) are presented as percentage of untreated cells (100%) and shown as the mean ± 1 SD (** $p<0.005$; * $p< 0,05$). Data are representative of 4 experiments.

and therefore favors parasite survival (Liew *et al.*, 1989; Liew, 1991). BALB/c mice are susceptible to the disease due to development of a Th2 type response after infection with *Leishmania major.* Low doses of gentamicin as part of a successful topical treatment for murine cutaneous Leishmaniasis have been described by Grogl *et al.* (1999), but gentamicin treatment alone did not induce healing of the lesions. Hence the effect of gentamicin on both disease progression (footpad swelling) and immunological parameters was assessed in *L. major*-infected BALB/c mice.

6.5.1 High doses of gentamicin prevent *Leishmania* disease progression (trial 1)

Using doses of gentamicin that are applied clinically to treat bacterial infections (3 and 6 mg/kg body weight, BNF, 1997; Sakaeva *et al.*, 1998), no effect was observed on *L. major*-mediated disease progression (data not shown). To examine the possibility that the low dose of gentamicin did not overcome the high dose infection of parasites, higher concentrations of gentamicin were used. Three groups of 6 BALB/c mice were infected with $1x10^5$ *L. major* parasites/mouse by footpad injection and treated i.p. with PBS as a control, 10 mg/kg gentamicin or 50 mg/kg gentamicin for 12 days, twice a day. At 10 mg/kg, gentamicin did not inhibit disease progression. However treatment with 50 mg/kg gentamicin completely inhibited disease progression over 80 days (Fig. 6.12 A). Two mice treated with 50 mg/kg of gentamicin were kept to observe the long-term effect of gentamicin

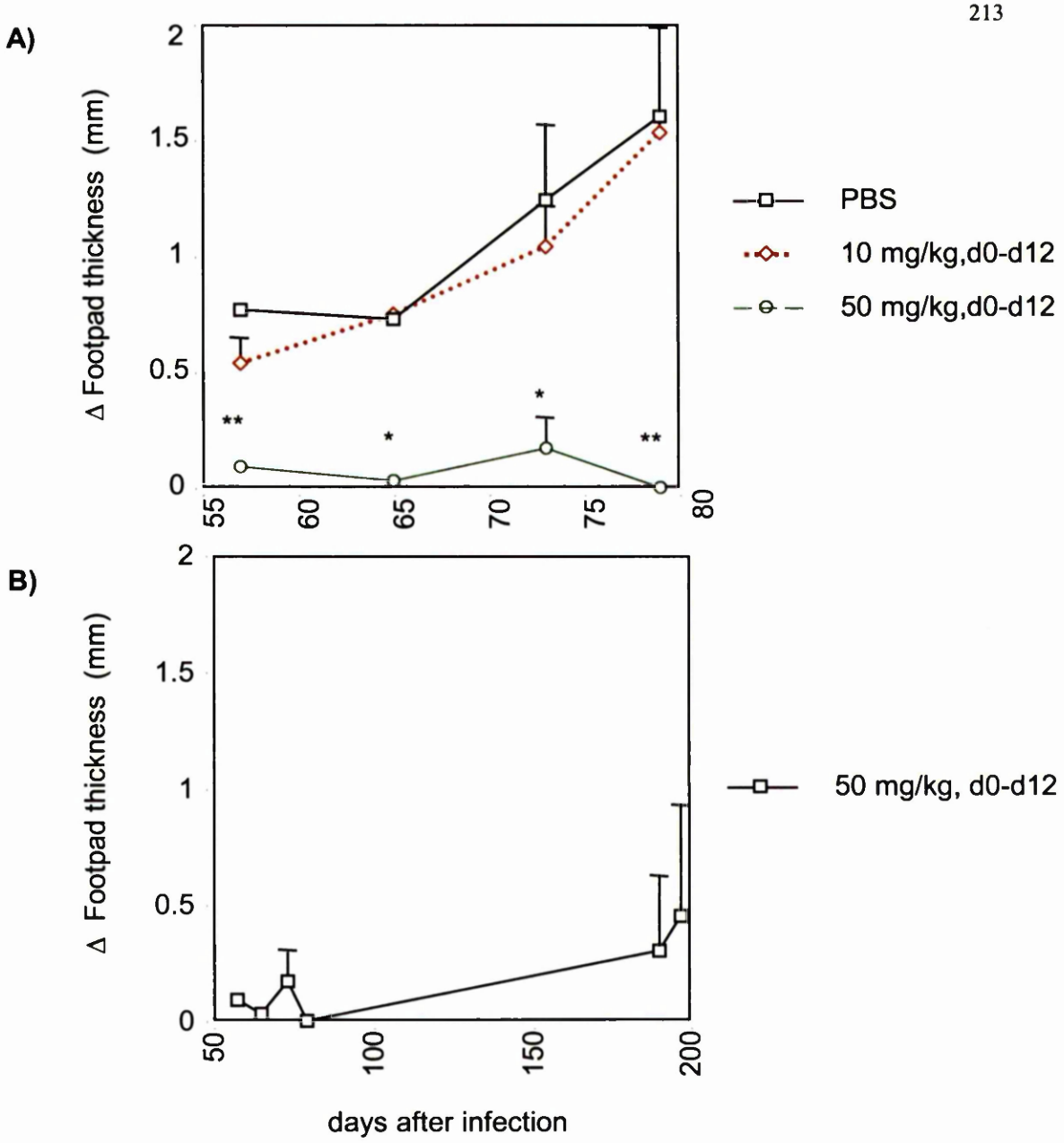


Fig. 6.12 Gentamicin treatment of *L. major*-infected BALB/c mice (trial 1)

Three groups of 6 BALB/c mice were infected in the left footpad on day 0 with $1x10^5$ parasites/mouse. From day 0 - 12 they received i.p. injections of PBS, 10 mg/kg or 50 mg/kg of gentamicin twice per day. The footpad swelling data show the difference between the infected left and the uninfected right footpad and are presented as the mean ± 1 SD (** $p<0.005$, * $p< 0.05$ comparing PBS control with 50 mg/kg treatment). **A)** shows the early development of foot pad swelling of 6 mice per group till 80 days after infection, **B)** shows the late development till 200 days after infection, monitoring footpad swelling of 2 mice which had been treated with 50 mg/kg of gentamicin.

injections. One mouse developed obvious lesions 180 days after the parasite infection, while the second mouse showed no signs of swelling (Fig. 6.12 B).

6.5.2 Investigation of the mechanism of the protective effect of high dose of gentamicin in Leishmaniasis during trial 1

After establishing that high doses of gentamicin prevented *L. major* infection in BALB/c mice, I next addressed the mechanism by which this occurred. Hence, I assessed the immunological parameters after *L. major* infection (with or without gentamicin treatment). The parasite load in the infected foot, antigen specific antibody production, cell proliferation in response to *L. major* antigen and cytokine production were measured. Further, the effect of gentamicin on leukocyte subsets in BALB/c mice was analysed by flow cytometry to determine which cell types were affected by this treatment regime.

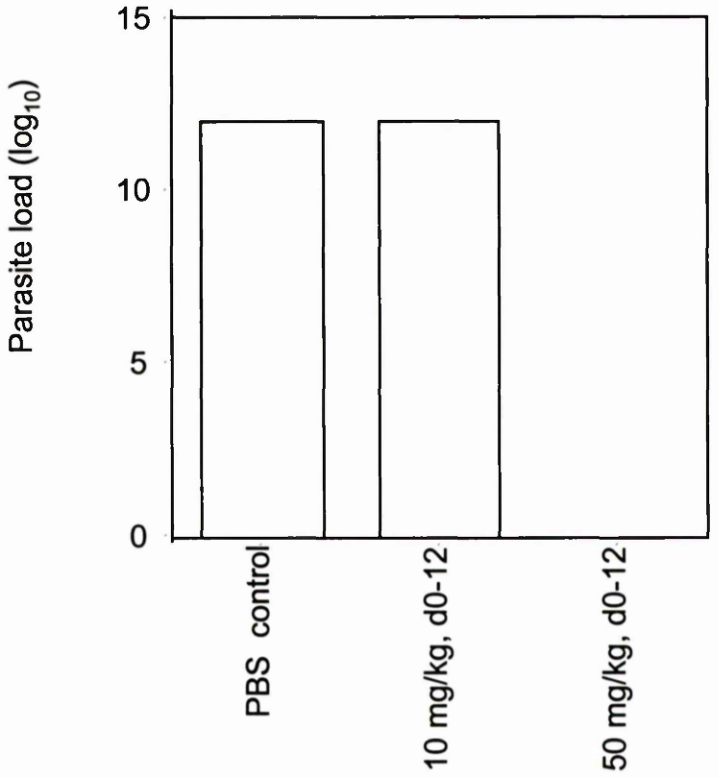
Three groups of BALB/c mice had been infected with *L. major* parasites and injected i.p. twice daily with PBS, 10 mg/kg or 50 mg/kg of gentamicin between day 0 and day 12. On day 79, three out of the six mice in each group were sacrificed, the popliteal lymph node cells, spleen cells, serum and the infected footpads were pooled and analysed for parasite antigen-specific responses.

The analysis of the parasite load by serial limiting dilution assay (section 2.15) confirmed that no parasites were found in the infected footpad of mice treated with 50 mg/kg gentamicin. Comparable numbers of parasites were seen in the footpads of mice treated with the lower dose of gentamicin and PBS (Fig. 6.13 A). Despite this, neither dose of gentamicin had any effect on the *L. major* antigen-

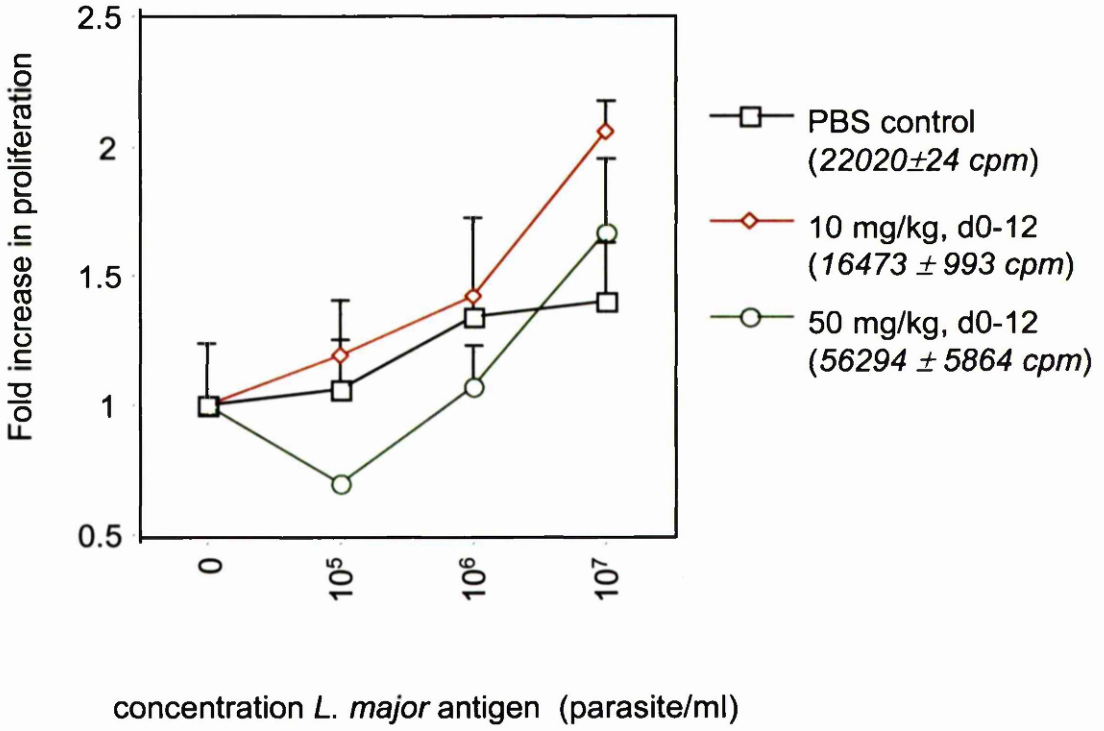
Fig 6.13 Assessment of parasite load and parasite antigen-induced proliferation at a late state of gentamicin-treated *L. major* infection in BALB/c mice

Three mice of trial 1 were sacrificed at day 79. The infected footpads were analysed for parasite load **(A)** by serial limiting dilution assay (section 2.15), culturing the parasites for 3 days in dilutions in complete Schneider's medium. Proliferation **(B)** was assessed by stimulating $1x10^5$ spleen cells with increasing doses of parasite antigen for 3 days and pulsing them with 1µCi ^{3}(H)-Thymidine. *Basal levels* are given in brackets. These results (n=3) are shown as the mean ± 1 SD and presented as fold increase in comparison to untreated cells.

A)


Parasite load (log_{10})
15
10
5
0
PBS control
10 mg/kg, d0-12
50 mg/kg, d0-12

B)


Fold increase in proliferation
2.5
2
1.5
1
0.5
0
10^5
10^6
10^7
concentration *L. major* antigen (parasite/ml)
PBS control
(*22020±24 cpm*)
10 mg/kg, d0-12
(*16473 ± 993 cpm*)
50 mg/kg, d0-12
(*56294 ± 5864 cpm*)

specific proliferation response of spleen cells *in vitro* (Fig. 6.13 B). The cytokine response of spleen cells to parasite antigen was analysed by ELISA. Infected mice injected with PBS responded to increasing parasite antigen doses with increasing production of IFN-γ as well as IL-4, which agrees with previous findings by Morris *et al.* (1992). Spleen cells of mice treated with 10 mg/kg of gentamicin responded with a higher IFN-γ and IL-4 cytokine production than spleen cells from control mice. Spleen cells of mice treated with 50 mg/kg gentamicin responded to increasing parasite antigen doses with no alteration in IFN-γ production, while the IL-4 production was slightly reduced in the presence of the antigen, reaching only 34% of the level of control spleen cells when stimulated with the highest antigen dose (Fig. 6.14).

L. major-specific IgG1 and IgG2a antibodies in the serum were also measured. Th1 cells promote IgG2a antibody production while Th2 cells are capable of stimulating B cells to produce IgG1. Therefore the IgG1/IgG2a ratio is generally accepted as an approximate measure for Th1/Th2 cell activity. As figure 6.15 shows, both isotypes were detectable in serum from the PBS-treated group. Levels of IgG1 were higher than levels of IgG2a. Serum from 10 mg/kg gentamicin-treated mice contained similar amounts of both antibodies (data not shown). Serum of mice that had been treated with 50 mg/kg gentamicin contained significant lower amounts of both IgG1 and IgG2a than the serum from control mice.

At this very late state of disease no sign for a biased Th1 or Th2 response could be found and the defective T cell response could reflect either the lack of sufficient

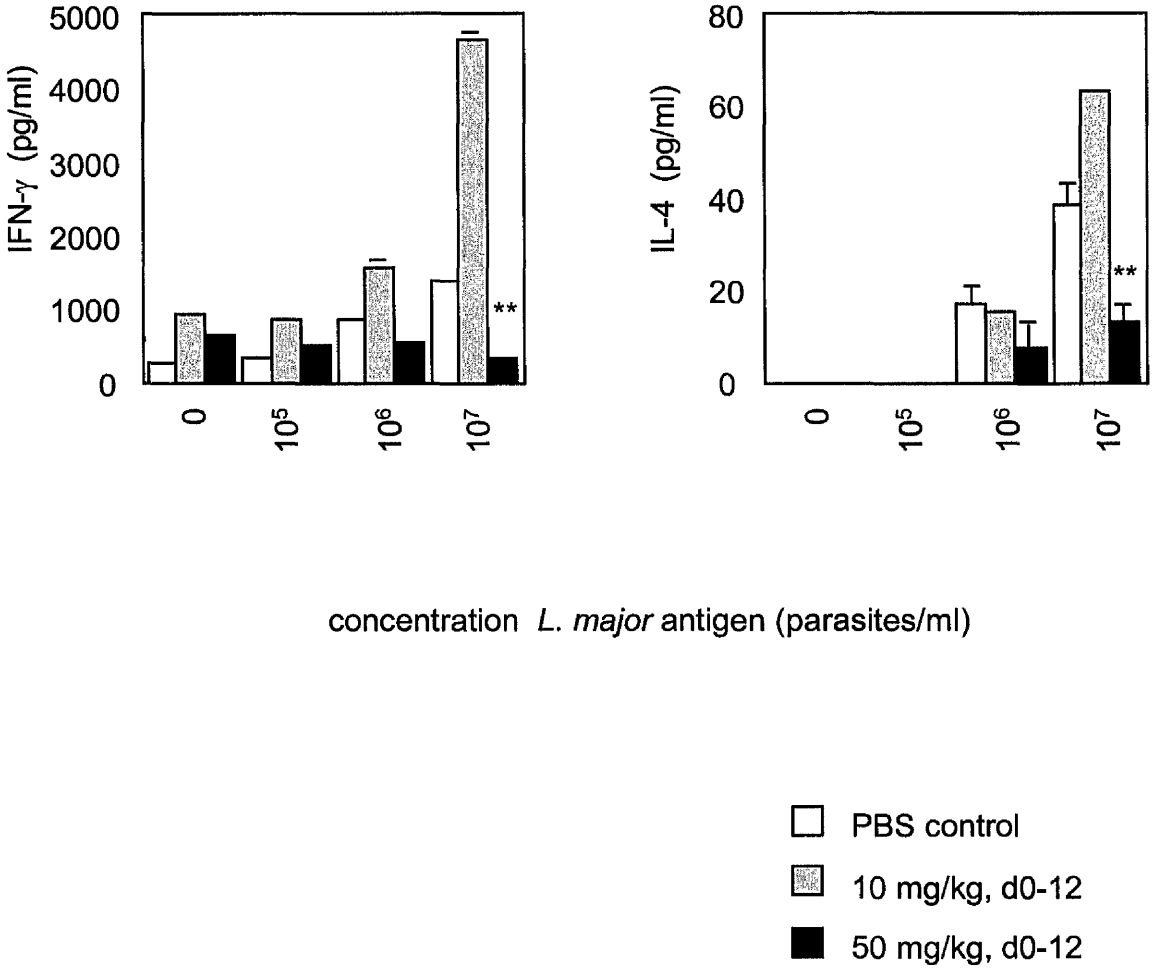


Fig. 6.14 Cytokine production of gentamicin-treated *L. major*-infected spleen cells taken at a late state of disease in response to parasite-antigen

Three mice from each treatment group of trial 1 were sacrificed on day 79 and spleens were collected and pooled. Spleen cells ($1x10^6$/well) were stimulated with increasing doses of parasite antigen for 3 days. Cytokine production was analysed by ELISA. Results (n=3) are shown as mean ± 1 SD. (** $p<0.005$, comparing PBS control with 50 mg/kg gentamicin treatment)

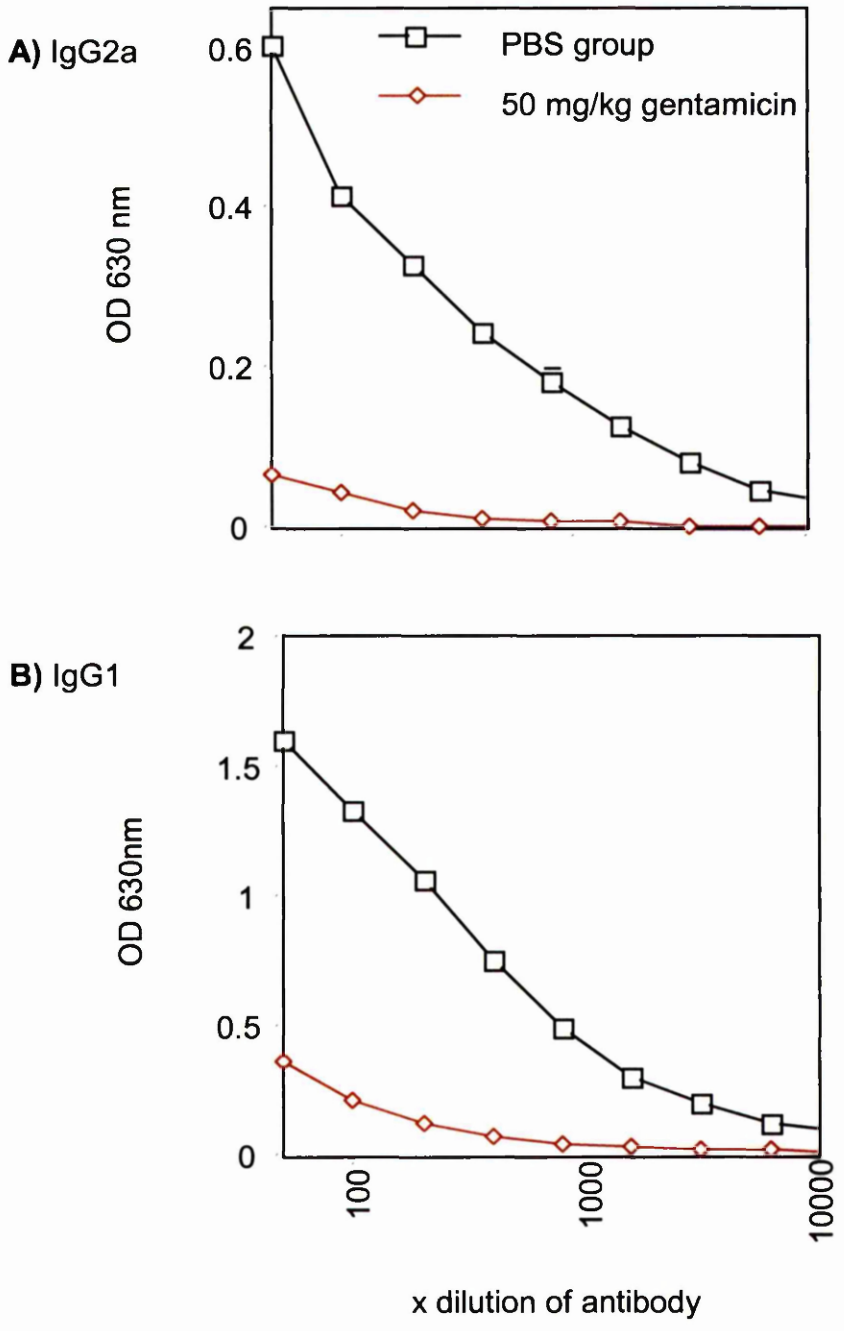


Fig. 6.15 Parasite antigen-specific serum antibody production of a late state of *L. major* infection, treated with gentamicin

Three mice from each treatment group of trial 1 were sacrificed on day 79 and serum was collected, pooled and analysed for parasite antigen-binding IgG2a **(A)** and IgG1 **(B)** antibodies by ELISA (section 2.23). The error bars represent the standard deviation between ELISA triplicates.

antigen to stimulate effector T cells differentiation or toxicity of 50 mg/kg gentamicin to T cells. Therefore, during the following experiment the effects of lower gentamicin concentrations and delayed treatment with the aminoglycoside were analysed during a more acute stage of the disease.

6.5.3 The effects of high doses of gentamicin applied at different stages of *Leishmania major* infection (trial 2)

After establishing that 50 mg/kg reproducibly prevented the onset of *L. major*-mediated disease progression in BALB/c mice, the ability of gentamicin to act as a therapeutic agent after *L. major* infection was examined next. Most successful treatments of *L. major* infection in mice start each prior to or at the time of infection suggesting that they function to influence initial events in T cell differentiation, rather than reversing established T cell responses. For example, IL-12 has to be administered within one week after the infection but not later, leading to a complete cure of Leishmaniasis in susceptible BALB/c mice (Sypek *et al.*, 1993).

Here, five groups of 6 BALB/c mice were treated i.p. as follow:

1. From the day of infection for 12 days with PBS.
2. From the day of infection for 12 days with 25 mg/kg of gentamicin.
3. From the day of infection for 12 days with 50 mg/kg of gentamicin.
4. From day 7 post-infection for 7 days with 50 mg/kg of gentamicin.
5. From the day that swelling became visible for 12 days with 50 mg/kg of gentamicin.

50 mg/kg of gentamicin prevented the growth of *L. major* if administered from the day of infection (Fig. 6.16 A). A lower dose (25 mg/kg) controlled the infection over 100 days, after which rapid swelling of the footpads (indicative of parasite multiplication) occurred (Fig. 6.16 B).

If Th1 cells had been involved in protection elicited by gentamicin to *L. major* parasites, one might expect that memory Th1 cells could prevent a reinfection. Therefore, mice that had been treated with 50 mg/kg of gentamicin from day 0 onwards and had overcome an initial challenge were reinfected with the parasite on day 91 (Fig. 6.16 B). After 3 weeks an increase in swelling was apparent, which increased steadily to the level seen in the PBS-treated mice, suggesting the absence of *L. major* specific memory Th1 cells.

A transient protection was achieved if mice were injected with 50 mg/kg of gentamicin from day 7 after infection. This treatment regime delayed detectable footpad swelling for 55 days, but after this period the increase in footpad swelling was similar to the PBS group (Fig. 6.17 A). Next, the ability of gentamicin to act therapeutically was examined. Mice were infected with the parasite and treated with gentamicin only after footpad swelling had reached 0.5 mm. The treatment decreased the swelling dramatically, but after the end of injections the disease progressed again as shown in Fig. 6.17 B. The trial was repeated using a 5 x higher parasite dose (trial 3). As shown in Fig. 6.18, the results were nearly identical. Gentamicin injections, started when open lesions had already appeared, decreased the swelling dramatically and closed the lesions. However, the swelling

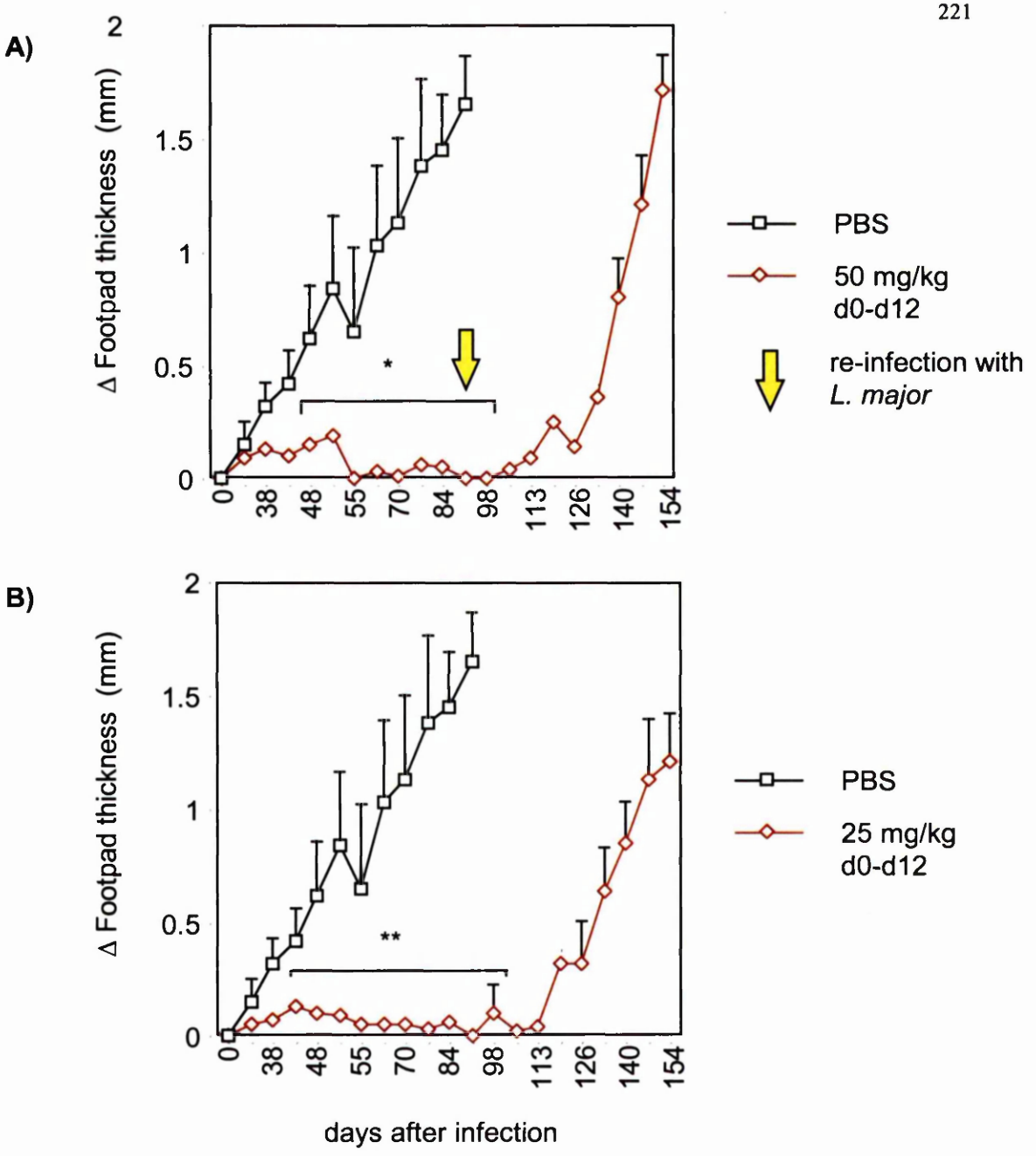


Fig. 6.16 Gentamicin treatment of *L. major*-infected BALB/c mice (trial 2)

Three groups of 6 mice were infected in the left footpad on day 0 with $1x10^5$ parasites/mouse and injected (i.p.) from day 0 - 12 with PBS, 50 mg/kg **(A)** or 25 mg/kg of gentamicin **(B)** twice a day. After 87 days, the group treated with 50 mg/kg of gentamicin was reinfected with the same parasite dose. The footpad swelling data show the difference between the infected left and the uninfected right footpad and are presented as the mean ± 1 SD (** $p<0.005$, * $p< 0.05$ comparing PBS control with gentamicin treatment).

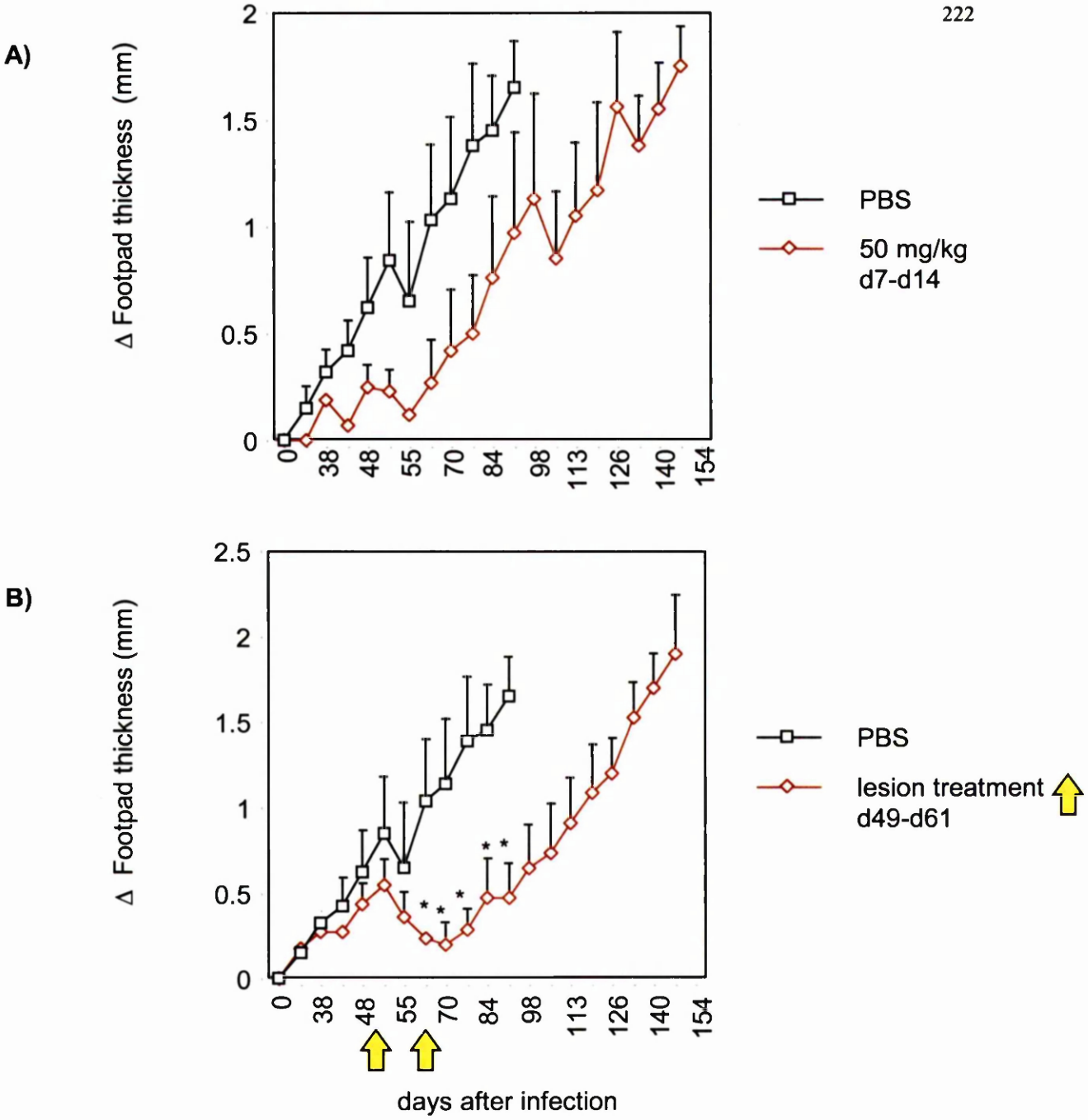


Fig. 6.17 Gentamicin treatment of *L. major*-infected BALB/c mice (trial 2)

Three groups of 6 mice were infected in the left footpad on day 0 with $1x10^5$ parasites/mouse and injected (i.p.) twice per day from day 7 - 14 with PBS and 50 mg/kg gentamicin **(A)** or when swelling appeared for 12 days with 50 mg/kg of gentamicin **(B)**. The footpad swelling data shown are the difference between the infected left and the uninfected right footpad and the mean ± 1 SD (* $p< 0.05$ comparing PBS control with gentamicin treatment).

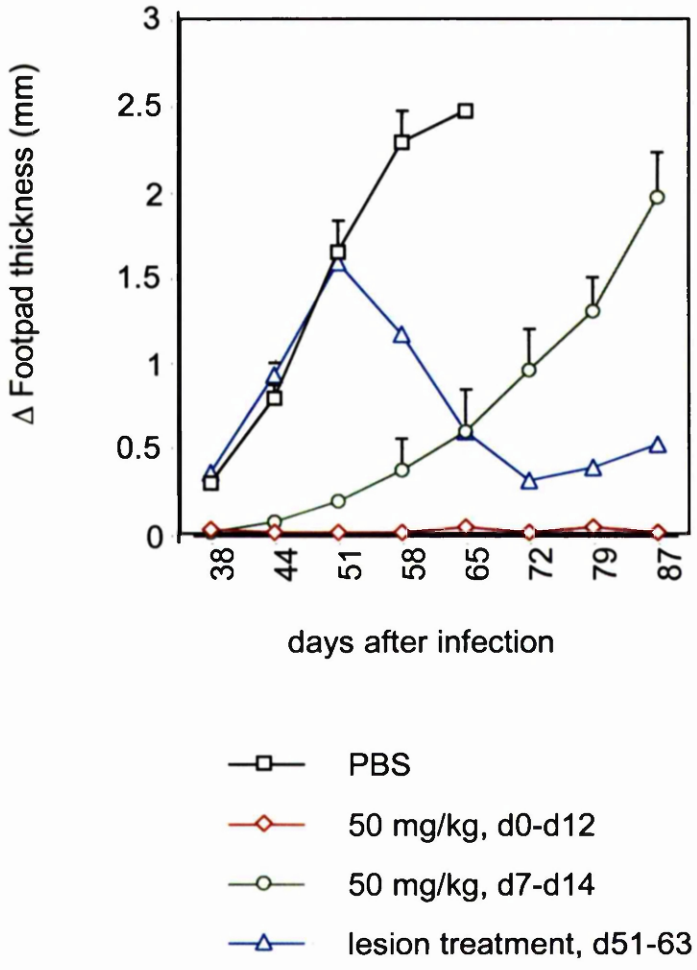


Fig 6.18 Gentamicin treatment of *L. major*-infected BALB/c mice (trial 3)

Four groups of 12 mice were infected in the left footpad on day 0 with $5x10^5$ parasites/mouse and injected (i.p.) twice per day from day 0 - 14 with PBS or 50 mg/kg gentamicin or from day 7 - 14 with 50 mg/kg gentamicin or when lesions appeared for 12 days with 50 mg/kg gentamicin twice a day. The footpad swelling data show the difference between the infected left and the uninfected right footpad and are presented as the mean ± 1 SD.

increased and the lesion relapsed soon after cessation of the gentamicin treatment.

6.5.4 Assessment of immunological parameters during the acute state of gentamicin-treated Leishmaniasis (trial 3)

BALB/c mice were infected with *L. major* and treated twice daily with PBS or gentamicin (50 mg/kg) from day 0-12 or from day 7-14 or when the swelling became visible (day 51-63). On day 54, three mice of the PBS group, 50 mg/kg (d0) group and the 50 mg/kg (d7) group were sacrificed and immunological parameter analysed. On day 67, three mice of the PBS group and the lesion treatment group were sacrificed and the response of spleen, lymph node cells and serum antibody levels against parasite antigen was analysed. Changes in cell populations (CD4(+), CD8(+) T cells, CD19(+) B cells or macrophages) under the influence of gentamicin were monitored by flow cytometry.

At this point, footpad swelling of the PBS group was strongly increasing, while footpad swelling of the 50 mg/kg gentamicin group was not detectable. Mice treated after day 7 of infection showed signs of footpad swelling. Mice with open lesions that had subsequently received gentamicin showed lesion healing and a reduction in swelling.

The parasite load in all gentamicin-treated groups was considerably lower than in the PBS control group. Mice that received 50 mg/kg of gentamicin from day 0 had a 10-fold lower parasite number than their control litter mates. When this treatment regime started from 7 day post-infection, a 3-fold reduction in parasites

was achieved. Gentamicin treatment given just after lesions had become apparent reduced the number of parasites 5-fold in comparison to the PBS control group (Fig. 6.19).

In Fig. 6.20, the response of popliteal lymph node cells to parasite antigen was examined. In a parallel experiment, spleen cells responded in a similar fashion (data not shown). No proliferative response to increasing doses of *L. major* antigen was seen with lymph node cells treated with 50 mg/kg gentamicin from day 0. Proliferation of lymph node cells from mice that were treated with gentamicin 7 day post-infection was similar to that of cells from PBS-treated mice (Fig. 6.20 A). Lymph node cells from mice that were treated with gentamicin after the appearance of open lesions had approximately a 3-fold increase in proliferation compared to cells from the control group (Fig. 6.20 B).

To test whether gentamicin treatment caused a general impairment of T cells proliferation, independent of their ability to react to parasite antigen, lymph node cells of mice treated with gentamicin or PBS were stimulated with 4 μg/ml plate bound anti-CD3 (Fig. 6.21). No difference in response to anti-CD3 between the PBS group and mice treated with 50 mg/kg from day 0 was detected. These data show that gentamicin had a selective effect on parasite antigen-reactive T cell expansion.

To exclude the possibility of a non-specific T cell response to parasite antigen, cells of uninfected mice were compared to cells of *L. major*-infected, PBS-treated mice (Fig. 6.22). Proliferation of lymph node cells from uninfected mice increased slightly in response to parasite antigen but reached only 10% of the proliferation

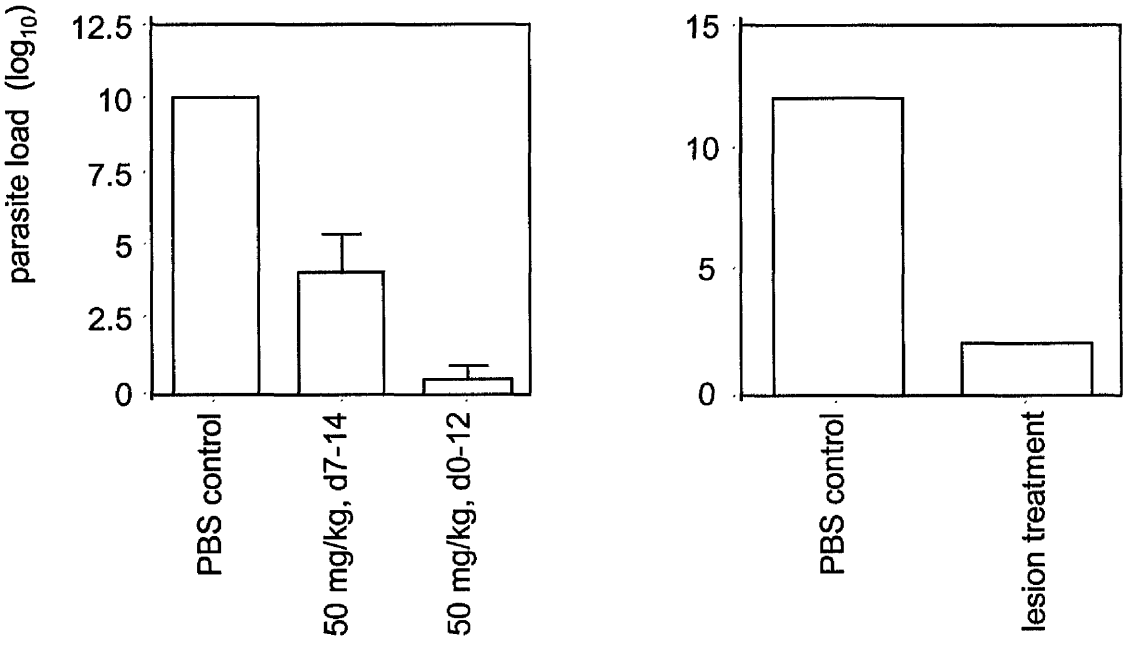


Fig. 6.19 Assessment of parasite load of gentamicin-treated *L. major*-infected BALB/c mice at a late state of infection (trial 3)

Three mice of each treatment group from trial 3 were sacrificed on day 54 and the parasites in the infected footpads were pooled and analysed by the serial limiting dilution assay (section 2.15). The results (n=3) are shown as the mean ± 1 SD.

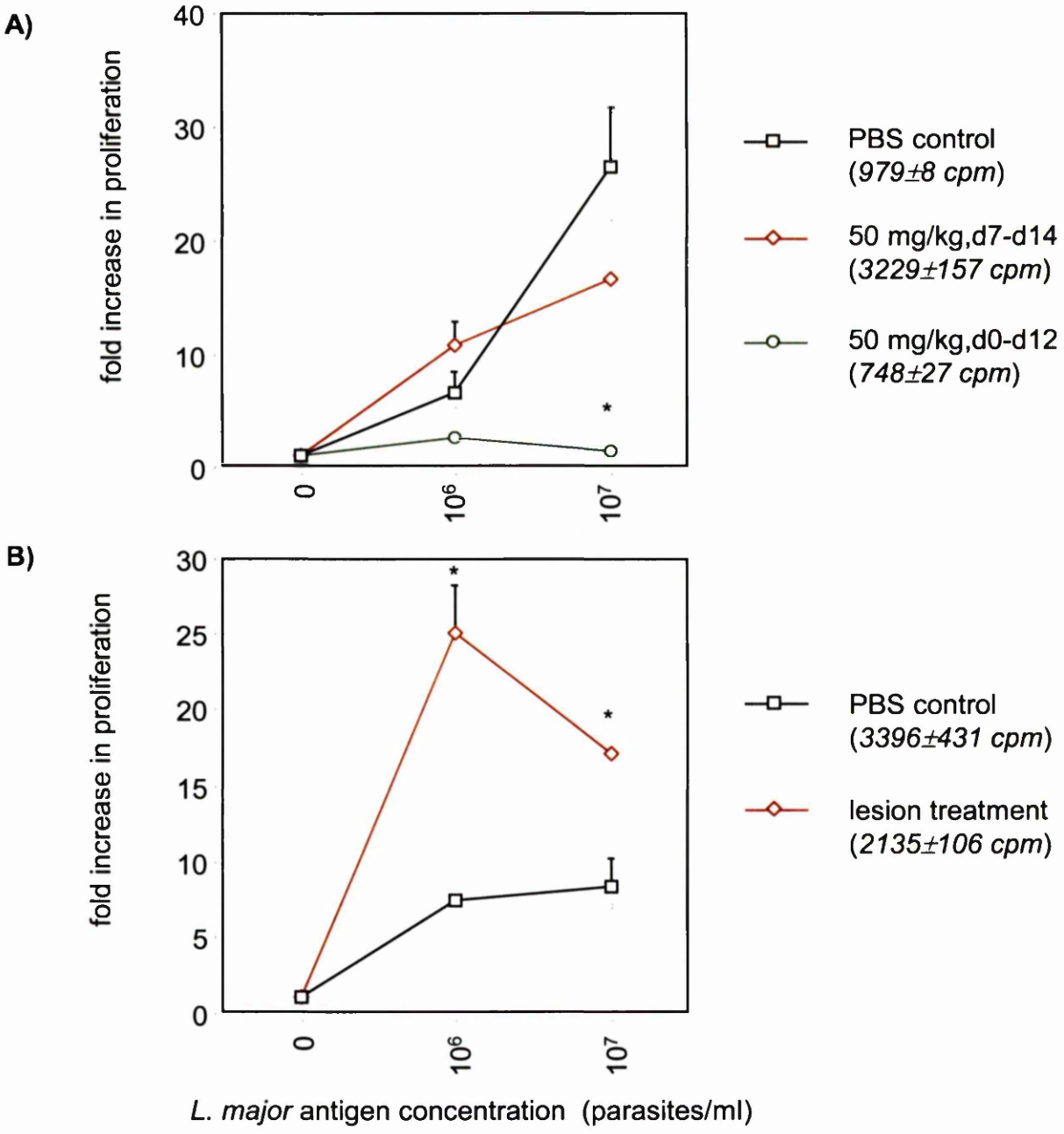


Fig. 6.20 T cell proliferative response of gentamicin-treated, *L. major*-infected BALB/c mice to parasite antigen (trial 3)

Three mice from each treatment group of trial 3 were sacrificed on day 54. The popliteal lymph node cells of each group were pooled and stimulated with increasing doses of parasite antigen for 3 days before ^{3}(H)-Thymidine uptake was measured. The *basal levels* of proliferation are shown in brackets. Results (n=3) are shown as mean ± 1 SD and as fold increase in comparison to unstimulated cells.

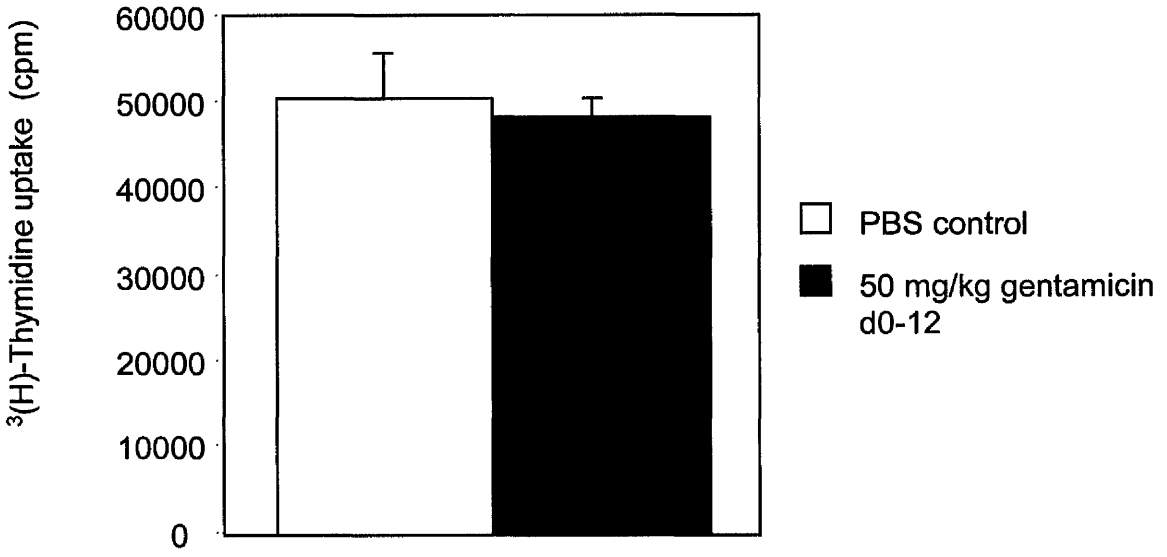


Fig. 6.21 T cell proliferative response of PBS versus gentamicin-treated popliteal lymph node cells derived *from L. major*-infected BALB/c mice (trial 3) to anti-CD3

Lymph node cells ($1x10^5$/well) of 3 mice, sacrificed on day 54 of trial 3, were pooled and stimulated with 4 µg/ml anti-CD3 Ab for 3 days before the cells were pulsed and ^{3}(H)-Thymidine uptake was measured. The results (n=3) are shown as the mean ± 1 SD.

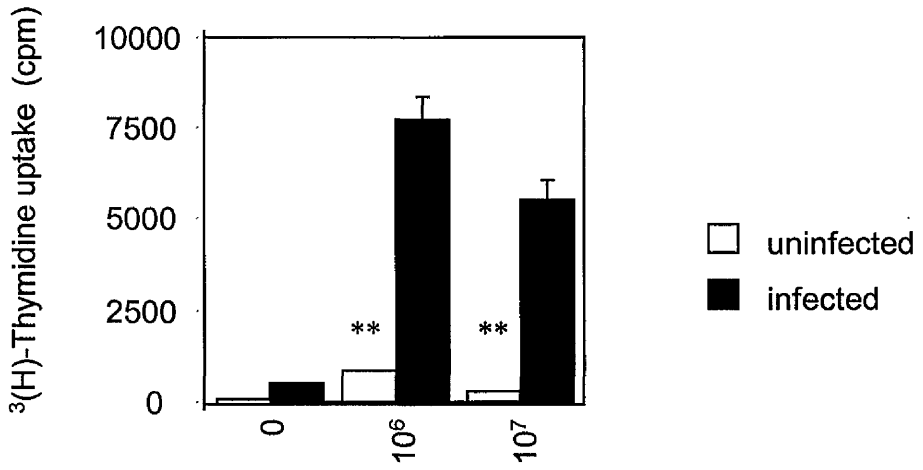


Fig. 6.22 Proliferative response of *L. major*-infected and uninfected lymph node cells to parasite antigen

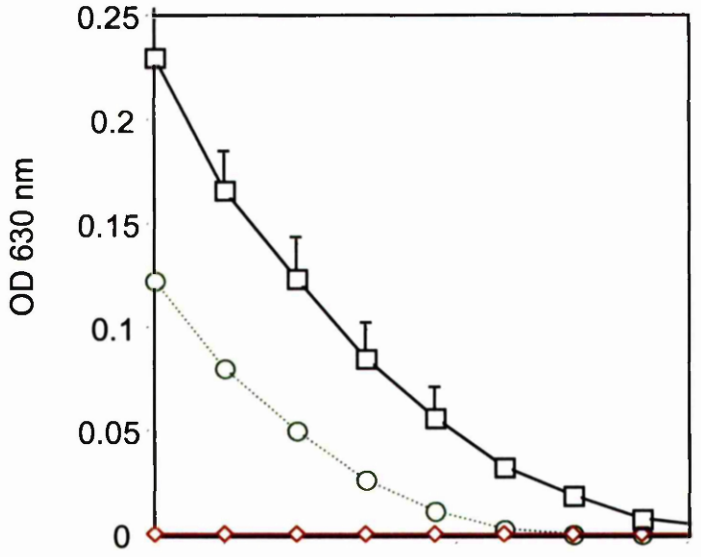
Lymph nodes of three uninfected and three *L. major*-infected BALB/c mice, sacrificed on day 54 of trial 3, were stimulated for 3 days with increasing doses of parasite antigen. Cell proliferation was measured by ^{3}(H)-Thymidine uptake. Data (n=3) are mean ± 1 SD (**$p<0.005$).

level of infected cells. This indicates a small non-antigen-specific response did occur but the magnitude of the response was not sufficient to interfere with parasite-specific responses.

Both parasite-specific IgG1 and IgG2a antibody levels were found in serum of the PBS group (Fig. 6.23). In serum from mice treated with the high dose of gentamicin from day 0 no IgG2a and significant lower amounts of IgG1 were detected compared to control serum or serum from mice treated from day 7. The serum of mice treated when lesions had appeared, showed no significant difference in IgG1 or IgG2a levels compared with the PBS controls (Fig. 6.24). Therefore, early gentamicin treatment diminished both IgG1 and IgG2a antigen-specific antibody responses.

To monitor the effect of gentamicin on the Th1/Th2 cell balance, Th1 and Th2 cell-specific cytokines were measured. All measured cytokines were produced by infected lymph node and spleen cells after culture with *L. major* antigen. Th1 cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-4 and IL-6) were expressed at similar levels in cells from the PBS control group and the 50 mg/kg gentamicin-treated group (d7). Only IL-10 expression was decreased by gentamicin treatment. However, cells from mice injected with 50 mg/kg gentamicin from day 0 showed no expression of the above cytokines. To investigate whether the suppression of cytokines was due to regulatory T cells (Tr1, Th3), the production of IL-10 and latent TGF-β were examined (Groux *et al.*, 1997). IL-10 was not expressed by lymph node cells treated early with gentamicin, whilst *de novo* TGF-β synthesis was not affected by an increasing *L. major* antigen concentration (Fig. 6.25).

A) IgG2a



B) IgG1

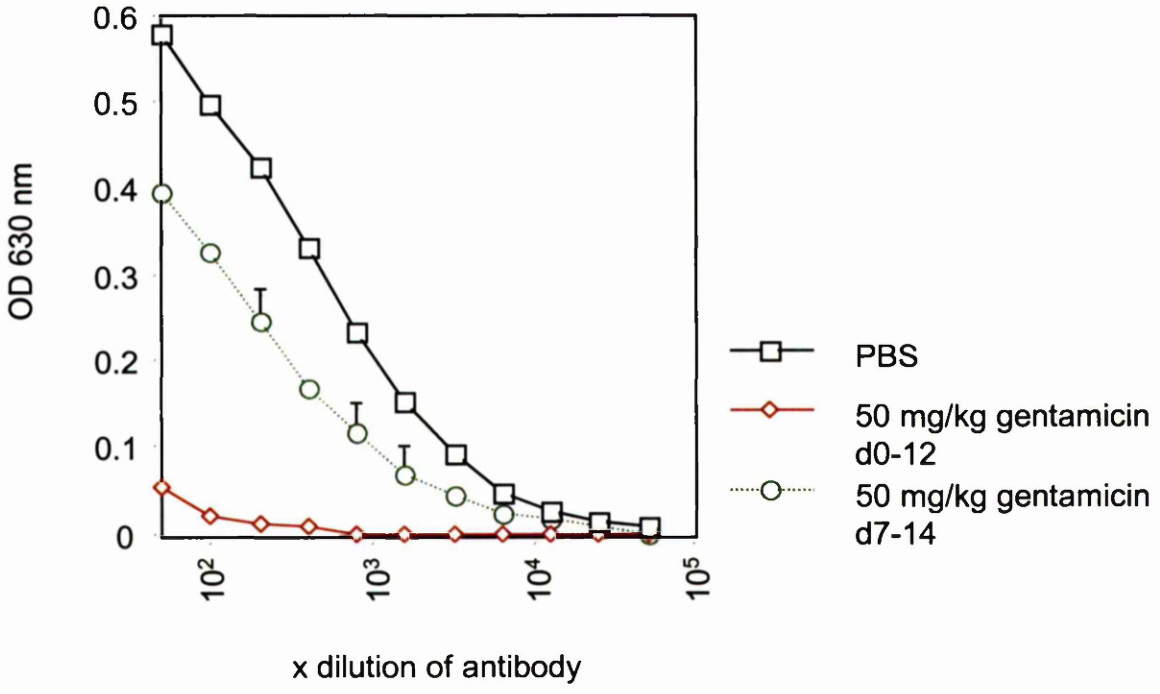


Fig. 6.23 Assessment of parasite-specific antibody production at an acute state of gentamicin-treated *L. major* infection in BALB/c mice (trial 3)

Sera of 3 mice of each indicated treatment group, sacrificed on day 54 (trial 3), were pooled and analysed for parasite-specific IgG2a **(A)** and IgG1 **(B)** antibodies (section 2.23). The error bars represent the standard deviation between ELISA triplicates.

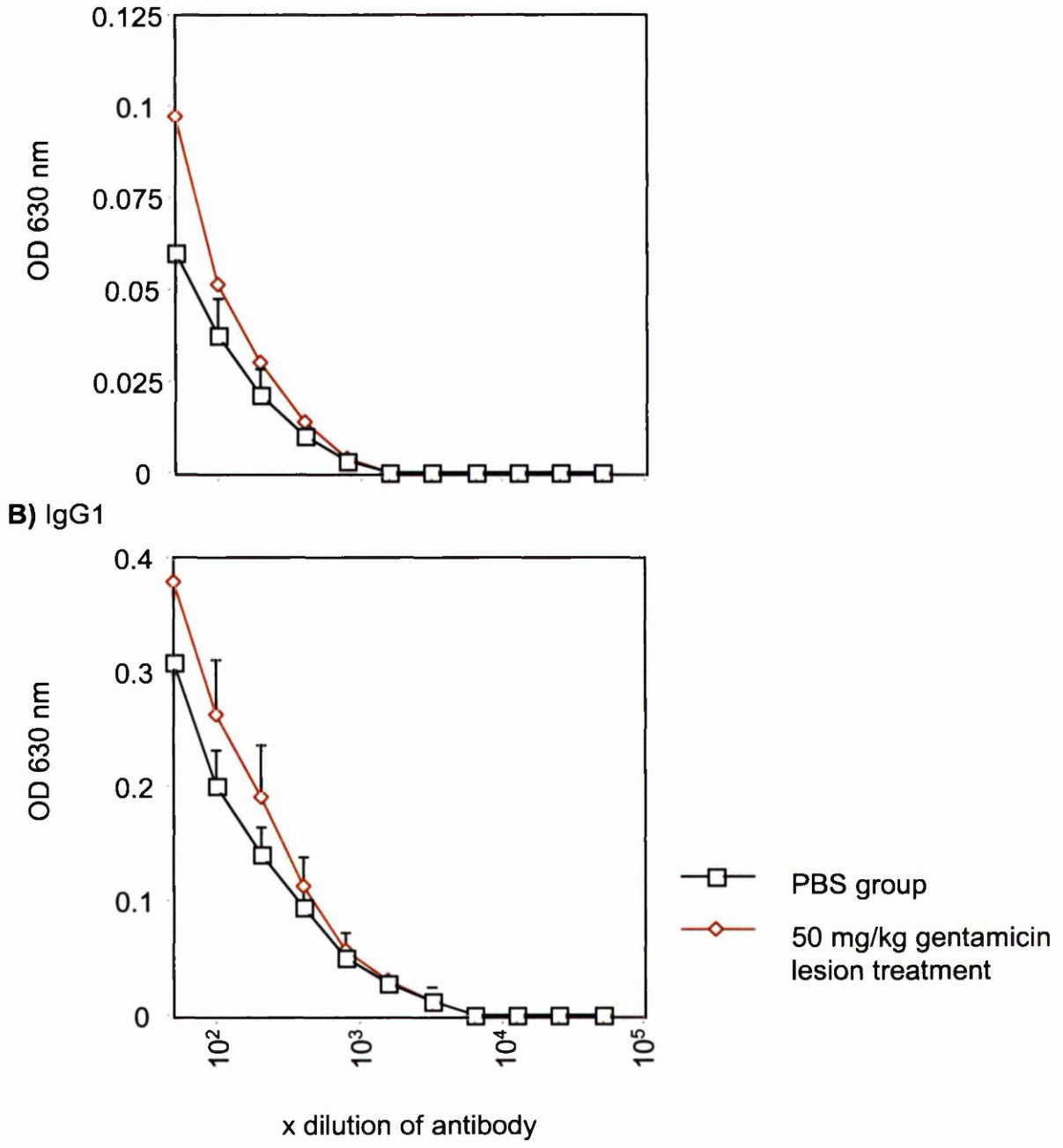


Fig. 6.24 Assessment of parasite-specific antibody production at an acute state of gentamicin-treated *L. major* infection in BALB/c mice (trial 3)

Three mice of each treatment group of trial 3 were sacrificed on day 67 and the sera pooled. The serum was analysed for parasite antigen-binding IgG2a **(A)** and IgG1 **(B)** antibodies by ELISA (section 2.23). The error bars represent the standard deviation between ELISA triplicates.

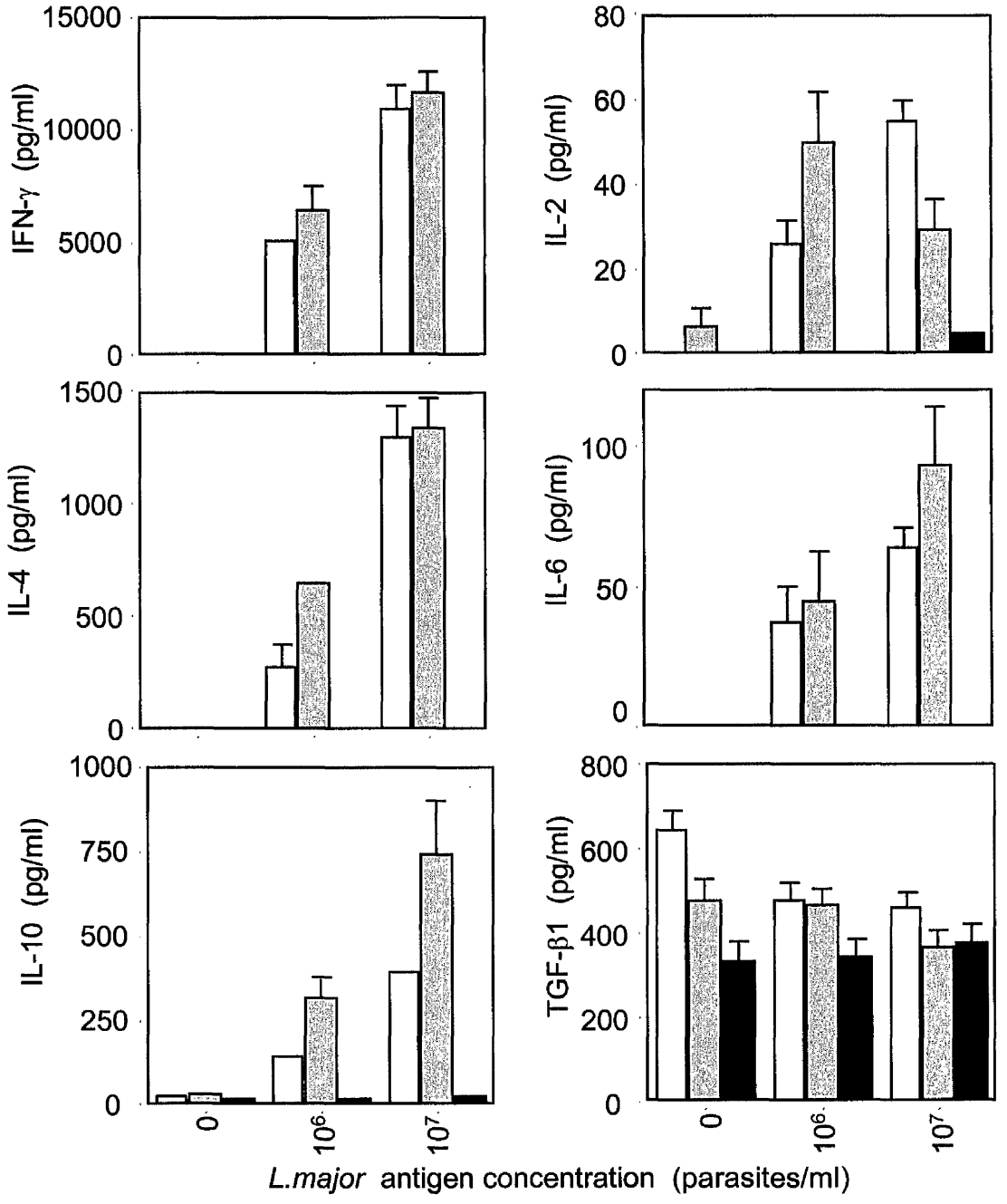


Fig. 6.25 Cytokine production of gentamicin-treated *L. major*-infected mice in response to parasite antigen (trial 3)

□ PBS control
▨ 50 mg/kg, d7
■ 50 mg/kg, d0

Three mice each treatment group were sacrificed on day 54 of trial 3 and popliteal lymph nodes and spleens were collected and pooled. $1x10^6$ lymph node cells or spleen cells (for IL-6) were stimulated with parasite antigen for 3 days and culture supernatants assayed for cytokines by ELISA. The error bars represent the standard deviation between ELISA triplicates.

These results suggest that the involvement of regulatory T cells in the suppression process was unlikely. Further, they suggest that treatment of mice with the high dose gentamicin at the time of infection eliminated parasite-specific T cells.

Parasite-specific IL-2 produced by lymph node cells from mice treated with gentamicin after lesion onset was found to be 1.4-2.6 times higher than the IL-2 level of the PBS-treated group. Th1 and Th2 type cytokines including IFN-γ, IL-4, IL-6, IL-10 were expressed to a lower level in the gentamicin-treated mice than in the PBS controls. TGF-β expression was not significantly different between the two groups and did not alter with an increasing antigen concentration (Fig. 6.26).

Early gentamicin treatment reduced *L. major*-specific proliferation and antibody response as well as cytokine production. Flow cytometry analysis was used to investigate whether these changes were due to changes in spleen cell subsets such as CD4(+) T cells, CD8(+) T cells, B cells (CD19(+)) or macrophages (FA11(+)). Pooled spleen cells from three PBS-injected and *L. major*-infected mice were compared with those from three *L. major*-infected mice treated with gentamicin from day 0. Gentamicin did not alter the percentage of CD4(+) cells or CD8(+) cells. The percentage of CD19(+) cells increased slightly by 5 % (38.74% in PBS-treated group compared to 43.76% in gentamicin-treated group), and the percentage of FA11(+) cells increased by 10% (20.06% in PBS-treated group compared to 30.49% in gentamicin-treated group) (Table 6.1). To check, whether gentamicin functioned by inducing cell death spleen cells were double stained for annexin V and cell surface markers. This revealed a 10% increase in apoptotic B

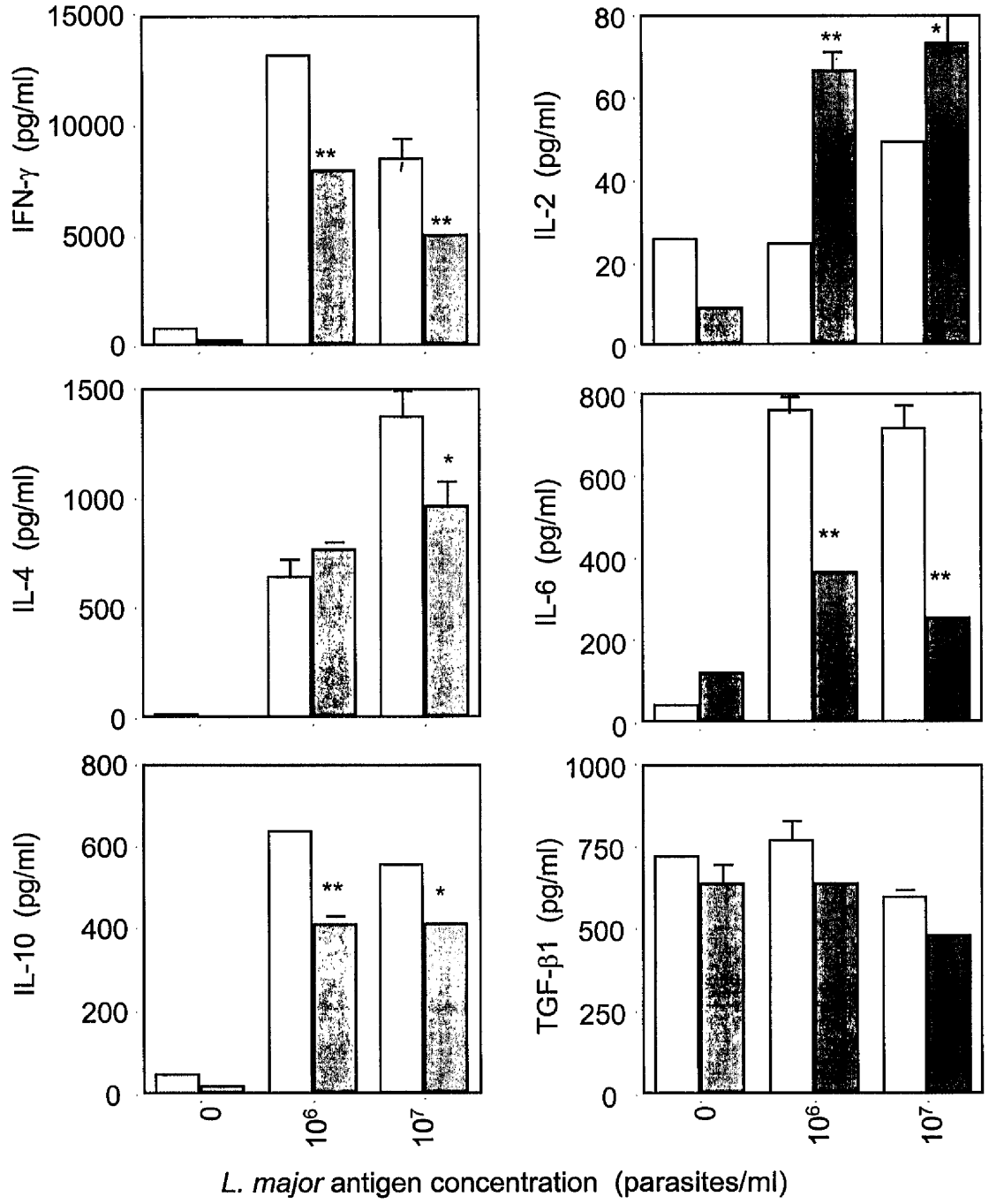


Fig. 6.26 Cytokine production of gentamicin-treated *L. major*-infected mice in response to parasite antigen (trial 3)

Three mice per group were sacrificed on day 54 of trial 3 and popliteal lymph nodes and spleens were pooled. $1x10^6$ lymph node cells or spleen cells (for IL-6) were stimulated with parasite antigen for 3 days before ELISA was performed. Data (n=3) are shown as mean ± 1 SD (**p<0.005; * p< 0.05).

Cell surface marker	PBS-treated	Gentamicin-treated
CD4(+)	9.48 %	10.19 %
CD8(+)	21.47 %	19.9 %
CD19(+)	38.74 %	43.76 %
CD19(+)/annexinV(+)	15.44 %	26.58 %
FA11(+)	20.06 %	30.49 %

Table 6.1. The effects of gentamicin on cell populations of *L. major*-infected BALB/c at an acute state of the disease (trial 3)

BALB/c mice were infected on day 0 with $5x10^5$ *L. major* parasites/mouse. Gentamicin (50 mg/kg) was injected i.p. twice daily from day 0 to day 12. On day 54, three mice were sacrificed and their spleen cells analysed for cell surface marker staining.

cells among gentamicin-treated cells (gated for $CD19^{high}$/$annexinV^{high}$, 15.44% in PBS-treated group versus 26.58% in gentamicin-treated group, Table 6.1).

When comparing size (FSC) and granularity (SSC) of the spleen cell population of gentamicin-treated versus PBS-treated cells, a lower number of bigger sized cells with higher granularity in region 2 (R2) and region 3 (R3) was observed in cells from gentamicin-treated mice (Fig. 6.27). In spleen cells from PBS-treated mice, three times more cells accumulate in R2 and R3 than in spleen cells from gentamicin-treated mice (14.59% in PBS-treated mice versus 5.51% in gentamicin-treated mice). According to the expression of their cell surface marker the population in R2 consisted mainly of MHCII(+) cells, CD4(+) and CD8(+) T cells and some CD19(+) B cells. Region 3 contained mainly MHCII(+) cells (Fig. 6.28). Taken together, these results suggest that gentamicin treatment might have suppressed proliferating MHCII expressing cells, CD4(+) and CD8(+) T lymphoblasts and CD19(+) B cells (Longobardi, 1992; Komai-Koma and Wilkinson; 1997).

6.5.5 Analysis of early events in gentamicin-treated *L. major* infection

The analysis of the late state of gentamicin-treated *L. major* infection suggested a general suppression of antigen-specific immune responses. To determine whether there was any *in vivo* evidence for selective suppression of Th2 over Th1 cells by gentamicin, mice were treated with the aminoglycoside for a shorter time period and were analysed at early timepoints after infection with *L. major* parasites. Two groups of 6 mice were infected with $1x10^5$ parasites per mouse and injected i.p.

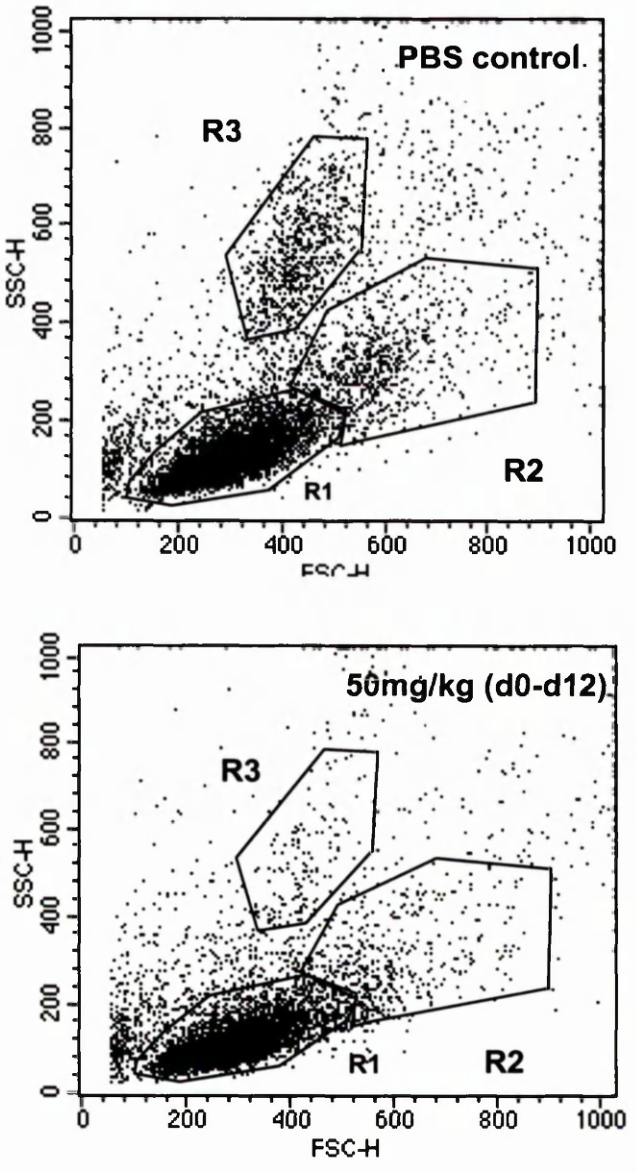
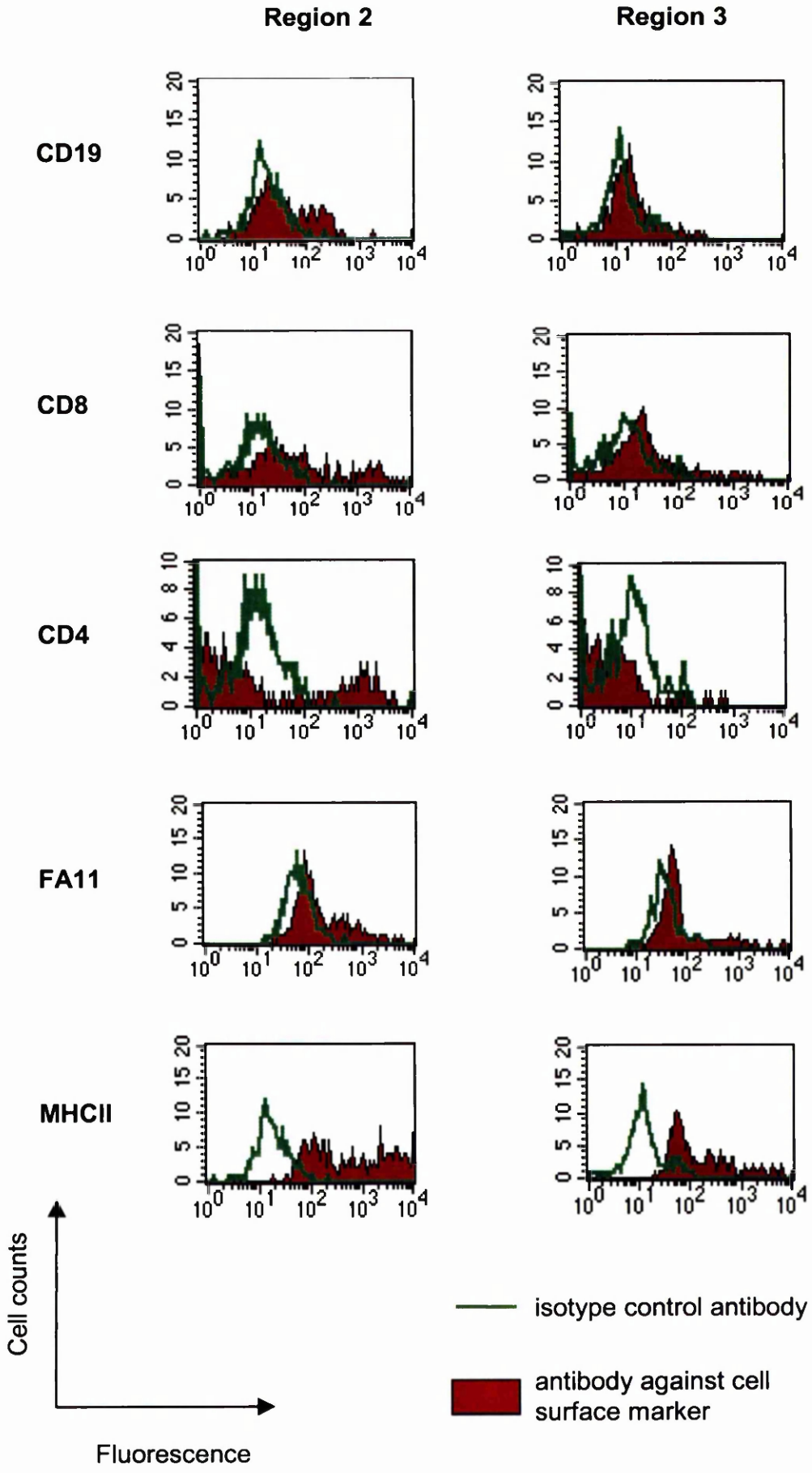


Fig. 6.27 Gentamicin treatment suppresses cell populations of bigger size and granularity

On day 54 after *L. major* infection (trial 3) pooled spleen cells of 3 mice treated with PBS or 50 mg/kg gentamicin (d0-12) were analysed by flow cytometry. The figure shows Forward Scatter (FSC) and Side Scatter (SSC) representing size and granularity of cells.

Fig. 6.28 Flow cytometry analysis of cell surface marker expression of cells in R2 and R3

Spleen cells, gated in region 2 and 3, were analysed for cell surface marker expression. Cells were analysed for CD19 (B cells), CD8 (Tc cells), CD4 (Th cells), FA11 (macrophages) and MHCII (APC) expression.


Region 2
Region 3
CD19
CD8
CD4
FA11
MHCII
Cell counts
Fluorescence
isotype control antibody
antibody against cell surface marker

with either gentamicin (50 mg/kg) or PBS for 7 days twice a day. Two mice of each group were sacrificed on day 3, 7 and 10. No footpad swelling was apparent at any of these timepoints.

When spleen and lymph node cells were stimulated with *L. major* antigen, no production of Th1/Th2 cytokines could be detected. However, when stimulated with 4 μg/ml plate-bound anti-CD3, proliferation levels of spleen cells, treated with gentamicin and taken on day 10 after infection, were similar to proliferation levels of PBS-treated spleen cells (23% increase in gentamicin-treated group). IL-2 levels in the gentamicin-treated group were twice that of the control group. IFN-γ levels were comparable between control and treated group. Th2 cytokines were downregulated in mice injected with gentamicin, IL-4 to 74% and IL-5 to 33% of their control levels (Fig. 6.29). Similar results were achieved with spleen cells taken after 7 days of gentamicin treatment (data not shown).

10 days after infection *L. major*-specific serum antibodies were detectable. IgG1 antibodies were impaired (to 36% of the control level at a 1/16 dilution), while IgG2a antibodies were expressed equally in both groups (Fig. 6.30).

As assessed by flow cytometry, gentamicin did not alter the number of CD4(+), CD8(+) T cells or B cells in the spleen 10 days after infection and only a slight decrease in the number of macrophages was apparent as a result of gentamicin treatment. Among popliteal lymph node cells, situated close to the infection, an 11% decrease in B cells and small decrease in macrophages was detectable (Table 6.2).

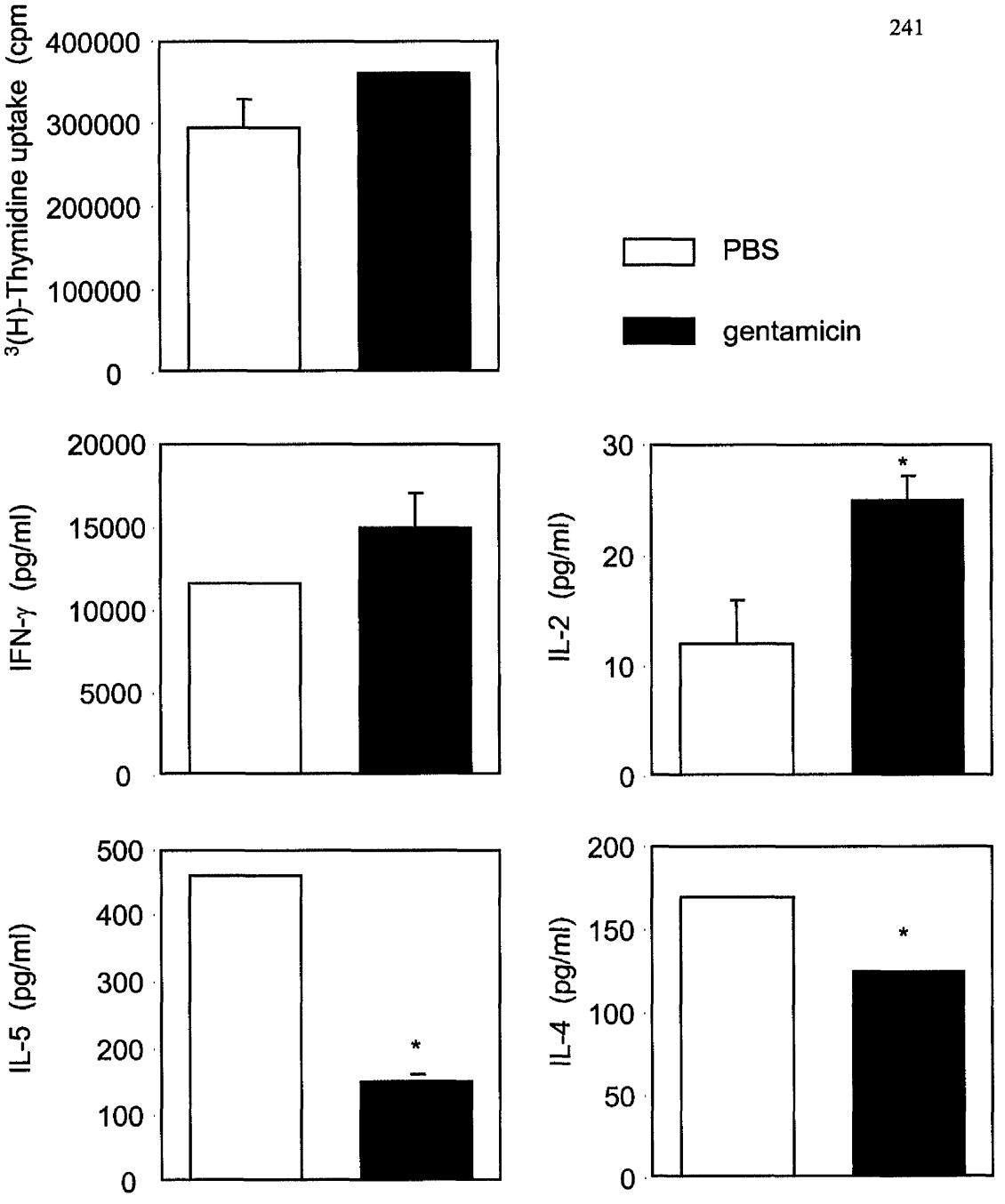


Fig. 6.29 Cytokine and proliferative response of gentamicin-treated *L. major*-infected BALB/c mice at an early state of disease (trial 4)

Spleen cells of 2 groups of three mice, infected with $5x10^5$ parasites/mouse and treated for 7 days twice a day with PBS or 50 mg/kg gentamicin, were collected on day 10, pooled and stimulated with 4 µg/ml anti-CD3 Ab for a further 3 days. Cytokines were assessed by ELISA and cell proliferation by ^{3}(H)-Thymidine uptake. Results (n=3) are shown as mean ± 1 SD (* $p<0.05$).

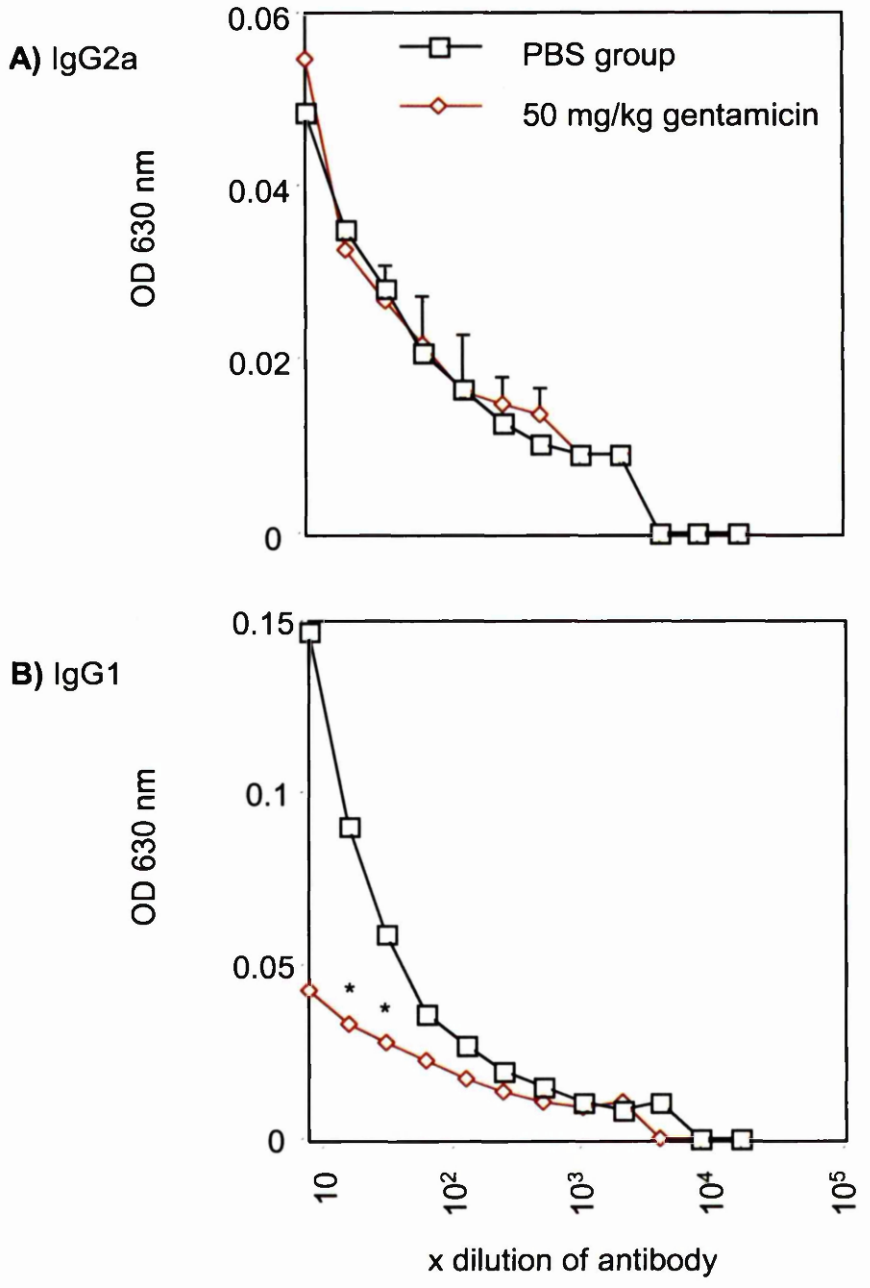


Fig. 6.30 Parasite antigen-specific antibody response of gentamicin-treated *L. major*-infected BALB/c mice at an early state of disease (trial 4)

Sera of two groups of 3 mice, infected with $5x10^5$ parasites/mouse and treated for 7 days twice a day with PBS or 50 mg/kg of gentamicin were collected on day 10 of infection and the IgG2a **(A)** and the IgG1 **(B)** antibody production were analysed by ELISA (section 2.23). Results (n=3) are shown as mean ± 1 SD (* $p<0.05$).

Cell surface marker	Uninfected spleen		Uninfected LN		Infected spleen		Infected LN	
	Gentamicin (%)		Gentamicin (%)		Gentamicin (%)		Gentamicin (%)	
	-	+	-	+	-	+	-	+
CD4(+)	22.61	26.24	NA	NA	27.29	26.76	41.0	44.86
CD8(+)	40.87	39.83	NA	NA	10.79	10.14	13.36	15.82
CD19(+)	10.43	10.65	NA	NA	45.91	48.34	40.87	29.81
FA11(+)	17.55	13.13	NA	NA	28.33	22.21	14.67	11.39

Table 6.2. Comparison of the effect of gentamicin on *L. major*-infected and uninfected BALB/c mice

BALB/c mice were infected with $5x10^5$ parasites/mouse or were kept uninfected. 50 mg/kg of gentamicin was injected twice daily for 7 days. On day 10, three mice were sacrificed and spleen and lymph node (LN) cells were pooled and analysed for the expression of cell surface markers by flow cytometry. (NA: not analysed)

Using size and granularity, a 56% smaller population of enlarged cells in lymph nodes from infected and gentamicin-treated mice was found (Fig. 6.31). Analysis of cell surface markers in this subset (R2) revealed, these cells were mainly CD19(+) and CD4(+) cells. Consequently, early after *L. major* infection gentamicin appeared to cause impairment in the recruitment of activated CD4(+) T lymphoblasts and B effector cells (Longobardi, 1992; Komai-Koma and Wilkinson; 1997).

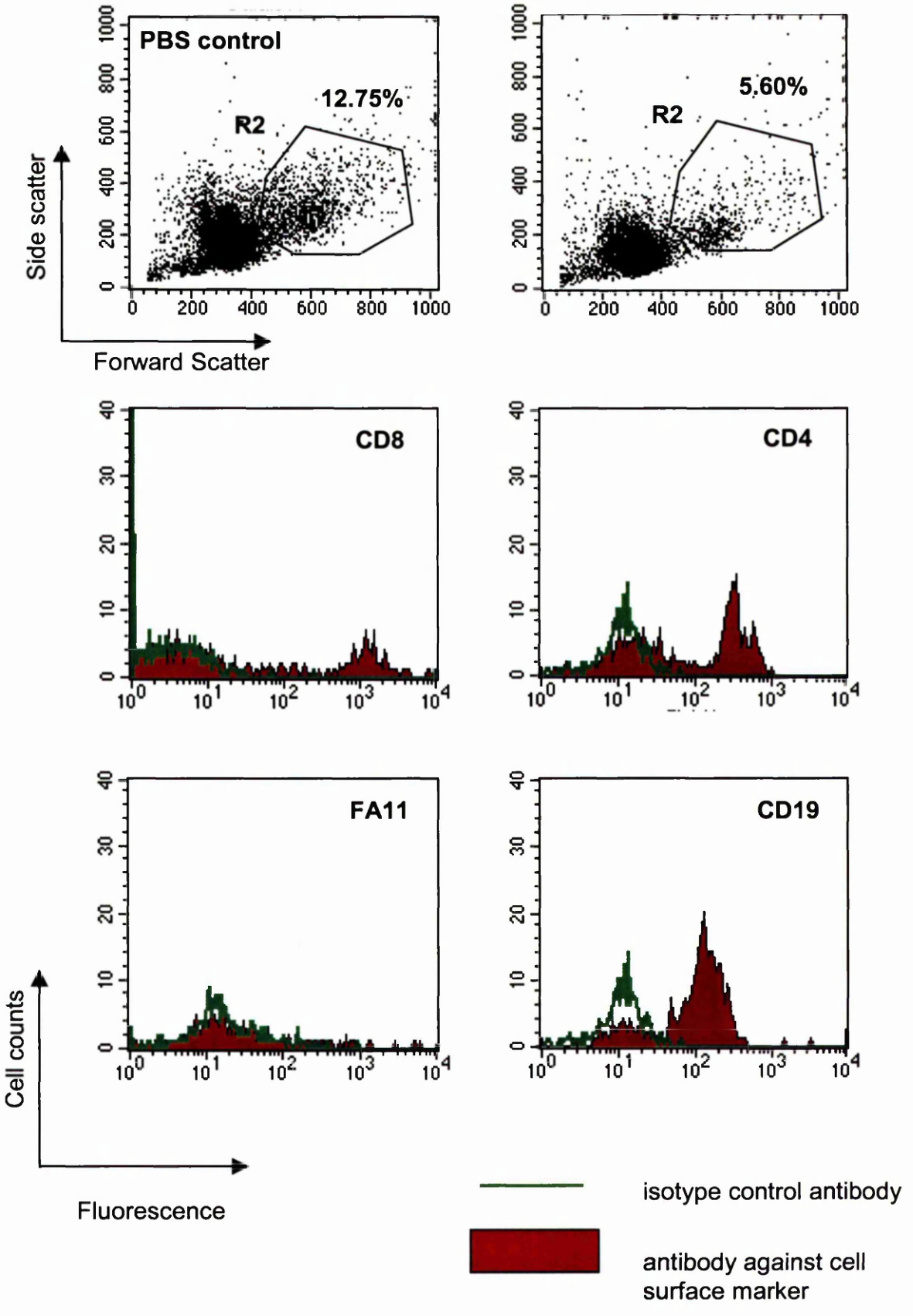
6.5.6 Analysis of early events in gentamicin-treated uninfected BALB/c mice

The analysis of early immunological parameters in *L. major*-infected BALB/c mice showed that gentamicin induced suppression of Th2 cell-associated markers (IgG1, IL-4, IL-5) and either enhanced or did not affect Th1 cell function. According to *in vitro* results presented earlier in this chapter, gentamicin selectively suppressed stimulated Th2 cells over Th1 cells. To address the question of whether gentamicin can suppress immune cells in the absence of an infection, two groups of 3 BALB/c mice were injected with 50 mg/kg of gentamicin or PBS for 7 days twice a day. On day 10, the spleens were pooled and analysed. Spleen cells were stimulated with 4 μg/ml anti-CD3 and cytokine release and proliferation responses were assessed. The effect of gentamicin on different cell population was also analysed by flow cytometry.

The proliferation and the IFN-γ expression in response to anti-CD3 were similar between spleen cells from PBS-treated and gentamicin-treated mice. However, spleen cells treated with gentamicin expressed significantly less IL-4 (decrease by

Fig. 6.31 Gentamicin treatment of *L. major*-infected mice suppresses CD4(+) and CD19(+) cell population of bigger size and granularity

Pooled popliteal lymph node cells, taken on day 10 after *L. major* infection from three mice which had been treated with PBS or 50 mg/kg of gentamicin, were analysed by flow cytometry. Cells gated in region 2 (R2) were stained for CD4, CD8, CD19 and FA11.


PBS control
12.75%
R2
5.60%
R2
Side scatter
Forward Scatter
CD8
CD4
FA11
CD19
Cell counts
Fluorescence
isotype control antibody
antibody against cell surface marker

34%) and marginally less IL-5 (decrease by 18%) (Fig. 6.32). Assuming, that the BALB/c mice used were free of infections and therefore did not contain any proliferating, antigen-specific Th1 and Th2 cells, other sources of IL-4 such as NK1.1(+) CD4(+) T cells (Skold *et al.*, 2000) might have been suppressed by gentamicin treatment.

Gentamicin did not alter the number of CD4(+) T cells, CD8(+) T cells and B cells in spleen cells of treated mice whilst the number of macrophages was reduced marginally by 4.4% (Table 6.2). The analysis of size and granularity of gentamicin treated and untreated cells revealed no differences (data not shown). These results suggest, that gentamicin mainly affected the antigen-specific immune responses.

6.6 Metabolism of serum gentamicin

It was vital to establish the levels of gentamicin that were present *in vivo* after gentamicin administration to BALB/c mice so similar doses could be used in *in vitro* experiments. Therefore, 6 mice were injected with a single dose of gentamicin (50 mg/kg) and the sera of 2 mice were collected after 4 h, 10 h and 24 h. The serum content was analysed for levels of gentamicin by fluorescence polarisation immunoassay as described in section 2.3. As shown in Fig. 6.33 the gentamicin concentration had decreased after 4 h to 45 μg/ml and after 10 h only 19 μg/ml were detected. One day after the injection the concentration decreased further to 13.5 μg/ml gentamicin. In human individuals with normal renal function the estimated half-life in serum is 2-3 h (BNF, 1997). A maximum of two half-lifes

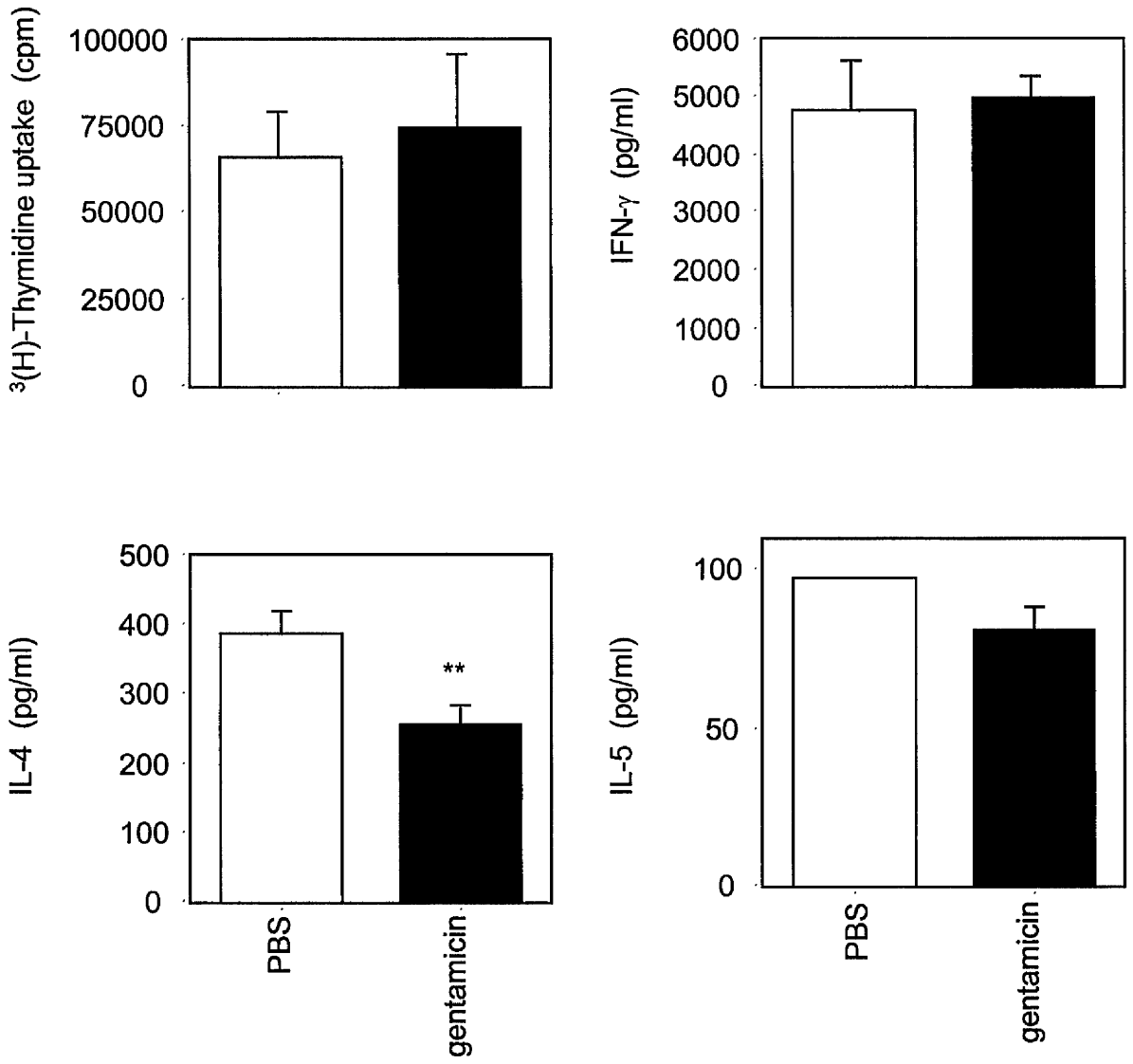


Fig. 6.32 The effect of gentamicin treatment on cytokine production of spleen cells from uninfected BALB/c mice

Three BALB/c mice were injected for 7 days twice daily with PBS or 50 mg/kg gentamicin and sacrificed on day 10. Spleen cells were pooled and stimulated with 4 µg/ml anti-CD3 Ab for 3 days. Cytokine release was assessed by ELISA and the cell viability by ^{3}(H)-Thymidine uptake. Results (n=3) are shown as mean ± 1 SD. (** $p<0.005$; *$p<0.05$)

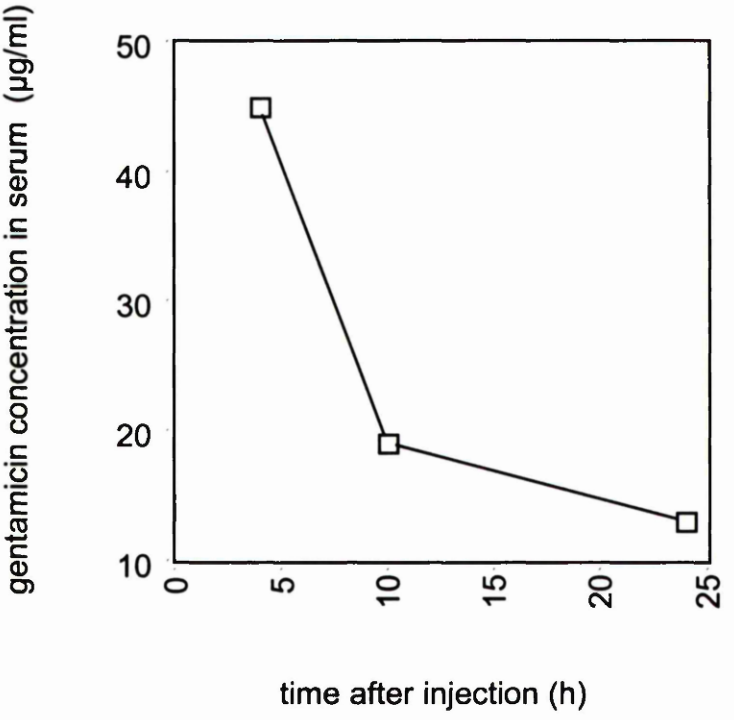


Fig. 6.33 Gentamicin serum concentration decreases over time.

BALB/c mice (n=9) were injected with 50 mg/kg gentamicin and three mice were sacrificed after 4, 10 and 24 h. The serum of 3 animals was pooled and the concentration of gentamicin was determined by fluorescence polarisation immunoassay (section 2.3).

passed before the first measurement was undertaken. Consequently, a maximal concentration of about 200 μg/ml gentamicin reached the serum and spleen cells. After 6 h, when the next injection was administered, only 10-20 μg/ml gentamicin remained in the circulation.

6.7 *In vitro* analysis of effects of gentamicin on parasites and cells of the immune response

After observing that gentamicin prevented the infection with *L. major* in the BALB/c mouse model, *in vitro* analysis was performed to determine whether the effect of gentamicin was a direct effect on parasites or affected specific cells of the immune system such as macrophages and Th cells.

6.7.1 The effect of gentamicin on *L. major* parasites *in vitro*

As shown in *L. major*-infected BALB/c mice, gentamicin reduced the parasite number in the infected footpad independently of the state of disease. To further investigate the function of gentamicin on *L. major* parasites, parasites in their log phase were cultured with increasing concentrations of gentamicin for three days in complete Schneider's medium, fixed and counted afterwards. To document any effects of gentamicin on shape and activity changes, photographs of the parasites were taken every 2 seconds under x 63 magnification. Gentamicin (1000 μg/ml) reduced the number of *L. major* parasites to 15% of the control level, while 100 μg/ml did not affect the parasite number significantly (Fig. 6.34 A). Untreated parasites moved quickly in direction of their flagellum and had a slim, elongated

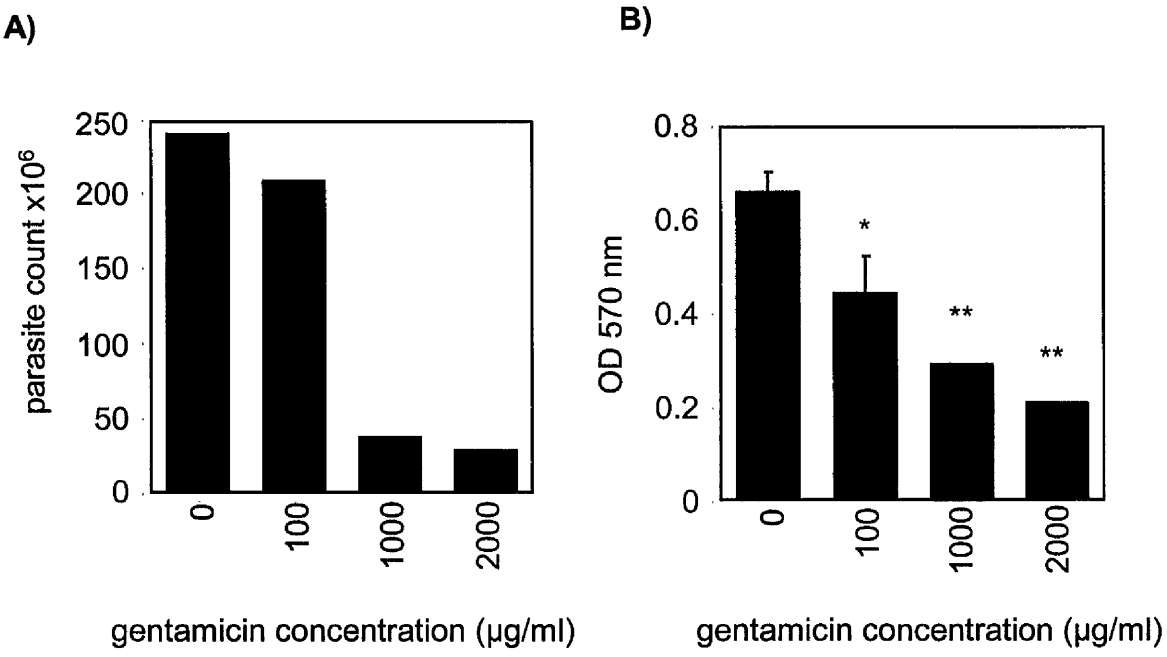


Fig. 6.34 The effect of gentamicin on mature BMMs and *L. major* parasites

A) *L. major* promastigotes were grown in complete Schneider's medium and cultured for 3 days with a concentration range of gentamicin, before being counted.

B) BMMs, matured with 10 ng/ml CSF-1 for 6 days, were cultured for 3 days with a concentration range of gentamicin and the MTT activity was assessed. The results (n=3) are shown as mean ± 1 SD (* $p<0.05$; ** $p<0.005$, comparing treated and untreated cells).

shape (6.35 A). Parasites incubated with 100 μg/ml gentamicin had a much shorter round body, a shorter flagellum and moved very slowly (Fig. 6.35 B). 1000 μg/ml gentamicin drastically altered morphology to a round body without flagellum, and greatly inhibited parasite mobility (Fig. 6.35 C). Cell debris was apparent, implying that a great percentage of parasites died in this concentration of gentamicin.

Zakai *et al.* (1998) reported that changes in *Leishmania* parasite morphology occurred at a lower pH of 5.5 increasing the infectivity of the parasites. To assess whether the observed shape and activity changes were due to lowering the pH in the medium, the pH changes caused by gentamicin were assessed. Only a concentration of 5 mg/ml gentamicin, which was never reached in *in vivo* experiments, altered the pH by one unit (Fig. 6.36). Therefore, gentamicin as an active component caused the changes described above.

6.7.2 The effect of gentamicin on the infectivity of *L. major* parasites *in vivo*

To examine whether gentamicin had a direct effect on the infectivity of *L. major*, parasites were incubated with 0, 50 and 500 μg/ml gentamicin for 48 h and then injecting into the footpad of 5 BALB/c mice. The swelling of the footpads was monitored over 70 days. Fig. 6.37 shows that parasites treated with different doses of the drug were similarly efficient in infecting BALB/c mice according to footpad swellings, suggesting that observed shape and activity changes at these drug concentrations given over 2 days did not affect the infectivity of parasites *in vivo*. These *in vitro* treatments were done with high doses of gentamicin for a short

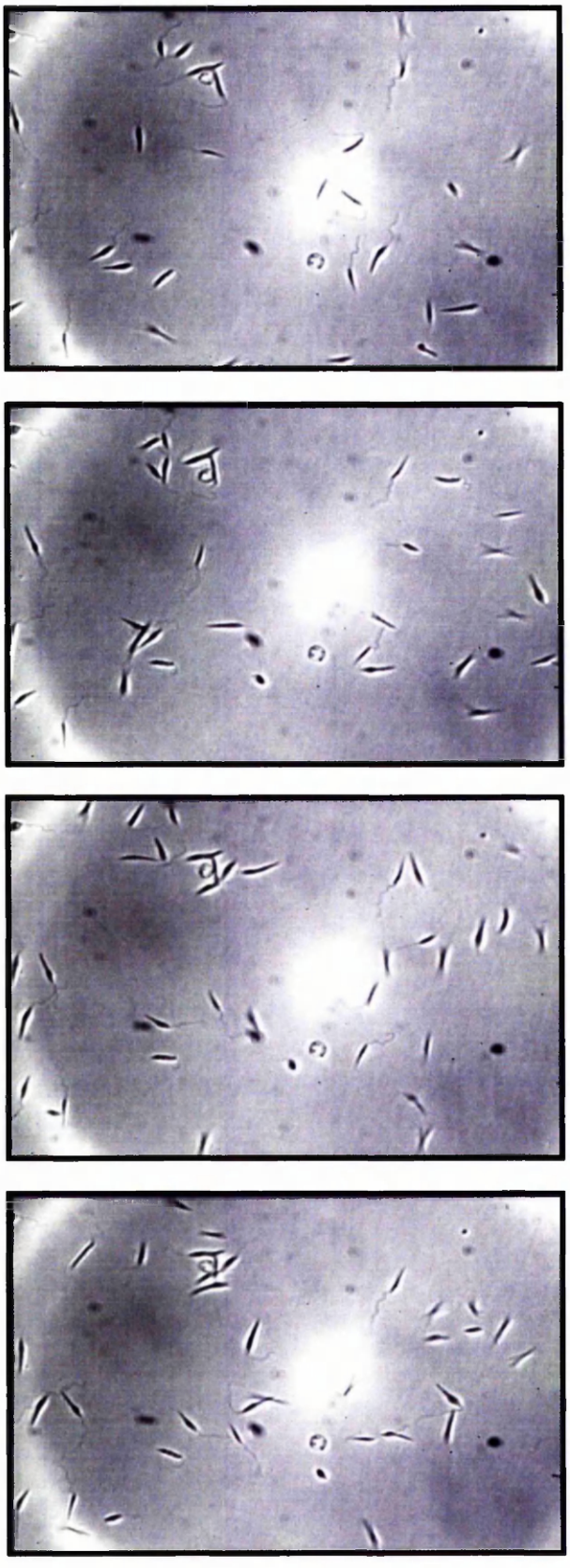


Fig. 6.35 A

Untreated *L. major* parasites

L. major parasites were grown for 3 days in complete Schneider's medium. Pictures were taken every 2 s with x 63 magnification.

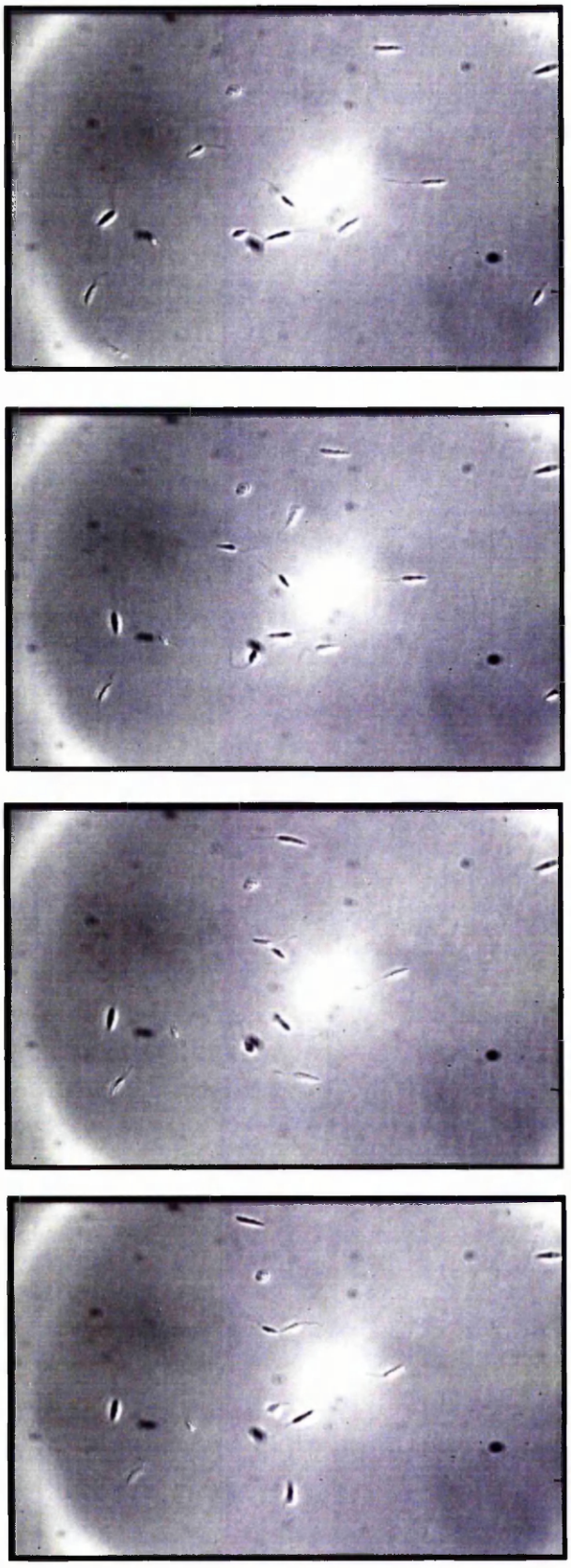


Fig. 6.35 B

Gentamicin-treated *L. major* parasites

L. major parasites were grown for 3 days in complete Schneider's medium and incubated with 100 µg/ml of gentamicin. Pictures were taken every 2 s with x 63 magnification.

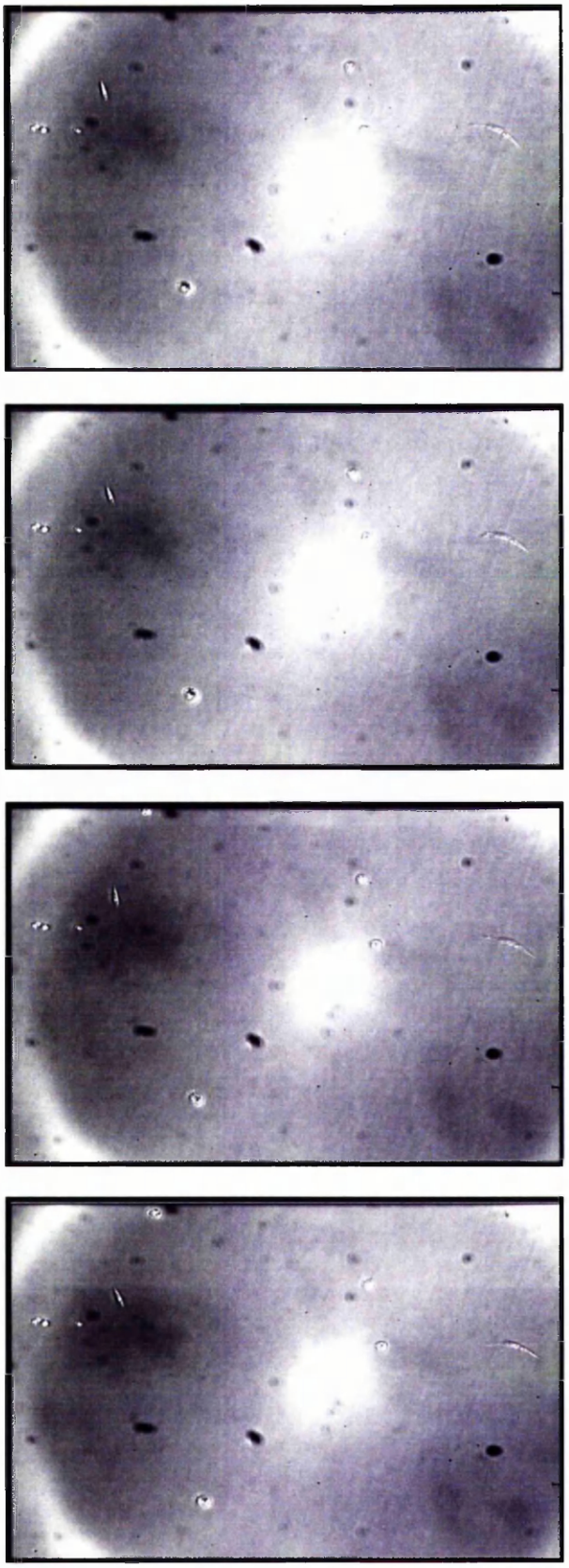


Fig. 6.35 C

Gentamicin-treated *L. major* parasites

L. major parasites were grown for 3 days in complete Schneider's medium and treated with 1000 µg/ml of gentamicin. Pictures were taken every 2 s with x 63 magnification.

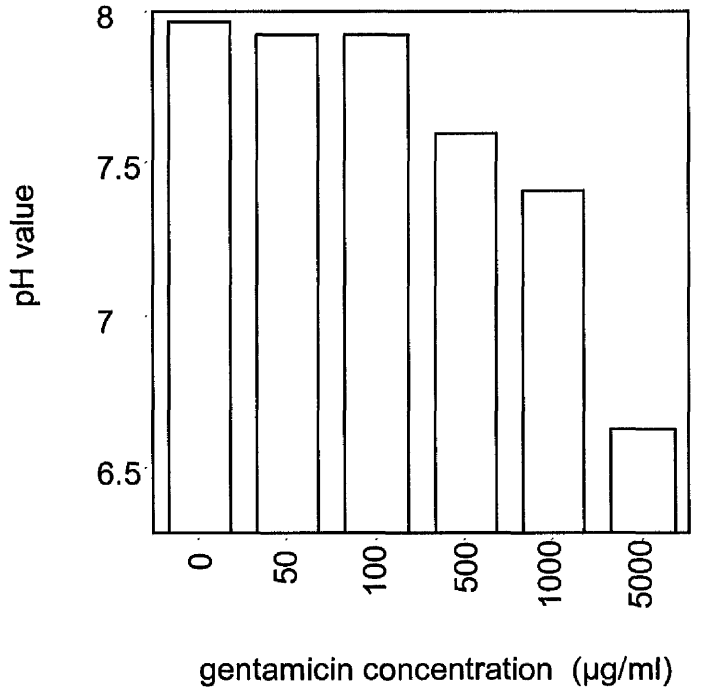


Fig. 6.36 Influence of gentamicin on the pH value of RPMi1640 medium

A concentration range of gentamicin was added to complete RPMI medium and the pH value was measured by pH meter.

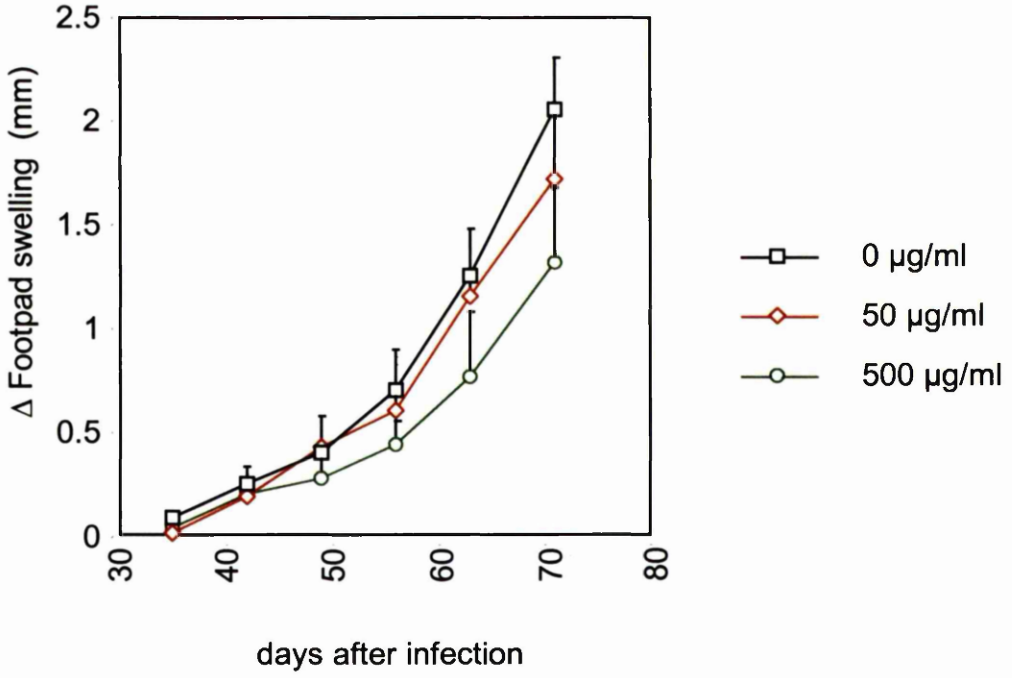


Fig. 6.37 Short-term gentamicin pretreatment does not affect infectivity of *L. major* parasites.

L. major parasites were cultured for 48 h with 0, 50 and 500 µg/ml gentamicin *in vitro*. Afterwards 5 x10^5 parasites of each group were injected into the left footpad of 5 mice. The foot pad swelling was measured starting from day 35. Results (n=5) are shown as the mean ± 1 SD.

period of time. During the *in vivo* trials, the parasites were exposed to gentamicin concentrations up to 200 μg/ml twice per day for 12 days, which might have affected the infectivity much more dramatically than Fig. 6.37 suggests.

6.7.3 The effect of gentamicin administration on T helper cells

Next, cells of the immune system involved in Leishmaniasis disease progression were examined for effects of gentamicin. The suppressive effect of gentamicin on polarised T helper cells had been investigated in chapter 6.2. Gentamicin concentration of 200-300 μg/ml had marginal effects on the proliferation and cytokine production of Th1 cells, but decreased Th2 cell proliferation and cytokine production (Fig. 6.10 and 6.11).

6.7.4 The effect of gentamicin on macrophages

As macrophages are hosts for *L. major* parasites, an effect of gentamicin on these cells could strongly influence the progression of the disease. Therefore, peritoneal macrophages, obtained from BALB/c mice, were stimulated *in vitro* with 10 ng/ml LPS and 0, 100, 500 and 1000 μg/ml gentamicin for 2 days. The mitochondrial activity as well as the cytokine expression (IL-6, IL-12 and TNF-α) was analysed. LPS stimulation of macrophages was chosen, because no cytokine release was detected in response to infecting parasite (data not shown), which has also been described by Chakkalath *et al.* (1994) and Belkaid *et al.* (1998).

Gentamicin reduced the cell viability of peritoneal macrophages stimulated with LPS in a dose-dependent manner (Fig. 6.38). The addition of 500-1000 μg/ml

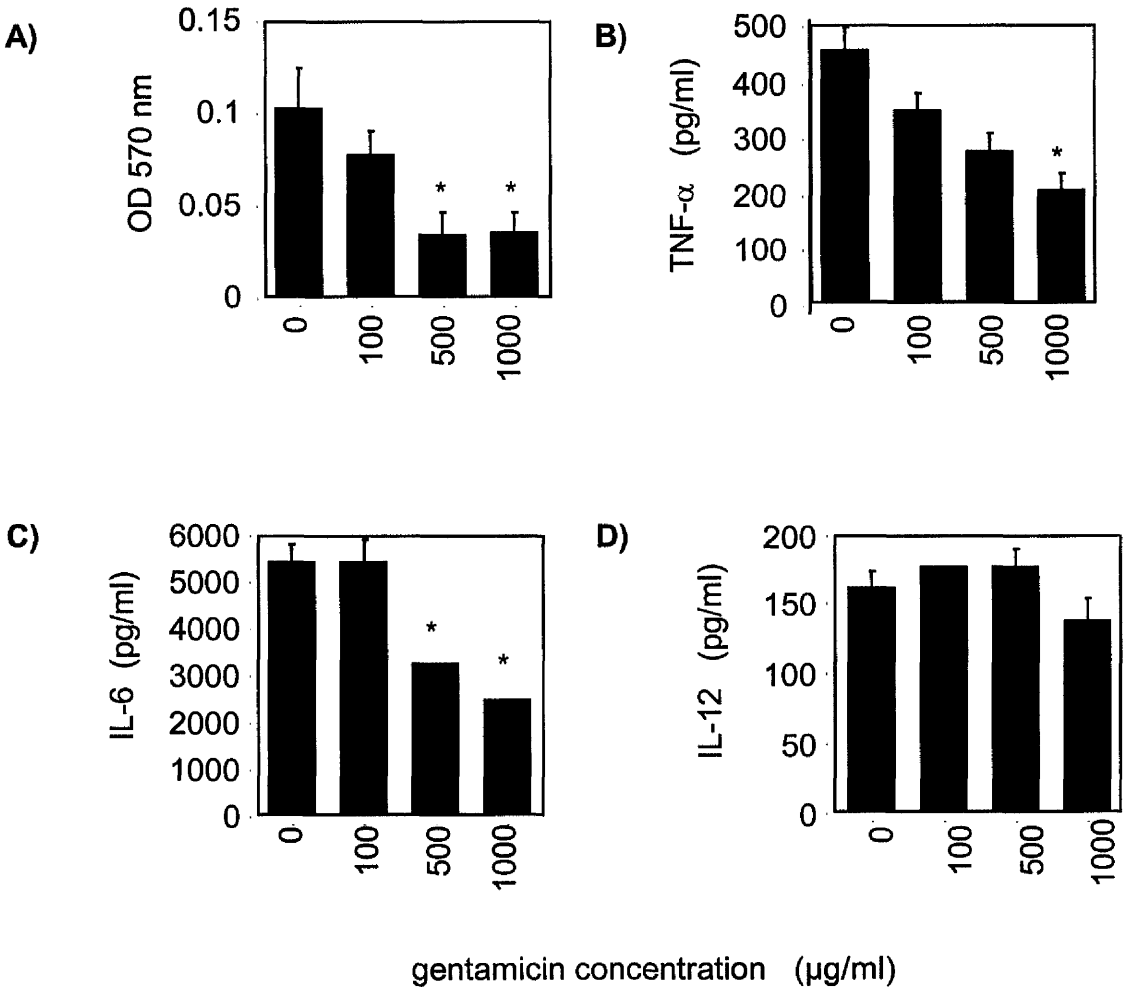


Fig. 6.38 Suppression of LPS- stimulated peritoneal macrophage effector functions by gentamicin

Peritoneal macrophages were obtained from BALB/c mice (section 2.12). $1x10^6$ cells were stimulated with 100 ng/ml LPS and a concentration range of gentamicin for 2 days. The cell viability was assessed by MTT assay **(A)** and the cytokine release by ELISA **(B-D).** The results (n=3) are shown as mean ± 1 SD (* $p<0.05$).

impaired the succinate dehydrogenase activity by 77%. Unstimulated and parasite infected macrophages cultured with gentamicin showed similar pattern in MTT assays (Fig. 6.34 B, data not shown). LPS activates inflammatory cytokine production in peritoneal macrophages. Gentamicin decreased IL-6 and TNF-α expression in a dose dependent manner (Fig. 6.38). Incubation with 500 μg/ml gentamicin caused a 60% decrease in IL-6 expression and TNF-α expression. The IL-12 production was not affected by gentamicin treatment, only 1000 μg/ml gentamicin decreased the IL-12 release marginally. NO was not detectable by Griess reaction due to requirement for additional IFN-γ combined with LPS stimulation. Unstimulated peritoneal macrophages and parasite-infected macrophages did not produce detectable cytokine levels. A primary function of the macrophages is to process and present antigen in the context of MHC class II molecules to T cells. MHC class II expression on viable cells did not change by the treatment with gentamicin, suggesting that the activation state of these cells was not affected (data not shown). In summary, gentamicin reduced viability and cytokine production of LPS-stimulated macrophages but did not affect their MHC II expression.

6.7.5 The effect of gentamicin on parasite-infected macrophages

Gentamicin is as a polycationic molecule water soluble, but poorly lipid soluble. It is therefore relatively ineffective at crossing biological membranes (Lutwyche *et al.*, 1998). Hence, cells might use active pump mechanisms or pinocytosis to absorb the drug. To determine whether sufficient amounts of gentamicin can enter

macrophages to affect intracellular parasites, macrophages were infected with parasites in a ratio of 1:10 (macrophage : parasite). Gentamicin was either added 24 h prior infection, at the time of infection or 24 h after infection with *L. major*. After 3 days the macrophages were lysed and the parasite number assessed by serial limiting dilution assays as described in sections 2.15 and 2.17. As a control, *L. major* parasites were kept under macrophage growth conditions (RPMI 1640 + 10% FCS, 37°C, 5% CO2) and treated with 10 - 2000 μg/ml gentamicin. Similar results as shown in Fig. 6.34 A were obtained. 10 and 100 μg/ml gentamicin did not reduce the parasite number, but the parasites appeared less active, while 1000 μg/ml seemed to have killed most parasites (data not shown).

Fig. 6.39 A shows that even 100 μg/ml gentamicin added to *L. major*-infected macrophages for 3 days killed all intracellular parasites. In photographs of the cells treated with 0 or 100 μg/ml gentamicin, taken before the parasites were washed away, no extracellular parasites were found in the 100 μg/ml culture, while numerous parasite were observed in the untreated culture (Fig. 6.40). To assess the ability of gentamicin to kill intracellular parasites, macrophages were infected with parasites for 24 h and remaining parasites were washed off before gentamicin was added (Fig. 6.39 B). No parasites were found in cells treated with 100 μg/ml or higher concentrations of gentamicin. Finally, when macrophages that had been pretreated with gentamicin for 24 h, were washed and challenged with parasites for further 3 days, 100 μg/ml of the drug did not change the number of invading parasites in macrophages. 1000 μg/ml gentamicin decreased the

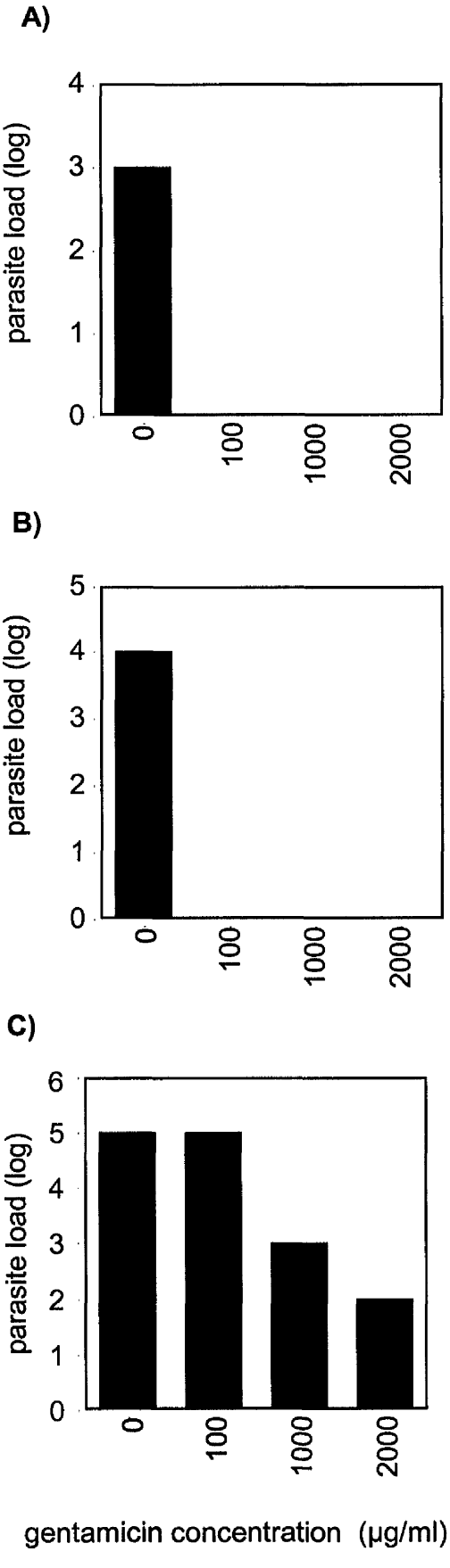


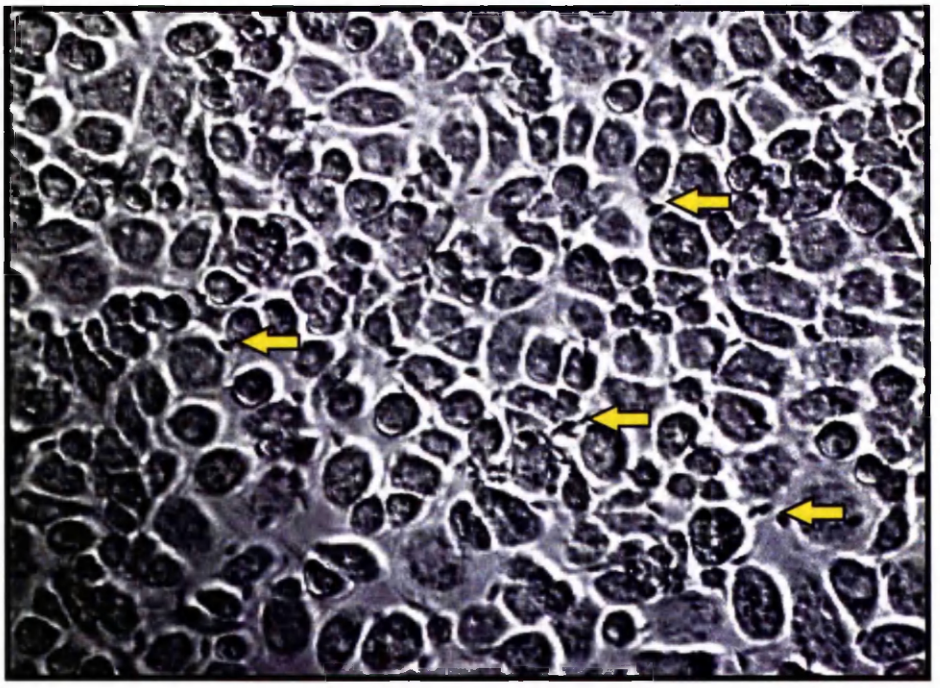
Fig.6.39 The effect of gentamicin on macrophage infecting *L. major* parasites

A) Mature BMMs were obtained as described in section 2.11. Cells were infected with parasites (1:10) and different gentamicin concentrations were added for 3 days. Macrophages were lysed and parasite dilutions were cultured in complete Schneider's medium for 2 days and assessed by serial limiting dilution assay (section 2.15).

B) Mature BMMs were pretreated with *L. major* (1:10). Free parasites were washed off after 24 h. Different concentrations of gentamicin were added for 3 days. The cells were lysed and the remaining parasites were counted by serial limiting dilution assay.

C) Mature BMMs were pretreated with gentamicin for 24 h, then washed and infected with *L. major* (1:10) and incubated for 3 days. The cells were lysed and the intracellular parasites were analysed by serial limiting dilution assay.

A)



B)

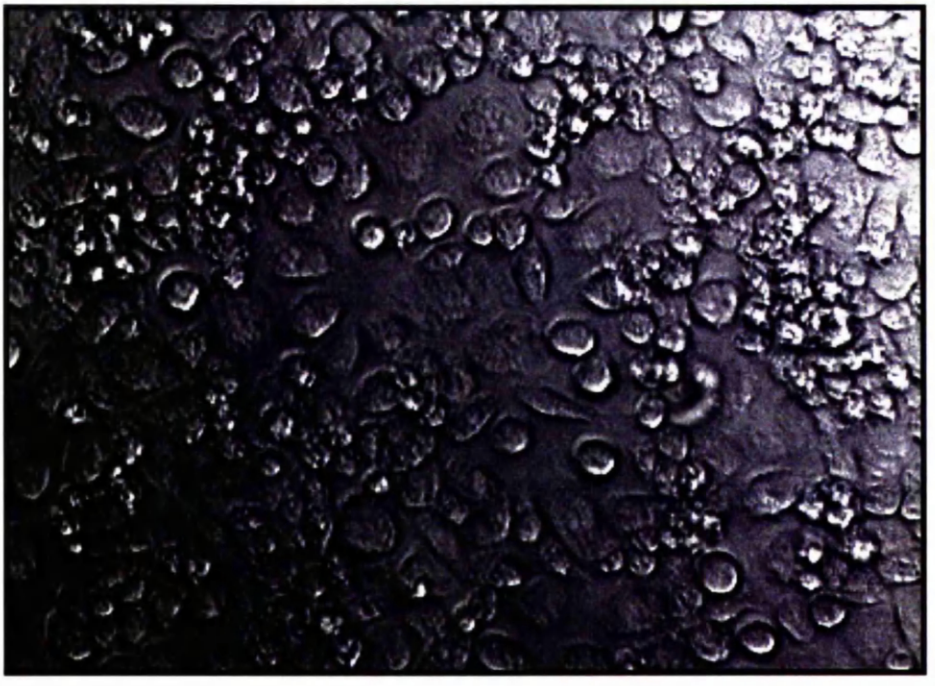


Fig. 6.40 The effect of gentamicin on macrophages infected with *L. major*

Mature BMMs were incubated with *L. major* (1:10) and without **(A)** or with 100 µg/ml of gentamicin **(B)** for 3 days. Arrows indicate extracellular parasites. (x 40 magnification)

parasite number by 40 %, 2000 μg/ml decreased the number further to 40% (Fig. 6.39 C).

Discussion

G418 and gentamicin - potential tools to select Th1 over Th2 cells

The effect of G418 on T helper cell subsets

G418 is known to have varying effects on different eukaryotic cell types. In order to use this selection marker on transfected cells, a dose response of toxicity must be performed on untransfected cells to determine the minimum dosage required for such selection. In the course of these experiments, it was found that in response to G418, Th1 cells exhibited a significantly higher survival rate than Th2 cells, as shown on Th cell clones and polarised Th cells (Fig. 6.2-6.7). Furthermore, higher Th1 type cytokine levels in comparison to Th2 type cytokines were observed after exposure of activated spleen cells to G418 (Fig. 6.8). Th1 type cytokines are also expressed by NK1.1(+) T cells and CD8(+) Tc1 cells upon TCR stimulation (Skold *et al.*, 2000; Harty *et al.,* 2000), while Th2 type cytokines are expressed by NK1.1(+) T cells and mast cells (Skold *et al.*, 2000; Bradding *et al.*, 1995). Thus, these cell types may also be affected by the gentamicin treatment. However, the results on Th cell clones and polarised Th cells suggest strongly a selective suppression of gentamicin on T helper cell subsets. Propidium iodide and annexin V staining of G418-treated T cell clones implied that G418

induced cell death either by apoptosis, supported by shrunk appearance of cells (Fig. 6.3), or necrosis more effectively in Th2 cells than in Th1 cells. Although the mechanism of Th1 cell resistance to G418 was not investigated, the most likely explanation, given the ability of aminoglycosides to interfere with the protein synthesis of eukaryotic cells (Manuvakhova *et al.*, 2000), is that the Th2 cell proliferation rate is higher than that of Th1 cells. This was evident on Th cell clones (Fig. 6.4, *basal levels*), where BALB/c mice derived Th1 cells (X9) proliferated 3-4 fold less than Th2 cells (X12) from the same genetic backround. Polarised Th2 cells, stimulated with anti-CD3 proliferated 5 days after stimulation much stronger than polarised and anti-CD3 stimulated Th1 cells (Fig. 6.6). Indeed, it is known that stimulation with anti-CD3 antibodies inhibits IL-2 dependent proliferation in Th1 but not in Th2 cells (Gajewski *et al.*, 1989; Williams *et al.,* 1990).

Another explanation may be that Th1 cells express a transport activity, which exports intracellular G418. One possible candidate of a membrane transport system promoting the efflux of cytotoxic drugs on Th cells is the P-glycoprotein, encoded by the *mdr* (multiple drug resistance) genes. Indeed, the expression of P-glycoprotein has been reported to identify a subset of activated CD4(+) T cells characterized by an augmented rate of proliferation and increased IL-2 and IFN-γ secretion in response to various polyclonal stimuli (Bommhardt *et al.*, 1994). However, it was also reported that the multiple drug resistance protein 1 (MDR1 or MRP1) transmembrane pump, which is encoded by *mdr1*, is highly expressed by resting Th2, but not by Th1 cells and is induced to equal expression level in both T

cell subsets after antigenic stimulation *in vivo* (Lohoff *et al.*, 1998; Prechtl *et al.*, 2000). Blockage of MDR1 with a monoclonal antibody did not increase the cytotoxicity of G418, suggesting that G418 resistance may not be associated with *mdr1* expression (Metchetner *et al.*, 1992). Additionally, when using Taxol and Doxorubicin, which are substrates of the *mdr1* coded P-glycoprotein (Sweet *et al.*, 1996), no selective suppression of cell viability of Th2 cells over Th1 cells was observed (data not shown). Although the expression pattern of this particular MDR member does not correlate with the selective resistance of Th1 cells but not Th2 cells to G418, it may be that other MDR members are expressed in a Th2-specific manner. Indeed, others have reported complimentary patterns of expression of the ABC transporters CFTR and MDR1 *in vivo* (Anderson *et al.*, 1991; Trezise *et al.*, 1992). Hence, it is conceivable that certain MDR members are expressed in a Th1-restricted manner while other family members, such as MDR1, are expressed in a Th2-specific manner.

LPS stimulated macrophages (like TCR stimulated Th1 cells) exhibit resistance to G418 (Sweet *et al.*, 1996), which might be mediated by an increased expression of the P-glycoprotein. An antibody blockade of P-glycoprotein on alloreactive CD4(+) T cells and CD14(+) monocytes resulted in diminished IFN-γ secretion by CD4(+) T cells and in diminished IL-12 export and/or secretion by activated monocytes (Frank *et al.*, 2001). IFN-γ upregulates the expression and activity of P-glycoprotein in human peripheral macrophages (Puddu *et al.*, 1999) and may thus increase IL-12 secretion of stimulated macrophages which then leads to further Th1 cell development. These findings link stimulated macrophages and Th1 cells

and suggest similar regulation of the P-glycoprotein expression in the two cell subsets.

Resistance to aminoglycosides and specifically to G418 is known from aerobic bacteria through the expression of enzymes from three classes: phosporylases, acetylases and adenyl transferases. These can inactivate aminoglycosides by cleavage or modification (Coppoc, 1996). No resistance is known from eukaryotic cells thought to express similar enzymes.

The observation that Th1 cell are more resistant to G418 killing than Th2 cells is of technical interest to immunologists. A refined method might be used to isolate Th1 cells out of mixture of activated T helper cells, or Th cells could be biased towards Th1 phenotype by adding low doses of G418 to the culture.

The in vitro effect of gentamicin on T helper subsets

Gentamicin, commonly used as antibiotic, has well known function against prokaryotic cells (Schroeder *et al.*, 2000). Side effects such as damage to sensory cells of the cochlea, the vestibular apparatus and on proximal tubular cells of the kidney on patients during long-term treatment suggest that gentamicin might also have adverse effects on eukaryotic cells (Lima da Costa *et al.,* 1998; Godber *et al.*, 1995; English *et al.*, 2001). Several groups reported an impairment of the immune response by gentamicin, suppressing the production of different cytokines, DTH responses, lymphocyte proliferation, NK cell activity and phagocytosis (Van Vlem *et al.*, 1996). Rats treated with 225 mg/kg of gentamicin twice per day for 12 days showed a decrease in body weight, especially the

thymus weight. DTH response and IFN-γ production were suppressed in a dose dependent manner (Exon *et al.*, 1989). Gentamicin (50 μg/ml) decreased the IL-2 production by mouse spleen cells *in vitro* (Ibrahim *et al.*, 1988). The findings outlined in this chapter corroborate these previous reports. That is, gentamicin affects T lymphocytes negatively at higher dosage by decreasing cell viability and cytokine production. The data in this thesis have further extended the knowledge about the function of gentamicin on T lymphocytes. It selectively suppressed Th2 type cytokine production and proliferation at moderate dosage and has therefore a potential as an agent to modulate Th1/Th2 function without universal toxicity to eukaryotic cells. Interestingly, 10-fold higher levels of gentamicin than of G418 were needed to achieve a similar suppression pattern. This finding correlates with the results of Manuvakhova *et al.* (2000), that 2-14-fold higher levels of gentamicin were needed to achieve the amount of G418-induced suppression of translation termination signals. The mechanism of the selective suppression of gentamicin is unclear. It is likely that, given the similarities of G418 and gentamicin, the same mechanisms - differential protein synthesis rate or differential expression of an ABC transporter protein - which directs Th2-selective toxicity of G418 are also responsible for the effects of gentamicin.

The in vivo effects of gentamicin on L. major infected BALB/c mice

L. major infections in susceptible BALB/c mice lead to the development of locally severe lesions that do not resolve spontaneously. This is due to the inability of their immune system to mount an effective protective Th1 response (Bradley,

1977). The disease progresses because antigen-specific Th2 cell response dominates due to an early outburst of IL-4 and other, partly genetic factors. The high IL-4/IFN-γ ratio prevents the activation of infected macrophages to destroy the parasites (Lehn *et al.*, 1989; Liew *et al.*, 1989). Resistant mice, such as CBA mice, exhibit a dominant Th1 cell response when infected with *L. major* parasites and are able to resolve the locally small lesions that occur (reviewed by Launois *et al.*, 1997). Therefore, progress of cutaneous leishmaniasis mainly depends on the Th1/Th2 cell ratio and their cytokine production. CD8(+) T cells can support Th1 cells by producing IFN-γ and TNF-α (Fong and Mosmann, 1990), that enables macrophages to kill the parasites in their phagosomes by increased NO production (Liew *et al.*, 1990). B cells have not been reported to play a major role in *L. major* infections (reviewed by Liew, 1986). Successful treatments of *L. major* in BALB/c mice have been based on a direct suppressive effect on the parasite, the alteration of the Th1/Th2 balance, the activation or suppression of macrophages or the general suppression of the early immune response (reviewed by Bogdan and Roellinghoff, 1998).

Gentamicin was shown to be a potential tool to alter the Th1/Th2 type balance *in vitro*. To test whether gentamicin could alter the Th1/Th2 balance *in vivo* the murine model for *L. major* infections in susceptible BALB/c mice was applied, where gentamicin has been reported as part of a successful topical treatment (15% paromomycin, 0.5% gentamicin, Grogl *et al.*, 1999). However, the mechanism of gentamicin in parasite killing had not been addressed.

Consistent with the result of other groups I found that low dose gentamicin treatment of *L. major*-infected mice did not prevent the onset of the disease (data not shown; Carter and Alexander, 1989; Neal and Croft, 1984). Therefore, high doses of gentamicin, administered at different stages of the disease, were used to treat parasite-infected BALB/c mice. BALB/c mice, injected with 50 mg/kg of gentamicin from the time of *L. major* infection for 12 consecutive days, did not show footpad swelling. When gentamicin treatments were started later in the disease progression, the onset of the disease was delayed or the swelling of the lesions was reduced but these treatments were unable to prevent the disease progression. Analysis of immunological parameter at this acute state of infection revealed a general suppression of the parasite-specific T and B cell immune response by gentamicin when administered from the day of infection (Fig. 6.20-6.25). Further, the number of B and T lymphoblasts was reduced by gentamicin treatment (Fig. 6.28 and 6.31) which might provide an explanation for the lack of *L. major*-specific antibody and cytokine response. Gentamicin could have prevented the onset of the disease by either shifting Th2 cell to Th1 cell dominance and therefore creating a Th1 cell memory phenotype or by nonspecific depletion of antigen responding CD4(+) T cell, which were mainly of a Th2 phenotype. Mice that had been cured of an initial infection by treatment with high dose gentamicin still succumbed to a subsequent reinfection, which could not be prevented by potentially existing memory Th1 cells, suggesting that gentamicin might not favor Th1 cell survival but rather selectively deplete Th2 cells. In such a case, disease progression would be prevented as long as the drug was present

but would not allow for development of a protective memory. Therefore, gentamicin was most effective at preventing disease progression only when administered in high doses at the time of infection with parasites.

In conclusion, long-term treatment with a high dose of gentamicin led to a complete suppression of the *L. major*-specific immune response. However, when *L. major*-infected BALB/c mice were treated with gentamicin from the time of infection for a shorter period (7 instead of 12 days) and analysed soon after the treatment finished, it was possible to show that gentamicin can shift the Th1/Th2 type cytokine balance *in vivo* as *in vitro* results had already suggested (Fig. 6.29). In gentamicin-treated *L. major*-infected BALB/c mice a decreased Th2 cytokine and IgG1 antibody production and a reduction of the CD4(+) T and B lymphoblast population could be detected. It is likely, that the ability of gentamicin to suppress Th2 type responses occurred dependently of *L. major* infection. Further experiments have to be performed to ensure that this treatment regime still efficiently cures *L. major* infections by altering the Th1/Th2 type balance. When BALB/c mice were treated from day 7 to 14 post-infection, a delayed onset but no prevention of the disease was achieved (Fig. 6.14 A and 6.15). At this stage, the treatment might have been unable to suppress the already strong Th2 immune response and the growing number of parasites. A treatment, starting at the time of infection stands better chances to interfere with the disease progress as shown for example with IL-12 (Sypek *et al.,* 1993). Spleen cells isolated from gentamicin-treated mice up to 10 days after the *L. major* infection responded only to anti-CD3 stimulation, not to stimulation with *L. major* antigen. Additionally, when uninfected

mice underwent similar treatment, a suppression of IL-4 production by gentamicin was observed (Fig. 6.32). Therefore it can not be excluded that other IL-4 producing cells such as splenic NK1.1(+) CD4(+) T cells and Fcγ(+) CD4(+) cells with intermediate expression levels of TCR (TCR ^{int}) also contributed to the cytokine profile upon primary TCR stimulation (Moodycliffe *et al.*, 1999). A possible suppression of these cells by gentamicin would have to be investigated.

The achieved prevention of leishmaniasis could be also due to a direct effect of gentamicin on the parasite or its ability to infect its host cell and/or suppress the immune response of macrophages. First, the *in vivo* concentration of gentamicin had to be measured to be able to test this concentration on the parasites. The estimated serum concentration in mice for gentamicin peaked at 200 μg/ml and deceased quickly, but a potential damage of the renal absorbency could lead to higher local concentrations and more damage to the immune cells.

Gentamicin was able to reduce dramatically the parasite number in the infected footpads (Fig. 6.13 A, 6.19 A). Grogl *et al.* (1999) observed a 24% suppression of *L. donovani* parasites in mice liver after injection with 700 mg/kg of gentamicin for 4 days. Further, in collaboration with Dr. Carter (University of Strathclyde, Glasgow), the high dose treatment of gentamicin (day 0 – day 12, i.p. injections of 50 mg/kg twice per day) was tested on hamsters infected with *L. donovani*. Gentamicin reduced the parasite number in spleen and bone marrow by 50%, in liver by 78%, while sodium stibogluconate, a clinically used drug, was unable to reach parasites in the bone marrow but reduced the parasites in the liver to 2%. As demonstrated in this chapter, gentamicin at high concentrations was able to kill

L. major parasite *in vitro*, modulating body shape and activity at lower doses (Fig. 6.34 A, 6.35 A-C). This might have been caused by gentamicin interacting with ribosomes of *Leishmania* and so promoting the association of the subunits which prevents protein synthesis as reported for paromomycin, another structural related aminoglycoside (Maarouf *et al.*, 1995). However, *L. major* parasites, treated prior infection for 48 h with gentamicin concentrations, which exceeded the estimated concentrations used for *in vivo* experiments, did not loose their infectivity in BALB/c mice. One could argue that during the *in vivo* trial the parasites were exposed to gentamicin for a longer period, which might then interfere with the protein synthesis causing the parasites to loose their vital functions. Other findings suggest that the success of gentamicin treatment in *L. major* infections was not only due to its suppressive effect on parasites. If high gentamicin concentrations had only led to a reduction in the number of parasites, BALB/c mice would have developed resistance and Th1 type responses as shown following the inoculation of very low numbers of *L. major* promastigotes (Li *et al.,* 1997). Furthermore, dead parasites are used as a vaccine to induce a protective Th1 response (Bebars *et al.,* 2000). A reinfection of mice, which had been successfully cured by high dose gentamicin treatment, was not precluded by a memory Th1 cell response (Fig. 6.16 A).

Macrophages act as host cells for *L. major* parasites and are therefore an attractive target for anti-*Leishmania* drugs. The induction of Th1 type cytokine IFN-γ can confer resistance by activating macrophages to destroy their intracellular parasites (Nacy *et al.*, 1985; Doherty and Coffman, 1999). In contrast to this

mechanism of resistance, suppression of the host cell viability by gentamicin would destroy the parasite's ability to transform into amastigotes and to replicate. Gentamicin reduced the cell viability of uninfected, parasite-infected and LPS-stimulated macrophages in a dose-dependent manner (Fig. 6.34 and 6.38). Although the estimated *in vivo* doses did not decrease macrophage viability to a great extent, the long-term treatment during the *in vivo* trial might have done so. The antibiotic, given at 40 mg/L, has been reported to significantly inhibit macrophage activation in response to filtrates of lymphocytes in culture (Sacha *et al.*, 1999). Whether defense mechanism of macrophages such as oxygen burst and NO production are also negatively affected by gentamicin treatment remains to be investigated. When *L. major*-infected macrophages were treated with the estimated *in vivo* doses of gentamicin (100 μg/ml), which would harm neither parasites nor macrophages alone, a complete clearance of intracellular parasites was observed (Fig. 6.39 A). This might have been caused by the altered ability of the parasite to enter the macrophage or by the increased ability of the macrophage to take up gentamicin and therefore enhance the local concentration inside the cell. Bray *et al.* (1983) reported an increased ability of macrophages to pinocytose when infected with amastigotes of *Leishmania mexicana mexicana*. Macrophages, when preincubated with gentamicin, which was removed before infection, showed less ability to clear parasites. This result could be interpreted with the reduced ability of macrophages to pinocytose in the absence of parasites. Consequently, the presence of *L. major* parasites could increase the uptake of

gentamicin into the host cell where high local concentrations could destroy the parasite.

Several parasite surface molecules (lipophospoglycan, promastigote surface protease) are involved in the attachment of promastigotes to macrophages via CR1 or CR3 (Puentes *et al.*, 1988). Entering the macrophage via these receptors does not trigger an oxygen burst (Wright and Silverstein., 1983). Interference of gentamicin as a charged molecule with the attachment process or with the synthesis of the cell surface molecules could prevent the entry of the parasite without inflammatory response from the host cell and rather promote phagocytosis and clearance.

In agreement with others I observed a suppressive effect of gentamicin on activated B cells and antigen-specific antibody production (Villa *et al.*, 1986; Van Vlem, 1996). Extensive gentamicin treatment caused suppression of both Th1 and Th2-specific antibody isotypes which might have been due to the lack of antigen or activated helper T cells or the killing of B cells by gentamicin. Shorter treatment favoured Th1 cell-specific IgG2a over IgG1 production, which corresponds with the findings on Th cells.

However, healing of an established *L. major* infection in mice is thought to be independent of the specific anti-leishmanial antibody response elicited during infection (reviewed by Liew, 1986). As shown in mice lacking B cells due to a disruption of the IgM locus, B cells do not have vital functions for the development of polarised T helper response by direct antigen presentation or costimulation or

antibody-mediate effector functions during a *L. major* infection (Brown *et al.*, 1999).

Chapter 7 General Discussion

This thesis is separated into two major parts, both investigating molecules that are able to modulate Th1/Th2 type responses. The balance between Th1 and Th2 cells responses determines the outcome of many infections and autoimmune diseases. Hence, the possibility to modulate this balance is crucial for a successful treatment. Firstly, ST2-Fc was expressed in insect and mammalian cells and used to investigate the function of ST2 on T helper cells. Then, the regulation of ST2 and ST2L expression by Th1 and Th2 cytokines was assessed. The second part of this project focused on the ability of gentamicin to modulate Th1/Th2 responses *in vitro* and *in vivo*.

The orphan receptor ST2L, with which ST2 shares a common extracellular domain, is preferentially expressed on the surface of Th2 cells and is important for Th2 effector functions *in vivo* (Kropf *et al.*, 1999; Xu *et al.*, 1998, Loehning *et al.*, 1998; Coyle *et al.*, 1999; Chan *et al.*, 2001). Evidence was obtained from studies with anti-ST2L antibodies and ST2-Fc fusion protein, mimicking the effects of ST2. Both treatments decrease Th2 type responses (Xu *et al.*, 1998; Coyle *et al.*, 1999). One hypothesis suggests that anti-ST2L antibodies and ST2-fusion protein block the binding of the ligand and ST2L signaling and therefore prevent crucial Th2 cell effector functions, such as Th2 type cytokine production (Meisel *et al.*, 2001). The effect of ST2 on ST2L expressing cells has been studied and the search for a soluble ligand is continuing. Only recently first attempts were made to reveal the effect of ST2 on ST2L(-) cells. An anti-inflammatory effect of ST2 on LPS-stimulated macrophages has been described (Sweet *et al.*, 2001), which was also seen with fixed Th2 cells expressing ST2L, but not Th1 cells. This thesis accumulated more evidence for a function of ST2 on ST2L(-) T helper cells. Naïve and

primed T helper cells do not express ST2L (own observations; Meisel *et al.*, 2001; Carter *et al.*, in press EJI, 2001). Here I show that ST2-fusion protein can suppress cytokine expression of antigen stimulated naïve T helper cells, but not on re-stimulated Th cells. As with macrophages, this effect was mediated by binding of ST2 to an unidentified cell surface protein on naïve Th cells. Coyle *et al.* (1999) also reported an effect of ST2 on ST2L(-) cells. Naïve Th cells were antigen stimulated in the presence of ST2 for three days, followed by a restimulation, which led to a dominant Th1 type cytokine expression. Hence, ST2 treatment results in cytokine gene expression changes and Th2 and Th1 type cytokine promoters might be differently affected.

Apart from macrophages and naïve Th cells, ST2 was found to bind to activated B cells and dendritic cells (DC) (Yanagisawa, 1997; Coyle *et al.*, 1999; Lambrecht *et al.*, 2000). The expression profile of one ligand candidate, the membrane bound SBP (Gayle *et al.*, 1996), in Th1 and Th2 cells is currently being investigated. ST2 may bind to antigen presenting cell subsets such as B cells and DCs and activate cell signaling. The obvious experiment to perform would be to assess the effect of ST2 on effector function of the B cells and DCs by measuring the cytokine and antibody production as well as the change of surface activation markers. Preliminary data suggest that ST2 increases IL-12 and TNF-α production by DCs while activation markers such as MHCII, CD80, CD86 and CD40 were not significantly affected (data not shown). Further, ST2 injections in ST2L-deficient mice (Townsend *et al.*, 2001) will show whether ST2 has an effect on ST2L(-) cells. As seen on macrophages and Th cells the expression of the ST2 binding molecule can be altered by LPS stimulation and TCR stimulation, suggesting an activation-

dependence. Similar binding studies with and without activation stimulus ought to be performed on B cells and DCs.

Another unsolved question is whether ST2 and ST2L act as agonists, which bind to a membrane-bound receptor and induce signaling as suggested on ST2L(-) cells (macrophages and naïve Th cells), or as antagonists, competing for a soluble ligand as assumed on ST2L(+) cells (Th2 cells) (Fig. 4.1). Soluble cytokine receptors (sCR) act mainly as antagonists to their membrane-bound homologue (reviewed in Fernandez-Botran *et al.*, 1996a). On the other hand, soluble cytokine receptor can enhance the concentrations of active cytokines *in vivo* via several mechanisms including increasing cytokine stability, decreasing proteolytic degradation and altering pharmacokinetics (e.g. prolonged half life) (Aderka *et al.*, 1992; Ma *et al.*, 1996; Mohler *et al.*, 1993) and therefore act as agonists. Whilst it is clear that in the studies outlined in this thesis, the major effect of ST2-Fc appeared to be a direct one, it is also likely that in other situations ST2 may act as a sCR. Whether ST2 would act as an antagonist or agonist in such circumstances awaits the identification of a putative ligand/receptor for ST2.

I next asked whether cytokines produced by Th1 and Th2 cells influence ST2L and ST2 expression. ST2L is known as a selective stable cell surface marker for Th2 cells, but the mechanism of Th2 cell-specific expression has not been addressed. Here I show that IL-4, a main player in Th2 cell polarisation, induces ST2L expression on naïve Th cells and polarised Th2 cells. A crucial role for IL-4 on ST2L expression was also suggested by Carter *et al.* (2001) and Meisel *et al.* (2001), reporting that IL-4 expression proceeds ST2L expression on polarising Th2 cells. ST2L expression also increases due to other

Th2 cell proliferation stimuli such as IL-5, IL-6, IL-1 and TNF-α expressed by APC and Th2 cells (Meisel *et al.*, 2001; own data). Although ST2L expression can be modulated by Th2 cytokines, studies on IL-4-, IL-5 and IL-10-deficient mice demonstrated that ST2L expression is not dependent on the presence of these cytokines. Double or triple knock-out mice might demonstrate the importance of Th2 cytokines. On the other hand IFN-γ, the main Th1 cytokine, is able to reduce ST2L expression on cloned Th2 cells. IL-2, another Th1 cell cytokine, decreased ST2L expression on cloned Th2 cells but to a much lesser extend than IFN-γ at similar concentrations (data not shown). These results suggest another way of cross-regulation between Th1 and Th2 cells. Interestingly, the expression of IL-18R, a cell surface marker for Th1 cells, is upregulated by IL-12 and IFN-γ, while the presence of IL-4 results in a downregulation of the IL-18R α chain (Sareneva *et al.*, 2000; Smeltz *et al.*, 2001).

By assessing the expression pattern of ST2 and ST2L in response to IL-4, I found evidence that the two forms are differentially regulated. This was not due to alternative promoter usage but might be explained by different mRNA stability, which will have to be assessed. Differential regulation of ST2 and ST2L expression had already been suggested in mast cells, where calcium ionophore stimulation resulted in a switch from the long to the short transcript (Gaechter *et al.*, 1998).

When more information on the biological activity and regulation of ST2 is revealed, ST2-Fc may move to be an attractive therapeutic agent due to its specificity, affinity and lack of immunogenicity. Further, the ST2-Fc fusion protein has the added advantage of an increased half-life as a result of its dimeric nature. Indeed, its ability to trigger anti-

inflammatory responses is of considerable interest as a therapeutic strategy for septic shock and autoimmune diseases such as rheumatoid arthritis.

Gentamicin, a broadly used antibiotic of the aminoglycoside family, had been reported to decrease the severity of *L. major* infection in mice when used topically in combination with paromomycin (Grogl *et al.*, 1999). However, the mechanism for this effect remained unclear. In the second part of this thesis I demonstrate that the aminoglycosides G418 and its structural analogue gentamicin are able to selectively suppress Th2 cells compared with Th1 cells. Gentamicin inhibits prokaryotic and to a lesser extend eukaryotic cells by binding to ribosomes and perturbing the elongation of the nascent chain by impairing the proof-reading process controlling translational accuracy (Mingeot-Leclercq *et al.*, 1999, Manuvakhova *et al.*, 2000). There are several possible mechanisms for the differential suppression of Th2 and Th1 cells. Firstly, presuming a faster rate of proliferation and therefore an increased protein synthesis of Th2 cells compared to Th1 cells, the same amount of gentamicin will lead to more nonsense mutation and loss of viability in Th2 cells. Secondly, Th2 cells might have an increased amount of gentamicin in the cell due to lower expression of an active pump, transporting gentamicin out of the cell. Members of the *mdr* family, coding for P-glycoprotein, are differentially expressed in Th1 and Th2 cells. Although *mdr* has yet to be shown differentially expressed in Th1 but not in Th2 cells, the increased expression of P-glycoprotein has been reported in Th1 type responses such as rheumatoid arthritis (Yudoh *et al.*, 1999). Additionally, IFN-γ upregulates the expression and activity of P-glycoproteins in macrophages (Puddu *et al.*, 1999).

Extrapolating these findings *in vivo*, high gentamicin concentrations prevented the onset of a *L. major* infection in BALB/c mice if the injections started at the time of infection. However, treatment with high concentrations of gentamicin caused a general suppression of *L. major*-specific Th and B cell immune responses. A suppressive effect of the applied gentamicin concentration was also observed on macrophage viability and parasite shape and activity. The latter might be explained by the fact that *L. major* ribosomes are also a target for aminoglycosides (Maarouf *et al.*, 1995). Although a short-term gentamicin treatment did not affect the infective ability of the parasite *in vivo*, a longer treatment might generate a parasite mutant unable to infect macrophages and can therefore be used for active immunisation.

When a shorter gentamicin treatment was used, gentamicin suppressed Th2 type responses while Th1 type responses were upregulated or not affected. Further experiments using a short term or lower dose of gentamicin treatments would have to establish whether the modulation of the Th1/Th2 balance is sufficient to prevent the development of leishmaniasis. This treatment regime should also limit the side effects of gentamicin on other cell types such as kidney cells. Attempts are made to find the active ring of gentamicin which is most important for binding to the ribosomes (Cashman *et al.*, 2001). This could lead to improved drug devoid of side effects such as oto- and nephrotoxicity.

Gentamicin and G418 could be useful tools to influence Th1/Th2 type balance in *in vitro* culture systems. Further, the *in vivo* ability of gentamicin to shift the Th1/Th2 type balance towards a Th1 biased phenotype and to decrease antigen-specific IgG1 antibody production might be used in murine disease models like CIA, schistosomiasis

mansoni or bacterial infections, where moderate doses of gentamicin may attenuate the disease (reviewed in Spellberg and Edwards, 2001; Boros and Whitfield, 1999). In contrast, helminth infections require a strong Th2 phenotype to clear the worm burden (Spellberg and Edwards, 2001). Here gentamicin might exacerbate the disease by promoting a Th1 phenotype.

Finally, the ability of gentamicin to prevent proof-reading during the protein synthesis has been used as a treatment for patients suffering from Duchenne and Becker muscular dystrophies (Wagner *et al.*, 2001; Karpati, 2001). A subset of these patients possess a nonsense mutation, causing the premature termination of dystrophin translation. Gentamicin successfully suppressed the nonsense mutations, allowing translation of full-length proteins.

Conclusions

The studies documented in this thesis were concerned with the modulation of Th1 type and Th2 type immune responses by ST2 and gentamicin.

ST2 has been shown to increase Th1 type responses and decrease Th2 type responses in ST2L expressing Th2 cells. This might be explained by a competition between ST2 and ST2L for their common ligand. Results obtained in this study suggest that ST2 is able to decrease cytokine responses on activated naive T helper cells, which do not express ST2L. ST2 mediates this function by binding to an unidentified cell surface molecule. Restimulated Th cells become resistant to ST2, which can not bind to these cells.

ST2L is expressed on Th2 but not on Th1 cells due to the cytokine environment during polarisation. IL-4 augments the ST2L expression, while IFN-γ decreases the ST2L expression. Further evidence found that ST2 and ST2L gene expression is differentially regulated.

Gentamicin, an aminoglycoside antibiotic, has been used as treatment for *L. major* infection. The success of this treatment might be due to its ability to selectively suppress Th2 type responses *in vitro* and *in vivo*. Excessive gentamicin treatment early during *L. major* infection causes a complete suppression of *L. major*-specific immune responses, while a shorter treatment selectively diminishes parasite-specific Th2 type responses. The sufficiency of this treatment to prevent the disease has to be further examined.

Additionally, gentamicin decreases macrophage function and might alter parasite behavior.

Thus, our understanding of the mechanisms of action of ST2 and gentamicin would contribute to our ability in modulating Th1/Th2 cell-mediated diseases.

Future studies

The following points provide a possible framework for future studies aiming at further investigating the role of ST2 and gentamicin in immune responses and to assess their potential as therapeutic agents.

- Identification of the ST2/ST2L binding molecule (soluble ligand or membrane-bound receptor).

- Demonstration of the influence of ST2 on effector functions of activated and non-activated B cells and DCs.

- Administration of ST2 to ST2L-deficient mice challenged with LPS or *L. major* to determine the effect of ST2 on ST2L(-) cells and investigate the immune response affected.

- Investigation of effect of short-term gentamicin treatment in Leishmaniasis

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Appendices

A.1 Suppliers

Abbott Laboratories	•TD_x system
American Type Culture Collection	•RAW264, D10, Dorris, Sf9, High5 cell line
Amersham-Pharmacia	•ECL detection kit •Rainbow protein molecular weight markers •Protein A Sepharose •nylon membrane •spectrophotometer
BDH	ammonium persulphate
Biogenesis Ltd.	•RNAzol^{TM}B
BioRad	•acrylamide •bis-acrylamide •nitrocellulose membrane
Becton Dickinson	•FACS Flow •FACS Lysing solution
Cadisch Precision Meshes	•nylon mesh
Costar	•all tissue culture flasks and plates •Eppendorf tubes (1.5ml; 0.5 ml)

	•all filter tips (1-1000 μl)
	•plastic scraper
Diagnostic Scotland	•all the HRP-conjugated secondary antibodies
	•Streptavidine Peroxidase
DIFCO	•thioglycollate broth
Dynatech	•TMB
Dynex	•MRX plate reader
	•Immunolon4 plates
Genosys	•all the oligonucloetides and peptide synthesis
Gibco-BRL	•1 kb DNA markers
	•agarose
	•dATP, dGTP, dCTP, dTTP
	•RPMI 1640
	•Schneider's medium
	•DMEM w Glutamaxl medium
	•Fetal Bovine Serum (FBS)
	•Fetal Calf Serum (FCS)
	•FBS low IgG
	•MEM (10x)
	•Formaldehyde

	•HEPES
	•L-Glutamine
	•Penicillin/Streptomycin
	•Phosphate Buffered Saline (PBS)
	•random primers
	•SuperScript II (reverse transcriptase)
	•geneticin sulphate (G418)
Harlan Olac	•DO.11.10 TCR transgenic mice
Hoeffer Scientific Ltd.	•whole electrophoresis system
Kodak	•X-ray film cassettes
	•X-omst AR autoradiography film
Kroeplin	•caliper
LKB Wallac, Turku, Finland	•Betaplate™96-well Harvester
	•Liquid scintillation counter
Milenyi Biotec Inc.	•CS columns
	•anti-rat-dyn-beads
Millipore Corp Ltd.	•stirred ultrafiltration cell 8010
	•YM30 membrane
Pharmacia	•Protein-A/Sepharose 4B beads
	•nylon membrane
	•spectrophotometer
Pharmingen	•Propidium iodide

	•Annexin V-FITC
	•most antibodies used for Flow cytometry
Pierce	•Coomassie® Protein Assay
Sigma	•Human IgG (Reagent Grade)
	•Lipopolysaccharide (LPS)
	•TEMED
	•mitomycin
	•ethidium bromide
	•gentamicin sulphate
	•Tryptan Blue
	•MTT
Weber Scientific International Ltd.	•counting chamber
West of Scotland Radionucleotide Dispensary, Western Infirmary,	•^{3}H-Thymidine

Gifts	•monoclonal anti-ST2L antibody (R. Klemenz, Basel)
	•IL-4 (Genzyme, Cambridge, MA)
	•IL-12 (Hoffman-LaRoche, Nutley, NJ)
	•IL-2 (Genosys)

- X4, X9, X12, group A streptococcal M protein (Dr JRobinson, University of Newcastle upon Tyne, UK
- CSF-1 (Professor David Hume, Brisbane, Australia)
- stably transfected CHO cell line (Dr. Daiwu Kang, Astra Zeneca, Sweden)

All the other chemicals used in this study are commercially available from SIGMA or BDH.

A.2. Table of antibodies used in flow cytometry

Antibody	Clone	Isotype	Host	Concen-tration	Company
m FITC anti-human IgG1	8cl6-39	IgG2a	Mouse	2.6 μg/ml	Sigma
m Biotin anti-mouse ST2	DJ8	IgG1, κ	Rat	2.5 μg/ml	Dr. R. Klemenz, Switzerland
m PE anti-mouse CD4 (L3T4)	H129.19	IgG2a	Rat	1 μg/ml	Pharmingen
m FITC anti-mouse CD4 (L3T4)	H129.19	IgG2a	Rat	2.5 μg/ml	Pharmingen
m FITC anti- mouse CD19	1D3	IgG2a, κ	Rat	0.5 μg/ml	Pharmingen
PE Annexin V				5 μl	Pharmingen
PI				0.5 μg	SIGMA
m PE anti-mouse CD8	53-6.7	IgG2a	Rat	0.1 μg/ml	Pharmingen
m FITC anti-mouse Pan-NK cells	DX5	IgM	Rat	2.5 μg/ml	Pharmingen
m Polyclonal anti-mouse macrosialin (FA/11)	FA/11	IgG2a	Rat	1:200 of culture sn	gift from Dr. Paul Crocker, Dundee (Lang *et al.*, 2000)
m Biotin anti-mouse KJ1-26	KJ1-26.1	IgG2a	Mouse	5 μg/ml	gift from Dr. Paul Garside, Glasgow
M FITC anti-mouse H-2D^d	34-2-12	IgG2a	Mouse	2.5 μg/ml	Pharmingen

m purified anti-mouse CD16/32/Fcγ receptor (Fc block)	2.4G2	IgG2b	Rat	2.5 μg/ml	Pharmingen
FITC Streptavidin				2.5 μg/ml	Pharmingen
FITC-conjugated $F(ab')_2$ fragment anti-rat IgG (H+L)			Donkey	6 μg/ml	Jackson ImmunoResearch Laboratories

m = monoclonal

p= polyclonal

A.3 Antibodies used for ELISA

A.3.1 Table of capture antibodies

Cytokine	Clone	Isotype	Host	Concentration (μg/ml)	Company
IFNγ	RA-642	IgG1	Rat	2	Pharmingen
IL-2	JES6-1A12	IgG2a	Rat	2	Pharmingen
IL-4	11B11	IgG1	Rat	2	Pharmingen
IL-5	TRFK5	IgG1	Rat	4	Pharmingen
IL-6	MPS-20F3	IgG1	Rat	1	Pharmingen
IL-10	JES5-2A5	IgG1	Rat	8	Pharmingen
IL-12	C15.6	IgG1	Rat	8	Pharmingen
TGFβ1	A75-2	IgG2a	Rat	1	Pharmingen
TNFα Duoset	polyclonal	IgG	Goat	0.3	R&D

A.3.2 Table of recombinant cytokines used in ELISA

Cytokine	Top Standard	Bottom Standard	Company
IFN-γ	20 ng/ml	20-40 pg/ml	Pharmingen
IL-2	10 ng/ml	10 pg/ml	R&D
IL-4	10 ng/ml	10-20 pg/ml	R&D
IL-5	10 ng/ml	40 pg/ml	R&D
IL-6	10 ng/ml	10-20 pg/ml	Pharmingen
IL-10	10 ng/ml	40 pg/ml	Genzyme
IL-12	10 ng/ml	10 pg/ml	Genzyme
TGFβ1	10 ng/ml	40-60 pg/ml	R&D
TNFα	10 ng/ml	20 pg/ml	Biosource international

A.3.3 Table of detection antibodies used in ELISA

Cyto-kine	Clone	Isotype	Conju-gate	Host	Concentra-tion (μg/ml)	Company
IFNγ	XMG1.2	IgG1	Biotin	Rat	0.5	Pharmingen
IL-2	JES6-5H4	IgG2b	Biotin	Rat	1	Pharmingen
IL-4	BVD6-24G2	IgG1	Biotin	Rat	1	Pharmingen
IL-5	RFK4	IgG2a	Biotin	Rat	4	Pharmingen
IL-6	MP5-32C11	IgG2a	Biotin	Rat	1	Pharmingen
IL-10	SXC-1	IgM	Biotin	Rat	4	Pharmingen
IL-12	C17.8	IgG2a	Biotin	Rat	1	Pharmingen
TGFβ1	A75-3	IGg2a	Biotin	Rat	0.5	Pharmingen
TNFα Duo-set	polyclonal	IgG	Biotin	Goat	0.3	R&D
IgG1	A85-1	IgG1	Biotin	Rat	1	Pharmingen
IgG2a	R19-15	IgG1	Biotin	Rat	1	Pharmingen