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THE ROLE OF IL-1 IN THE INDUCTION OF IL-17 PRODUCING T CELLS

CAROLINE EVA SUTTON

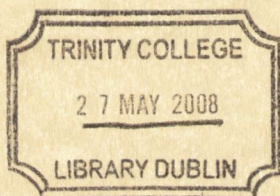
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Caroline Sutton

Caroline Sutton

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ABSTRACT

T helper cell responses have traditionally been divided into two distinct subsets, Th1 and Th2, producing IFN- γ and IL-4 respectively. However, more recently the IL-12 family member, IL-23, has been shown to have a role in the development of IL-17 producing T cells (T_{IL-17}), including Th cells. The initial aim of this project was to examine the adjuvant activity of heat labile enterotoxin from *Escherichia coli* (LT). In the course of these studies, designed to examine the role of IL-1 in the adjuvant effect of LT, I discovered a novel role for IL-1 in the induction of T_{IL-17} cells.

LT is potent adjuvant and enhances antigen-specific T helper (Th) cell responses to co-administered antigens in vivo. The adjuvant activity of LT has been attributed to enzyme activity and enhancement of intracellular cAMP concentrations. However, non-toxic mutants of LT, such as LTK63, retain adjuvant activity, suggesting alternative mechanisms to cAMP induction. This study demonstrated that LTK63 was capable of modulating dendritic cells (DC) activation, but with slower kinetics than the wild type toxin, with the exception of enhancement of LPS-induced IL-1 β production. IL-1 was found to be at least partially responsible for the adjuvant activity of LT and LTK63. Antigen-specific Th1 and Th2 responses were reduced, but antigen-specific and CD3-induced IL-17 was completely abrogated in IL-1RI $^{-/-}$ mice. This finding pointed to a novel role for IL-1 in the development of T_{IL-17} cells.

Upon further examination it was found that IL-23 did not induce IL-17 production by spleen cells or purified CD3 $^{+}$ T cells from IL-1RI $^{-/-}$ mice and was a weak stimulus for IL-17 production by T cells from wild type mice. However IL-23-induced IL-17 was substantially enhanced by the addition of IL-1, in both the presence and absence of anti-CD3 co-stimulation. The T cells that produced IL-17 in response to IL-1 and IL-23 stimulation, in the absence of anti-CD3, were found to be neither CD4 $^{+}$ nor CD8 α^{+} suggesting a possible role for un-conventional T cells. It was found that $\gamma\delta$ T cells, but not NK T cells, produced IL-17 in response to IL-1 and IL-23 in the absence of anti-CD3 co-stimulation. Furthermore, IL-1 and IL-23-activated $\gamma\delta$ T cells promoted IL-17 production from $\alpha\beta$ T cells, and this was enhanced by the presence of DC, which secreted pro-inflammatory cytokines in response to IL-17.

Application of these results to a model of autoimmunity showed that the capacity to induce experimental autoimmune encephalomyelitis (EAE) in mice, a model for multiple sclerosis (MS), was significantly reduced in IL-1RI $^{-/-}$ when compared with wild type mice. This defect correlated with a failure to induce autoantigen-specific T_{IL-17} cells. Adoptive transfer of total CD3 $^{+}$ T cells from wild type mice, but not purified $\gamma\delta$ T cells or $\gamma\delta$ T cell-depleted CD3 $^{+}$ T cells induced EAE in IL-1RI $^{-/-}$ mice. Thus, IL-1 is required to activate both $\alpha\beta$ and $\gamma\delta$ T cells in the development of EAE.

A link between PAMP-induced innate activation and development of autoimmune disease has not been firmly established, although development of EAE has been shown to require DC maturation. Moreover, LT provides its adjuvant activity in the absence of PAMP stimulation. Therefore studies were performed to link the role of danger associated molecular patterns (DAMPs) in innate immune cell activation. Elastase, HMGB1 and HRF promoted IL-23 production and maturation of DC in vitro in the absence of LPS, and in the case of HRF and HMGB1 had adjuvant properties in vivo. Furthermore, LT and LTK63 were both capable of enhancing DAMP-induced IL-1 β and IL-10 production by DC. Therefore, DAMPs may provide signals to innate immune cells required for the induction of pathogenic T cells and the development of EAE as well as the adjuvant activity of LT in vivo.

PUBLICATIONS

Sutton, C, Brereton, C, Keogh, B, Mills, KHG, Lavelle, EC. (2006). A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J. Exp. Med.* 2006 July; 203(7): 1685-1691

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ABBREVIATIONS

ADP	adenosine diphosphate
ANOVA	analysis of variance
APC	antigen presenting cell
ATP	adenosine triphosphate
BMDC	bone marrow-derived DC
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cHRF	cleaved histamine releasing factor
CpG	cysteine-phosphate-guanosine motif
cpm	counts per minute
CT	cholera toxin
CTL	cytotoxic T lymphocyte
CTLA-4	cytolytic T lymphocyte-associated antigen 4
d	day
DAMP	damage associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
DMSO	dimethyl sulphoxide
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorting

FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage-colony stimulating factor
h	hour
His	histamine
HMGB1	high-mobility group box 1
HRF	histamine releasing factor
HRP	horseradish peroxidase
i.n	intranasal
i.p	intraperitoneal
Ig	immunoglobulin
IL-	interleukin
ICAM	intracellular adhesion molecule
iDC	immature DC
IFN	interferon
KLH	keyhole limpet haemocyanin
L	litre
LPS	lipopolysaccharide
LT	heat labile <i>E. coli</i> enterotoxin
LTK63	LT possessing amino acid substitution at position 63 (Ser-Lys)
LTR72	LT possessing amino acid substitution at position 72 (Ala-Arg)
MΦ	macrophage
MAL	MyD88 adaptor-like

MCP	monocyte chemotactic protein
mDC	mature DC
MDP	muramyl dipeptide
mg	milligram
MHC	major histocompatibility complex
min	minute
MIP	macrophage inflammatory protein
MS	multiple sclerosis
NF-κB	nuclear factor- κ B
ng	nanogram
NOD	nucleotide binding oligomerization domain
NK	natural killer
OD	optical density
OVA	ovalbumin
OPD	o-phenyldiamine
PAMP	pathogen-associated molecular pattern
Pam3Cys	palmitoyl-3-cysteine-serine-lysine-4
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
pg	picogram
PGN	peptidoglycan
PMA	phorbol myristic acid
PMN	polymorphonuclear
PRR	pathogen recognition receptor

RA	rheumatoid arthritis
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	room temperature
RT-PCR	real time-PCR
s.c	sub-cutaneous
SD	standard deviation
TB	tuberculosis
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
TIR	Toll IL-1 receptor
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cell
TRIF	TIR-domain containing adaptor inducing interferon β
μg	micrograms

DECLARATION OF AUTHORSHIP	I
ACKNOWLEDGEMENTS	II
ABSTRACT	IV
PUBLICATIONS	V
ABBREVIATIONS	VI
CONTENTS	X

CHAPTER I: GENERAL INTRODUCTION

1.1 OVERVIEW OF THE IMMUNE SYSTEM	1
1.2 THE INNATE IMMUNE SYSTEM	2
1.2.1 CELLS OF THE INNATE IMMUNE SYSTEM	2
1.2.1.1 Dendritic Cells	3
1.2.1.2 Macrophages	4
1.2.1.3 Natural Killer Cells	5
1.2.2 PATHOGEN RECOGNITION RECEPTORS	5
1.2.3 TOLL-LIKE RECEPTORS	7
1.2.4 SURFACE EXPRESSED TLRs	8
1.2.4.1 TLR4 and LPS signalling	8
1.2.4.2 Co-operative TLRs: TLR1, TLR2 and TLR6	10
1.2.4.3 Intracellular TLRs	12
1.2.5 TLR SIGNALLING	12
1.2.5.1 The MyD88 dependent pathway	12
1.2.5.2 The MyD88 independent pathway	14
1.2.6 TLR EXPRESSION ON DC SUBPOPULATIONS	14

1.2.7	DC ACTIVATION OF THE ADAPTIVE IMMUNE RESPONSE	15
1.2.8	INNATE IMMUNE CELL ACTIVATION BY ENDOGENOUS DANGER SIGNALS	18
1.2.9	T HELPER CELL CYTOKINE PRODUCTION BY DC	19
1.2.9.1	IL-12	19
1.2.9.2	IL-23	20
1.2.9.3	IL-27	22
1.2.9.4	IL-10	23
1.2.10	THE ROLE OF CHEMOKINES IN LINKING INNATE AND ADAPTIVE IMMUNITY	24
1.2.11	CHEMOKINE CONTROL OF DC MIGRATION	25
1.2.12	THE INFLUENCE OF CHEMOKINES ON T CELL RECRUITMENT AND DEVELOPMENT	27
1.3	ADAPTIVE IMMUNITY	28
1.3.1	T HELPER CELLS	28
1.3.2	TH1 AND TH2 CELLS	29
1.3.2.1	IFN- γ	30
1.3.2.2	IL-4	30
1.3.2.3	TH _{IL-17} CELLS	31
1.3.2.4	IL-17	32
1.3.3	REGULATORY T CELLS	33
1.3.4	CYTOTOXIC T CELLS	36
1.3.5	INNATE LIKE LYMPHOCYTES	37
1.3.6	$\gamma\delta$ T CELLS	37
1.3.7	B CELLS	38

1.4 VACCINATION	39
1.4.1 ADJUVANTS	40
1.4.2 <i>E. COLI</i> HEAT LABILE ENTEROTOXIN	42
1.5 MOUSE MODELS OF HUMAN DISEASES	44
1.6 HYPOTHESIS	45

CHAPTER II: MATERIALS AND METHODS

2.1 MATERIALS	46
2.1.1 AMMONIUM CHLORIDE LYSIS SOLUTION, (0.87%)	46
2.1.2 BACTERIALLY DERIVED MOLECULES	46
2.1.3 CELL CULTURE MEDIUM	46
2.1.4 ELISA STOPPING SOLUTION, (1M H ₂ SO ₄)	47
2.1.5 ELISA WASH BUFFER (10L)	47
2.1.6 ELUTION BUFFER	47
2.1.7 FACS BUFFER	47
2.1.8 FACS BLOCKING BUFFER	48
2.1.9 LURIA BERTANI BROTH (LB)	48
2.1.10 LB AGAR	48
2.1.11 MINIPREP SOLUTION I	48
2.1.12 MINIPREP SOLUTION II	49
2.1.13 MINIPREP SOLUTION III	49
2.1.14 NICKLE COLUMN WASH BUFFER	49
2.1.15 PHOSPHATE-BUFFERED SALINE (PBS) 20X	49

2.1.16	PHOSPHATE CITRATE BUFFER	50
2.1.17	RECOMBINANT PROTEINS FOR IMMUNE CELL STIMULATION	50
2.1.18	TRIS, BORIC ACID, EDTA (TBE), 10X	50
2.1.19	WASH BUFFER 1	51
2.2	METHODS	52
2.2.1	ANIMALS	52
2.2.2	CULTURING CELL LINES	52
2.2.3	CRYOPRESERVATION OF CELLS IN LIQUID NITROGEN	53
2.2.4	IMMUNISATIONS	53
2.2.5	CELL COUNTS	54
2.2.6	DETECTION OF MURINE T CELL-RESPONSES EX VIVO	54
2.2.7	PROLIFERATION ASSAY	55
2.2.8	T CELL CYTOKINE CONCENTRATIONS BY ELISA	55
2.2.9	ANALYSIS OF KLH-SPECIFIC SERUM IgG SUBCLASS TITRES	57
2.2.10	GENERATION OF MURINE BONE MARROW- DERIVED DENDRITIC CELLS	58
2.2.11	ANALYSIS OF BMDC MATURATION AND CYTOKINE/CHEMOKINE SECRETION	59
2.2.12	IL-12p40 ELISA	61
2.2.13	IL-1 β , IL-12p70, TNF- α ELISA	62
2.2.14	IL-6 ELISA	63
2.2.15	GM-CSF ELISA	64
2.2.16	MIP-1 α ELISA	65

2.2.17 IL-23 ELISA	66
2.2.18 FLOW CYTOMETRY ANALYSIS	67
2.2.19 PREPARATION OF RECOMBINANT MURINE HMGB1	68
2.2.19.1 PCR amplification of murine HMGB1 DNA	68
2.2.19.2 Visualisation of DNA by gel electrophoresis	69
2.2.19.3 Purification of DNA from agarose gel	69
2.2.19.4 Ligation of DNA into cloning and expression plasmids	70
2.2.19.5 Growth of <i>E. coli</i>	70
2.2.19.6 Glycerol stocks of <i>E. coli</i>	71
2.2.19.7 Heat shock transformation of <i>E. coli</i>	71
2.2.19.8 Miniprep of plasmid DNA	72
2.2.19.9 Bacterial expression of recombinant murine HMGB1 protein	73
2.2.19.10 Purification of recombinant murine HMGB1 protein	73
2.2.20 INDUCTION AND ASSESSMENT OF EAE	74
2.2.21 DETERMINATION OF MOG-SPECIFIC IMMUNE RESPONSES	74
2.2.22 T CELL PURIFICATION	75
2.2.23 ISOLATION OF $\gamma\delta^+$ T CELLS AND $\gamma\delta$ T CELL DEPLETED CELLS	75
2.2.24 FACS ANALYSIS FOR INTRACELLULAR PROTEINS	76
2.2.25 REAL TIME PCR	77
2.2.26 STATISTICAL ANALYSIS	77
TABLE 2.1 ORIGIN AND SPECIFICITIES OF ANTIBODIES USED IN ELISA	79

TABLE 2.2	ORIGIN OF RECOMBINANT CYTOKINES USED AS ELISA STANDARDS	80
TABLE 2.3	FACS ANTIBODIES AND RELEVANT ISOTYPE CONTROLS	81

CHAPTER III: MODULATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES BY *E. COLI* HEAT LABILE ENTEROTOXIN

3.1	INTRODUCTION	82
3.2	RESULTS	85
3.2.1	WILD TYPE LT AND LTR72 PROMOTE A MIXED TH1/TH2 RESPONSE, WHEREAS LTK63 PROMOTE A POLARISED TH2 RESPONSE TO A CO-ADMINISTERED ANTIGEN	85
3.2.2	LT OR LTR72 PROMOTES MIXED IgG1/IgG2a ANTIBODY PRODUCTION, WHEREAS LTK63 GENERATES A POLARISED IgG2a ANTIBODY RESPONSE TO A CO-ADMINISTERED ANTIGEN	86
3.2.3	LT INDUCES THE MATURATION OF BMDC	86
3.2.4	MATURATION OF DC BY LT IS NOT DEPENDENT ON cAMP PRODUCTION	87
3.2.5	LT DOES NOT INDUCE DC CYTOKINE PRODUCTION BUT CAN MODULATE TLR LIGAND-INDUCED CYTOKINE RESPONSES	88

3.2.6	LT MODULATION OF DC CYTOKINE PRODUCTION IS NOT DEPENDENT ON ENZYME ACTIVITY OF THE TOXIN	89
3.2.7	IL-10 IS NOT RESPONSIBLE FOR THE INHIBITION OF LPS-INDUCED CD40, ICAM-1, IL-12 OR TNF- α BY LT	90
3.2.8	LT MEDIATED MODULATION OF LPS-INDUCED CYTOKINE PRODUCTION IS NOT DUE TO A SOLUBLE FACTOR	91
3.2.9	IL-1RI PLAYS A ROLE IN THE ADJUVANT PROPERTY OF LT AND LTK63	92
3.2.10	INDUCTION OF IL-1 β IS NOT RESPONSIBLE FOR THE ACTIVATION OF DC BY LT	93
3.3	DISCUSSION	95

CHAPTER IV: A CRUCIAL ROLE FOR IL-1 IN THE DEVELOPMENT OF IL-17 PRODUCING T CELLS

4.1	INTRODUCTION	120
4.2	RESULTS	126
4.2.1	IL-1RI IS REQUIRED FOR THE INDUCTION OF ANTIGEN-SPECIFIC T _{IL-17} CELLS	126
4.2.2	IL-1 SYNERGISES WITH THE T _{IL-17} POLARISING CYTOKINE IL-23 TO PROMOTE IL-17 PRODUCTION FROM T CELLS	127

4.2.3	KINETICS OF IL-17 PRODUCTION FROM CD3 ⁺ T CELLS	128
4.2.4	BOTH IL-1 α AND IL-1 β ARE CAPABLE OF SYNERGISING WITH IL-23 TO INDUCE IL-17 PRODUCTION FROM CD4 ⁺ AND CD8 ⁺ T CELLS	129
4.2.5	IL-23 ALONE INDUCES THE TRANSCRIPTION OF IL-17 AND THE IL-17 ASSOCIATED TRANSCRIPTION FACTOR ROR γ T mRNA	130
4.2.6	IL-1 AND IL-23 DO NOT REQUIRE ANTI-CD3 STIMULATION TO INDUCE IL-17 PRODUCTION	130
4.2.7	IL-18, TNF- α AND IL-6 BUT NOT IL-33, SYNERGISE WITH IL-23 TO PROMOTE IL-17 PRODUCTION IN THE ABSENCE OF ANTI-CD3 STIMULATION	131
4.2.8	LPS AND Pam3Cys SYNERGISE WITH IL-23 TO PROMOTE IL-17 PRODUCTION FROM SPLEEN CELLS IN AN IL-1RI-DEPENDENT MANNER	133
4.2.9	TNF- α , BUT NOT IL-1 AND IL-23, UP-REGULATES THE EXPRESSION OF IL-1RI	134
4.2.10	IL-6 AND TGF- β INDUCED IL-17 PRODUCTION BY SPLEEN CELLS IS IL-1RI-DEPENDENT	135
4.2.11	IN THE ABSENCE OF ANTI-CD3 STIMULATION, IL-1 AND IL-23 INCREASE THE PRODUCTION OF IL-17 FROM CD3 ⁺ BUT NOT CD4 ⁺ OR CD8 ⁺ T CELLS	136
4.2.12	$\gamma\delta$ T CELLS PRODUCE IL-17 IN RESPONSE TO IL-1 AND IL-23 STIMULATION	137
4.2.13	$\gamma\delta$ T CELLS CONDITION CD4/CD8 T CELLS TO	

PRODUCE IL-17 VIA BMDC	140
4.2.14 IL-17 PROMOTES IL-23, IL-1 β AND IL-12p40	
PRODUCTION FROM BMDC	141
4.2.15 PRO-INFLAMMATORY CYTOKINE STIMULATION	
OF BMDC INDUCES IL-6 AND IL-23 PRODUCTION	141
4.2.16 IL-1RI IS REQUIRED FOR THE DEVELOPMENT OF	
SEVERE EAE	142
4.2.17 INDUCTION OF ANTIGEN-SPECIFIC T _{IL-17} CELLS	
DURING EAE IS DEPENDENT UPON FUNCTIONAL	
IL-1RI	143
4.2.18 IL-1RI IS REQUIRED UP-STREAM OF T CELL	
ACTIVATION IN THE INDUCTION OF EAE	144
4.2.19 DEVELOPMENT OF EAE IN IL-1RI ^{-/-} MICE	
REQUIRES TRANSFER OF BOTH WILD TYPE $\alpha\beta^+$	
AND $\gamma\delta^+$ T CELLS	145
4.3 DISCUSSION	147

CHAPTER V: ACTIVATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES BY ENDOGENOUS DANGER SIGNALS

5.1 INTRODUCTION	198
5.2 RESULTS	201
5.2.1 ENDOGENOUSLY-DERIVED DANGER SIGNALS	
ACTIVATE BMDC	201

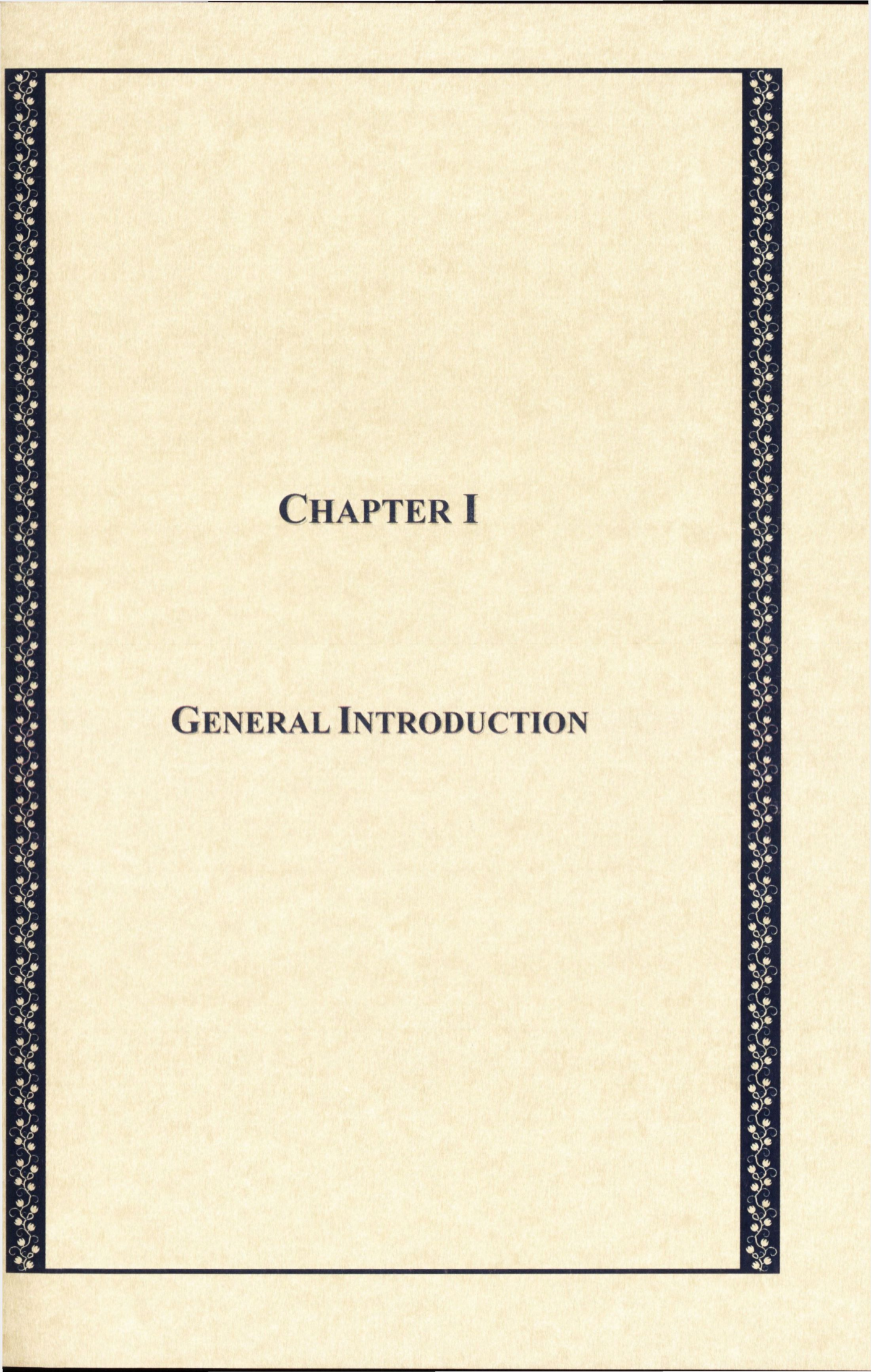
5.2.2	ENDOGENOUSLY-DERIVED DANGER SIGNALS INDUCE PRO-INFLAMMATORY CYTOKINE PRODUCTION FROM BMDC	201
5.2.3	IMMUNISATION WITH ANTIGEN IN THE PRESENCE OF HRF OR cHRF AS AN ADJUVANT INDUCES A TH2 TYPE RESPONSE	203
5.2.4	IMMUNISATION WITH KLH IN THE PRESENCE OF HRF OR cHRF ENHANCES ANTIGEN-SPECIFIC ANTIBODY PRODUCTION	204
5.2.5	IMMUNISATION WITH ANTIGEN IN THE PRESENCE OF HMGB1 AS AN ADJUVANT INDUCES ANTIGEN- SPECIFIC IL-10 AND IL-5 PRODUCTION	204
5.2.6	<i>E. COLI</i> HEAT LABILE ENTEROTOXIN MODULATES DAMP-INDUCED DC CYTOKINE PRODUCTION	205
5.2.7	ENZYMATICALLY INACTIVE LT MUTANTS CAN MODULATE ENDOGENOUS-INDUCED DC CYTOKINES	206
5.3	DISCUSSION	209

CHAPTER VI: GENERAL DISCUSSION

6.1	GENERAL DISCUSSION	231
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CHAPTER VII: REFERENCES

7.1	REFERENCES	241
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CHAPTER I

GENERAL INTRODUCTION

1.1 OVERVIEW OF THE IMMUNE SYSTEM

All multi-cellular organisms are required to mount some form of resistance to protect against potentially harmful invading pathogens. The skin and mucosal membranes are the first line of defence, providing a successful barrier against pathogen invasion. However, sporadically this barrier is broken necessitating a subsequent response to detect the invading 'non-self' entity quickly and remove it from the body and minimise damage. These actions are preformed by the immune system, a coordinated collection of proteins, cells and organs, whose sole aim is to detect and eliminate any threat from invading pathogens. There are two arms to the immune system, the first given the name 'innate', due to the non-specific response it provides, and secondly the 'adaptive' response, which adapts to target specifically any invading pathogen. The two arms act independently and also in concert with one another, to facilitate elimination of the threat, acting either as sentinels, to first encounter and alert the body to the non-self, or activators, to remove the non-self safely. Cells of the innate immune response circulate throughout the body, but are abundant at mucosal sites, allowing for early detection of invading pathogens. In contrast, cells of the adaptive immune response are located primarily in the lymphatic organs, the thymus, spleen and lymph nodes.

The adaptive immune response, mediated by T and B cells, was traditionally deemed the more important of the two arms of the immune response, given its complex nature of activation and its specific response. However, key to activation of the adaptive immune response is activation and maturation of cells of the innate immune system.

1.2 THE INNATE IMMUNE SYSTEM

This innate immune response provides the first line of defence against invading pathogens. It is comprised of innate cells, which target pathogens that have by-passed the primary barrier provided by the skin and mucous membranes. Unlike the adaptive response, which develops gradually, the innate response is quickly activated following pathogen invasion. Indeed innate responses occur within minutes and hours of pathogen invasion as opposed to days or weeks, which are required for the induction of the adaptive response. The various cells of the innate immune system include the antigen presenting cells (APC), macrophages (M Φ) and dendritic cells (DC), as well as natural killer cells (NK). These innate cells patrol the body in a resting or immature form, capable of rapid maturation upon recognition of an invading pathogen. Cells of the innate immune system employ complex, highly conserved receptors, known as pathogen recognition receptors (PRRs) (section 1.2.2) expressed either on the cell surface, intracellularly or secreted into the extra-cellular spaces, for recognition of invading pathogen.

1.2.1 CELLS OF THE INNATE IMMUNE SYSTEM

All cells of the immune system and blood are derived from a pluripotent hematopoietic stem cell contained within the bone marrow. This stem cell differentiates either to from a common lymphoid progenitor, which gives rise to natural killer cells (NK), as well as T and B lymphocytes of the adaptive immune system, the common myeloid progenitor, which gives rise to monocytes, DC and

polymorphonuclear (PMN) cells of the innate immune system, erythrocytes or megakaryocytes, which differentiate into platelets.

1.2.1.1 DENDRITIC CELLS

Dendritic cells, which are derived from monocytes, are the most potent APC of the innate immune system. DC are also responsible for linking the innate and adaptive immune responses by activation and polarising T helper and cytotoxic T cell responses. There are various populations of DC, which differ in their phenotype, location and function. These populations are primarily identified by differential expression of cell surface markers. DC sub-populations were originally discriminated on the basis of cell surface expression of the CTL associated receptor CD8 α . CD8 α ⁺ DC are referred to as lymphoid DC, while CD8 α ⁻ DC are known as myeloid DC and are also CD11c⁺ CD11b⁺ (Pulendran *et al.*, 2004). A third population of murine DC have also been described, which express high levels of the B cell markers B220 (CD45R) and GR-1 (Ly-49G/C), as well as CD11c. The murine CD11c⁺B220⁺GR-1⁺ subset closely resembles the human plasmacytoid DC population, therefore these DC are known as plasmacytoid DC (pDC) (Nakano *et al.*, 2001). CD11c⁺ DC are highly responsive to lipopolysaccharide (LPS) from Gram-negative bacteria, as they have a higher expression of the LPS receptor TLR4 on their surface. CD8 α ⁻, CD8 α ⁺, along with plasmacytoid B220⁺ DC share a common origin in that they can be generated from both myeloid and lymphoid precursors (Ardavin, 2003; Traver *et al.*, 2000), which migrate to various tissues, where they differentiate into immature DC (Banchereau *et al.*, 2000; Kalinski *et al.*, 1999; Moser and Murphy, 2000; Pulendran *et al.*, 2001).

In their immature form, DC recognise pathogens or pathogen associated particles via pathogen recognition receptors (PRR) (section 1.2.2), which are expressed in high numbers on their cell surface. Immature DC also exhibit potent endocytic activity. Following activation through PRR, DC change from an immature antigen-capturing role to T cell activation mode, in a process known as maturation, which is accompanied by translocation of DC to the lymphatic organs. Furthermore, DC maturation is associated with up-regulation of cell surface expression of co-stimulatory molecules, such as CD80, CD86 and CD40 (Banchereau *et al.*, 2000), as well as expression of MHC (Moll, 2004), which are necessary for priming T cell responses. Mature DC have enhanced cell surface expression of CCR7 and thereby their responsiveness to its ligands, CCL19 (MIP-3 β) and CCL21 (secondary lymphoid tissue chemokine, SLC), that are produced in the T cell areas of the lymph nodes (Dieu *et al.*, 1998; Sacki *et al.*, 1999; Vecchi *et al.*, 1999). As CCR7 also mediates homing of T cells to secondary lymphoid organs, it is a key receptor for bringing together antigen bearing mature DC and responder T cells (Willimann *et al.*, 1998).

1.2.1.2 MACROPHAGES

M Φ , similar to DC, are monocyte derived innate immune cells. However, activation of M Φ , in response to pathogen recognition, leads to a very different response to that described for DC maturation (section 1.2.1.1). Whereas DC main function is in antigen-presentation and activation of adaptive immune responses, M Φ have bactericidal properties as well as a function in antigen presentation. The bactericidal mechanism employed by the M Φ relies on phagocytosing the whole pathogen, with the help of the mannose and scavenger receptors. The pathogen is

subsequently destroyed within the M Φ , which leads ultimately to the expression of pathogen associated antigens on MHC molecules on the surface of the M Φ , as well as release of these pathogen-associated antigens into the extra cellular space. DC encountering these M Φ processed pathogen-associated antigens will then undergo maturation.

1.2.1.3 NATURAL KILLER CELLS

NK cells are derived from lymphoid progenitor cells, similar to T and B cells. However, unlike these latter two cell subsets, NK cells lack antigen specificity. They act as innate immune cells responding to interferon (IFN) stimulation, which is produced in response to viral and intracellular infection. NK cells mediate control of pathogens by cytotoxic mechanisms.

1.2.2 PATHOGEN RECOGNITION RECEPTORS (PRR)

PRRs are immune receptors, which recognise microbial molecular structures and trigger various effector responses (Medzhitov *et al.*, 1997). These receptors are expressed on a wide range of cells, including immune cells such as neutrophils, M Φ , DC, NK cells, B cells as well as non-immune cells, such as epithelial and endothelial cells. There are three main classes of PRR, the first of which consist of non-phagocytic receptors, expressed either on the cell surface or intracellularly, such as toll-like receptors (TLRs) (section 1.2.3) and nucleotide-binding oligomerization domain (NOD) proteins. The NOD family of intracellular proteins are characterised by a nucleotide-binding domain followed by a leucine rich repeat region (Bertin *et al.*,

1999; Inohara *et al.*, 2001; Ogura *et al.*, 2001). It has been suggested that NODs play a key role in host defence in tissues where TLRs are absent or expressed at low levels (Philpott *et al.*, 2001), such as on colonic epithelial cells. These cells are in constant contact with microbial flora, and TLR expression is down regulated in order to avoid stimulation of cells by commensal microorganisms, which would lead to unnecessary inflammation. The two best-characterised NODs are NOD1 and NOD2, containing one and two CARD domains respectively (Ogura *et al.*, 2001). It has been shown that NOD1 recognises purified peptidoglycan from gram-negative bacteria, such as *Escherichia coli* and *Shigella flexneri* (Girardin *et al.*, 2001). In contrast NOD2 is regarded as a general sensor for both Gram-positive and Gram-negative bacteria, as it recognises muramyl dipeptide (MDP), a motif found in all peptidoglycans (Girardin *et al.*, 2003; Inohara *et al.*, 2003). It has been demonstrated that a mutation in NOD2 is associated with Crohn's disease, a common inflammatory disease of the intestinal tract (Hampe *et al.*, 2002; Hugot *et al.*, 2001). Patients with this mutation have a reduced ability to produce the anti-inflammatory cytokines, IL-10 and TGF- β (Netea *et al.*, 2004). Furthermore, a NOD2 mutation has also been found to potentiate NF- κ B activation and IL-1 β processing (Maeda *et al.*, 2005).

A second class of PRR induce phagocytosis. These include the M Φ mannose receptor (MR) and the M Φ receptor with collagenous structure (MARCO). These phagocytic receptors recognise microbial ligands on the surface of pathogens, leading to engulfment into the phagocytic cell and ultimately destruction of the microbe. This destruction is due primarily to activation of a respiratory burst and the production of reactive oxygen intermediates, formed from the superoxide radical (O_2^-) as well as by reactive nitrogen intermediates.

A third class of receptor include the secreted PRR, which activate complement, opsonize microbial cells to facilitate phagocytosis and act as accessory proteins for microbial activator recognition by transmembrane receptors, such as mannan-binding lectin (MBL) and the peptidoglycan recognition proteins (PGRPs)

1.2.3 TOLL-LIKE RECEPTORS

TLRs are expressed, either on the cell surface or in the cytoplasm, primarily on cells of the innate immune system, and are crucial sensors of the innate immune response. The TLRs remain highly conserved throughout evolution, indicating both their efficacy and importance in alerting the body to the presence of invading pathogens. They take their name from the similarity to a fruit fly (*Drosophila melanogaster*) receptor named Toll, which was first noted for its role in the dorso-ventral polarisation of the embryonic fly. Upon further investigation, however it was noted that the receptor played a role in host defence in the adult fly, controlling fungal infection through the induction of the anti-fungal agent, drosomycin (Lemaitre *et al.*, 1996). Receptors with close homology to this Toll receptor were subsequently discovered in the genomes of other multi-cellular organisms and were conserved in organisms as diverse as plants and mammals.

The first mammalian TLR was cloned, in 1994, by Nomura and co-workers (Nomura *et al.*, 1994) and in 1996, the gene encoding this protein, later known as TLR1, was mapped to chromosome 4 in humans (Taguchi *et al.*, 1996). To date, mammalian TLRs comprise a family of at least 11 members. TLR1-9 are conserved between the human and the mouse (Takeda and Akira, 2005). TLRs 3, 7, 8 and 9 are all expressed in endosomes within the cell, whereas the others TLRs are expressed on

the cell surface. Surface expressed TLRs are type I transmembrane receptors. They consist of leucine rich repeats (LRRs) of varying length, on the extracellular domains, joined to an intracellular domain, which provides the signalling portion of the receptor. This intracellular region is homologous with the intracellular domain of IL-1 receptor family members and is therefore known as the Toll/IL-1 receptor (TIR) domain (Akira, 2001; Medzhitov, 2001).

1.2.4 SURFACE EXPRESSED TLRs

The TLRs 1, 2, 4, 5, 6, 10 and 11 are all expressed on the cell surface, where they recognise bacterially derived PAMPs present in the extracellular space.

1.2.4.1 TLR4 AND LPS SIGNALLING

TLR4 was first implicated in LPS signalling through studies with C3H/HeJ mice, which are highly resistant to the biological effects of LPS (Sultzter, 1968). Detailed genetic studies revealed that C3H/HeJ mice had a single amino acid substitution in a conserved residue of the cytoplasmic domain (P712H) of TLR4, rendering the mice hypo-responsive to LPS. However, TLR4 does not directly bind to LPS, accessory proteins are required to form a complex, which binds LPS and allows for signalling via TLR4. Studies with LPS binding protein (LBP)-deficient mice revealed a non-redundant role for LBP in the response to LPS (Jack *et al.*, 1997; Wurfel *et al.*, 1997). LBP was found to bind to the lipid A moiety of LPS and this LPS/LBP complex was shown to trigger association with another LPS-binding molecule CD14 (Tobias *et al.*, 1993). Indeed LPS-induced TNF- α production from

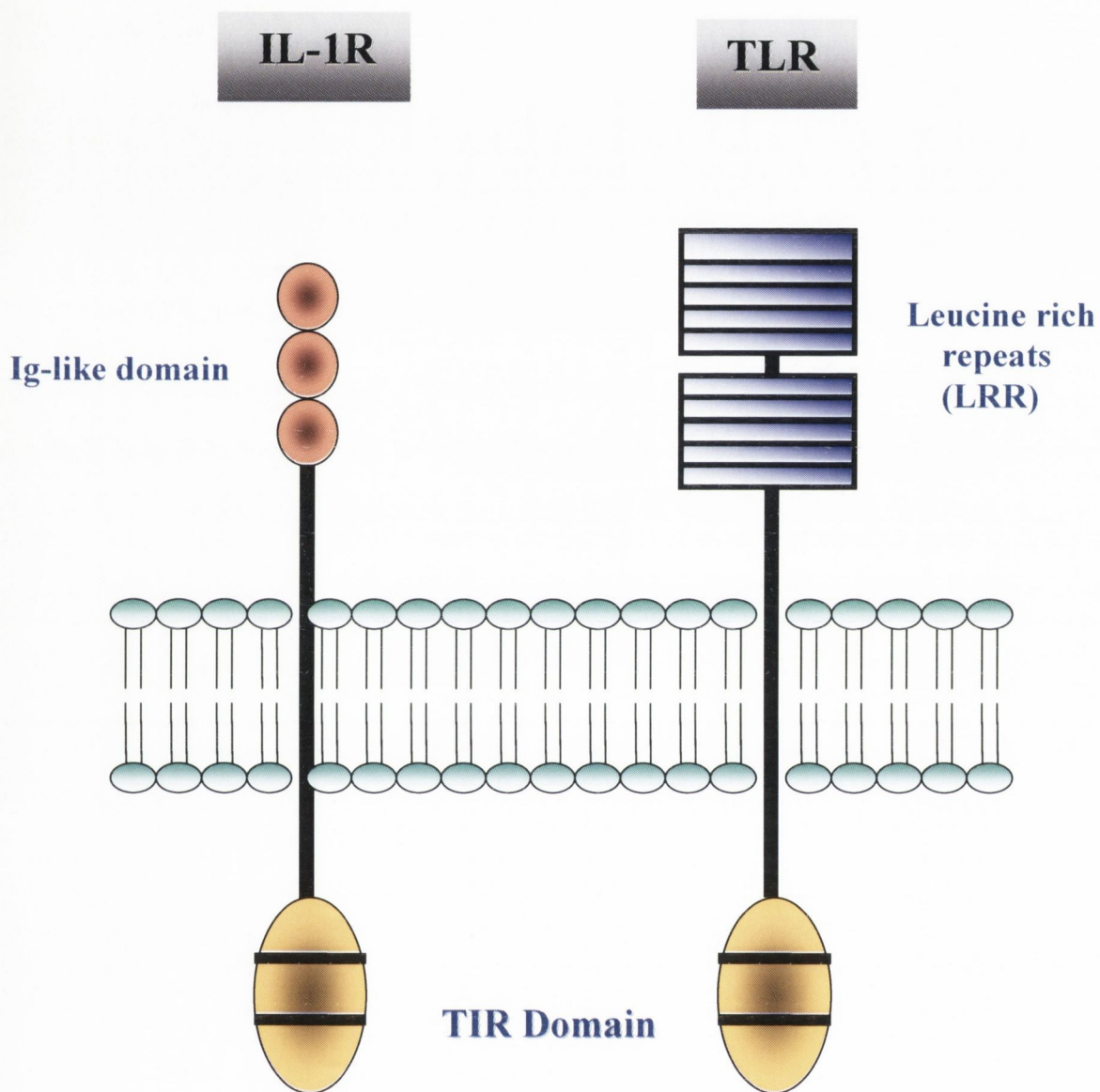


Figure 1.1: TLR and IL-1R families share a homologous intracellular TIR domain.

MD-2 has been shown to be entirely dependent upon CD14 (Chen *et al.*, 2004). The last, non-redundant member, of the LPS recognition complex was found to be MD-2, which links with LBP and CD14 to recognise and bind LPS in order to signal through TLR4 (Shimazu *et al.*, 1999).

However, it should be noted that TLR4 does not exclusively respond to LPS signalling, TLR4 can sense other PAMPs including the fusion protein from the respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000). Indeed TLR4-deficient mice cannot eradicate RSV infection efficiently. TLR4 has also been implicated in the recognition of damage associated molecular patterns (DAMPs). These are host-derived products, released during inflammation, tissue injury and necrosis, which trigger inflammatory responses. The extra domain A of fibronectin, heat shock protein (Hsp)-60, as well as small molecular weight fractions of hyaluronan, are DAMPs which have been linked with TLR4 signalling (Okamura *et al.*, 2001; Taylor *et al.*, 2004; Vabulas *et al.*, 2001). However, it has been suggested that DAMP signalling via TLR4 may be due to LPS contamination of the DAMP preparations (Gao and Tsan, 2004).

1.2.4.2 CO-OPERATIVE TLRs: TLR1, TLR2 AND TLR6

TLR2 recognises a wide range of microbial molecules, such as bacterial lipoproteins (Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999), a phenol soluble modulin from *Staphylococcus epidermis* (Hajjar *et al.*, 2001), zymosan from yeast cell walls (Underhill *et al.*, 1999) and glycosylphosphatidylinositol (GPI)-anchors, from the membrane of *Trypanosoma cruzi* (Campos *et al.*, 2001). In addition, TLR2 has been reported to recognise LPS from *Leptospira interrogans* and *Helicobacter pylori*

(Werts *et al.*, 2001). LPS from these bacteria differ from LPS from other bacteria in the number of acyl chains in their lipid A moiety, which is thought to explain this differential recognition (Netea *et al.*, 2002).

TLR2 forms heterophilic dimmers with other TLRs, such as TLR1 and TLR6, both of which are structurally related to TLR2 (Ozinsky *et al.*, 2000). TLR1 and TLR2 association has been shown to recognise triacyl lipoproteins, whereas TLR2 and TLR6 co-operate in the recognition of diacyl lipoproteins (Takeuchi and Akira, 2001).

1.2.4.3 INTRACELLULAR TLRs

The TLRs 3, 7, 8 and 9 are all internally expressed. TLR3 differs from the other TLRs in that it lacks a proline residue in its LRR region, which is conserved among the other TLRs. This receptor is preferentially expressed within mature myeloid DC (Muzio *et al.*, 2000) and responds to double stranded RNA (dsRNA) and synthetic dsRNA (poly I:C) (Alexopoulou *et al.*, 2001). dsRNA is associated with viral infections, as most viruses produce dsRNA at some point in the infection cycle. The TLRs 7 and 8 have been implicated in the response to several synthetic molecules (Hemmi *et al.*, 2002), which have been approved for the treatment of the human papillomavirus infection. It has also been reported that TLR7 and 8 recognise uridine-rich single- stranded (ss) RNA from viruses, such as human immunodeficiency virus and influenza virus (Heil *et al.*, 2004; Lund *et al.*, 2004).

TLR9 recognises unmethylated CpG motifs, which are contained in prokaryotic DNA (Hemmi *et al.*, 2000). Although eukaryotes possess these CpG motifs, the frequency is significantly reduced and the cysteine residues of the CpG

motifs are highly methylated (Wagner, 1999). Indeed, TLR9-deficient mice have been shown to be susceptible to mouse cytomegalovirus (MCMV) infection (Tabeta *et al.*, 2004).

1.2.5 TLR SIGNALLING

The TIR domain initiates downstream transcriptional events through the recruitment of adapter molecules, primarily via myeloid differentiation factor 88 (MyD88) and MAL (MyD88 Associated Ligand)/TIRAP (TIR domain-containing adapter protein), but also through the adapters TIR-domain containing adaptor inducing interferon- β (TRIF) (Oshiumi *et al.*, 2003; Yamamoto *et al.*, 2003) and TRIF-related adaptor molecule (TRAM) (Bin *et al.*, 2003; Fitzgerald *et al.*, 2003; Oshiumi *et al.*, 2003). Following ligation of the extra cellular TLR through either direct TLR/TLR ligand ligation or indirect ligation of the TLR and its ligand by intermediate protein components, adapter molecules are recruited, allowing ultimately for either gene transcription or suppression, principally through activation of the transcription factor NF- κ B.

1.2.5.1 THE MyD88 DEPENDENT PATHWAYS

MyD88 can interact with the TIR domain of all TLR family members, with the exception of TLR3. Following TLR ligation and subsequent activation of the TIR domain of the TLR, MyD88 is recruited through its TIR domain, located at its C-terminal portion. Thus allowing for the TLR associated TIR domain to bind with the MyD88-TIR domain. Following this ligation, and MyD88 activation, the N-terminal

death domain of MyD88 recruits a second family of signal transducers, known as IRAKs (IL-1 receptor-associated kinases) either IRAK-1, 2 or 3 (Muzio *et al.*, 1997; Wesche *et al.*, 1999). Activation of IRAK-1 by phosphorylation facilitates the interaction and activation of tumour necrosis factor receptor-associated factor 6 (TRAF-6) (Cao *et al.*, 1996). TRAF-6 in turn associates and activates TGF- β -activated kinase 1 (TAK1). TRAF-6-activated TAK1 subsequently phosphorylates IKK β , which leads to the activation of the transcription factors including NF- κ B and IRFs (Wang *et al.*, 2001). The NF- κ B family of transcription factors play a critical role in innate immunity (Silverman and Maniatis, 2001). In addition, TRAF-6 activated mitogen activated protein (MAP) kinases, with TAK1 potentially phosphorylating the MAP kinase kinase 6 (MKK6), which in turn phosphorylates the MAP kinase, JNK leading to the activation of the API pathway (Wang *et al.*, 2001). This signalling leads ultimately to either protein expression or suppression dictated by the ligating TLR agonist.

1.2.5.2 THE MyD88 INDEPENDENT PATHWAYS

The existence of a MyD88-independent pathway was revealed through the observation that M Φ from MyD88-deficient mice retained the ability to activate NF- κ B and MAP kinases, albeit with delayed kinetics, following stimulation with LPS (Kawai *et al.*, 1999). Furthermore, DC from MyD88-deficient mice showed some characteristics of maturation, such as enhanced expression of co-stimulatory molecules (CD40 and CD86) in response to LPS (Kaisho *et al.*, 2001). This was one of the first indications that MyD88 was not the only adapter molecule responsible for TLR signalling and that LPS signalling via TLR4 was not entirely MyD88-mediated.

Furthermore, since LPS-deficient activation of IFN regulatory factor (IRF)-3 is preserved in MyD88-deficient cells, this suggested that IRF-3 activation may contribute to the MyD88-independent pathway (Kaisho *et al.*, 2001). The MyD88-independent pathway is also mediated by the TIR-associated, MAL protein, which has been shown to interact specifically with TLR4 and is responsible for an alternative means of TLR4 activation of NF- κ B, as well as through the TIR-adapters, TRIF and TRAM. LPS-induced cytokine production and TLR2 signalling, in MAL-deficient M Φ , is severely impaired (Horng *et al.*, 2002; Yamamoto *et al.*, 2003), however, MAL is not required for TLR5, 7 or 9 signalling (Horng *et al.*, 2002). TRIF has been shown to link TLR3 and TLR4 to the kinase complex IKK ϵ /TBK1, which in turn activates the transcription factor, IRF3 (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). IRF3 is responsible for the induction of a group of genes specific for TLR3 and TLR4, such as interferon- β (IFN- β), or interferon producing protein 10 (IP-10) (Doyle *et al.*, 2002; Kawai *et al.*, 2001). The induction of inflammatory cytokines by LPS is also abolished in TRIF-deficient M Φ , suggesting that TRIF plays a role in both the MyD88-dependent and independent pathways. To date evidence suggests that MyD88, MAL, TRIF and TRAM all have a role to play in responding to LPS stimulation.

1.2.6 TLR EXPRESSION ON DC SUBPOPULATIONS

TLRs are differentially expressed on human and murine DC subsets. In mice, all DC subsets isolated from the spleen express TLR1, 2, 4, 6, 8 and 9 on their surface (Edwards *et al.*, 2003). Murine plasmacytoid B220⁺ DC do not express TLR3, while murine CD8 α ⁺ DC lack TLR5 and TLR7 expression (Doxsee *et al.*, 2003;

Edwards *et al.*, 2003). Expression of TLR7 has been linked solely with plasmacytoid DC (Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001), or both plasmacytoid and myeloid DC (Ito *et al.*, 2002; Krug *et al.*, 2001).

1.2.7 DC ACTIVATION OF THE ADAPTIVE IMMUNE RESPONSE

DC are the key APC required for priming adaptive immune responses. These cells are not only present antigen to naïve T cells, but also help to polarise T cell responses. Polarisation of adaptive immune responses by DC is influenced by the DC subpopulations, activation status of DC as well as DC to T cell ratio (Kalinski *et al.*, 1999; Maldonado-Lopez *et al.*, 2001; Maldonado-Lopez and Moser, 2001; Moser and Murphy, 2000; Pulendran *et al.*, 1999). Each DC population is capable of driving both T helper type I (Th1) and Th2 responses, depending upon the pathogen-derived signals and host-derived cytokines present (De Smedt *et al.*, 2001; MacDonald *et al.*, 2001; Maldonado-Lopez and Moser, 2001; Vieira *et al.*, 2000).

Activation of naïve T helper cells is dependent upon three signals, provided mainly by the pathogen activated DC. Firstly, major histocompatibility complex class II (MHCII) molecules, with bound pathogen peptides attached, are required to ligate the T cell receptor (TCR), located on the surface of the naïve Th cells. This interaction is strengthened by the presence of co-stimulatory molecules, such as B7 family members, CD80 and CD86, as well as CD40. Up-regulation of these co-stimulatory markers, on the surface of the pathogen-primed DC and interaction with CD28 on the T cell, constitute signal two (Cunningham and Lafferty, 1977). In the absence of the

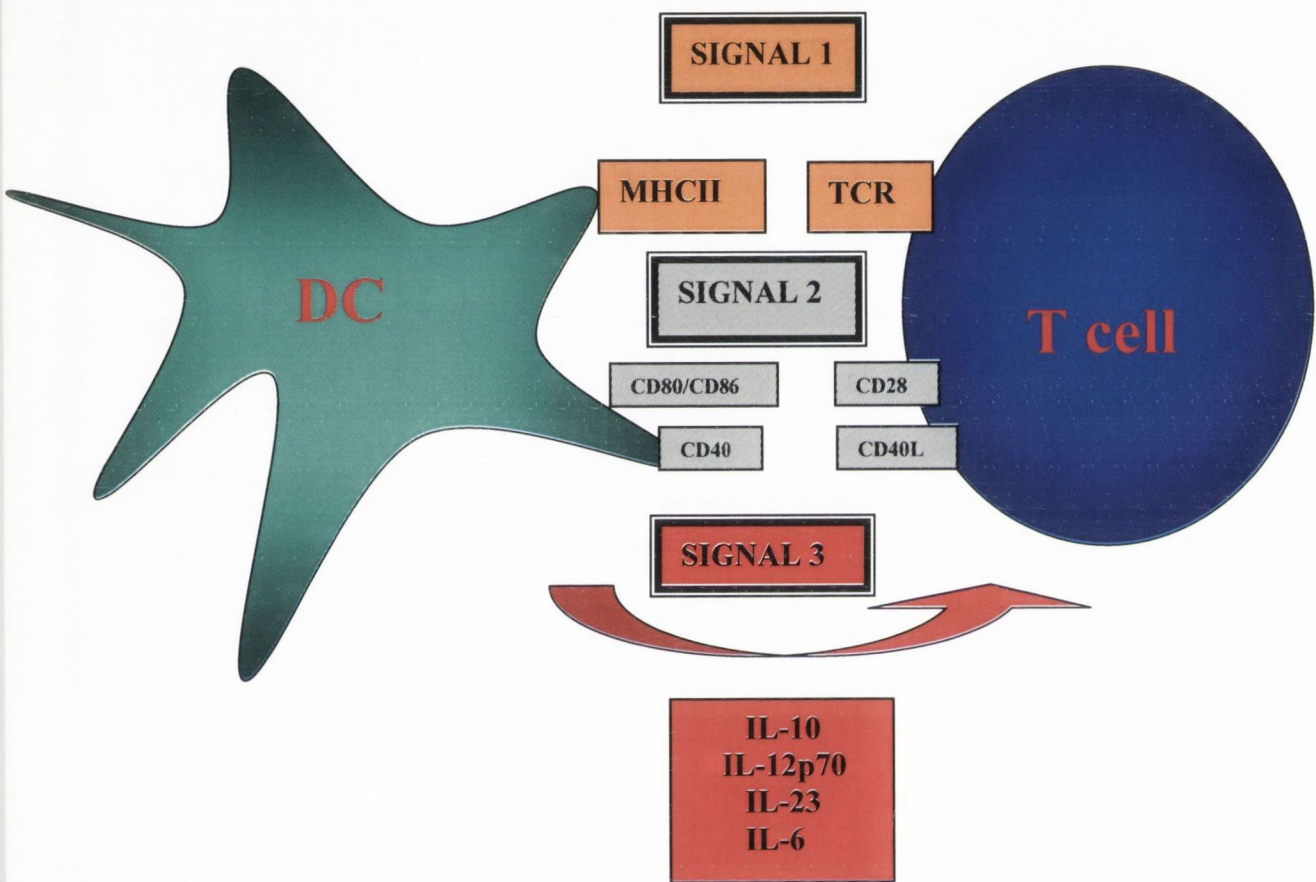


Figure 1.2: DC activation of naïve T cells via signals 1 (MHCII), 2 (co-stimulatory molecules) and 3 (cytokine production).

co-stimulatory signal two, Th cells become anergic. The third signal, required for full Th cell activation is innate cytokine production. The cytokine profile produced by cells of the innate immune system help to bias the T cell toward a specific response, such as a Th1, Th2, Treg or T_{IL-17}.

Mosmann and Coffman were the first to identify separate Th cell subsets, in 1986. They described two types of CD4⁺ Th cells, one of which induced IL-2, granulocyte macrophage-colony stimulating factor (GM-CSF) and interferon- γ (IFN- γ), and was termed Th1, and a second called Th2 cells, which secreted IL-4, IL-5 and IL-13 (Mosmann *et al.*, 1986). IL-12p70 was found to be required for Th1 cell development (Hsieh *et al.*, 1993; Manetti *et al.*, 1993), while Th2 responses have been attributed to production of IL-4 and monocyte chemotactic protein 1 (MCP1) (d'Ostiani *et al.*, 2000; Ohshima and Delespesse, 1997). However, it has been suggested that Th2 cells constitute the default Th response, induced by DC-derived IL-6 in the absence of IL-12p70 (Rincon *et al.*, 1997). Furthermore, development of Th2 responses may depend upon the co-stimulatory molecules CD86 and OX40 ligand on DC rather than cytokine production (Ohshima *et al.*, 1998; Ranger *et al.*, 1996). T regulatory (Treg) cell polarising factors have been identified as IL-10 and transforming growth factor- β (TGF- β) as well as the B7 family member B7-H1 (Dong *et al.*, 1999; McGuirk and Mills, 2002; Zeller *et al.*, 1999). Finally, IL-23 promotes the development of IL-17 producing Th_{IL-17} cells, although this IL-23-induced IL-17 response is not unique to the Th cell subset (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005).

1.2.8 INNATE IMMUNE CELL ACTIVATION BY ENDOGENOUS DANGER SIGNALS

Activation of innate immune cells is not confined to pathogen derived immunostimulatory molecules. It has recently been found that various endogenously derived factors are immunostimulatory under certain conditions (Matzinger, 1998). These damage associated danger signals (DAMPs) are specifically released during necrotic cell death, as opposed to apoptotic cell death, where the cell is degraded safely (Basu *et al.*, 2000; Fadok *et al.*, 1998). Heat shock proteins (Hsp) 70 and 60, the DNA binding protein high mobility group box 1 (HMGB1) and enzymes, such as pancreatic and neutrophil elastase have each been found to be immunostimulatory when aberrantly expressed (Andersson *et al.*, 2000; Chen *et al.*, 1999; Jaffray *et al.*, 2000). In addition, there are many more endogenous factors emerging with immunostimulatory properties. These immunostimulatory properties include activation of DC and M Φ as well as possessing adjuvant activity when co-administered with antigen in vivo (Fadok *et al.*, 1998; Rovere-Querini *et al.*, 2004).

DAMPs have also been associated with the development of autoimmune diseases. Increased macrophage elastase has been found in the synovial fluid of patients with rheumatoid arthritis (RA), while neutralising HMGB1 has been shown to ameliorate collagen-induced arthritis in both mice and rats (Andersson and Erlandsson-Harris, 2004; Liu *et al.*, 2004).

1.2.9 T HELPER CELL CYTOKINE PRODUCTION BY DC

The cytokine milieu that is established during the DC mediated innate response to pathogens sets the stage for the adaptive immune response. Key cytokines released by the pathogen primed DC and other innate immune cells are instrumental in shaping the antigen-specific T cell response (section 1.3, figure 1.3).

1.2.9.1 IL-12

IL-12 was first discovered in 1989, as a soluble factor that could stimulate natural killer (NK) cells to produce IFN- γ (Kobayashi *et al.*, 1989). It is a heterodimeric cytokine of 70 kDa, comprised of covalently linked IL-12p40 and IL-12p35 subunits (Kobayashi *et al.*, 1989) generating IL-12p70. It was subsequently discovered that IL-12p70 could also induce IFN- γ production from CD4⁺ Th1 cells (Hsieh *et al.*, 1993; Manetti *et al.*, 1993). The IL-1 family member IL-18 was originally described as an IFN- γ releasing factor (Dinarello, 2000), and was subsequently found to synergistically enhance IL-12p70-induced IFN- γ production, from NK cells and CD4⁺ T cells. The synergy between IL-12 and IL-18 is at least partially explained by the finding that each cytokine induces the receptor for the other (Lawless *et al.*, 2000; Smeltz *et al.*, 2001; Yoshimoto *et al.*, 1998). In addition, IL-12 alone or in combination with IL-18, acts on APC to induce IFN- γ and further IL-12 production (Frucht *et al.*, 2001; Fukao *et al.*, 2001; Grohmann *et al.*, 1998; Grohmann *et al.*, 2001). This acts to promote further Th1 differentiation and cell-mediated responses. The IL-12p40 subunit of IL-12 is expressed in excess over the IL-12p35 subunit. IL-12p40 can also associate with IL-12p19 to generate another IL-

12 family member, IL-23. IL-27 is a further member of the IL-12 family, which comprises EBI3 and p28.

TLR agonists, such as LPS, LTA and CpG, are potent inducers of IL-12p70 production from DC and M Φ (Gazzinelli *et al.*, 1993; Hochrein *et al.*, 2001; Pulendran *et al.*, 2001). Splenic DC have been shown to secrete IL-12p70 and stimulate Th1 cells (Macatonia *et al.*, 1993). However, within the DC subsets found in the spleen, the CD8⁺ subset was shown to secrete much higher concentrations of IL-12p70 than CD8⁻ DC (Pulendran *et al.*, 1997). In addition to DC, monocytes and M Φ , IL-12p70 is induced by the engagement of CD40 on APC with CD154 (CD40L) on activated T cells (Trinchieri *et al.*, 2003). IL-12 has pleiotropic functions that promote cellular responses through a variety of mechanisms (Farrar *et al.*, 2002).

The IL-12p70 receptor (IL-12R) is a member of the gp130 superfamily of cytokine receptors and is composed of IL-12R β 1 and IL-12R β 2 subunits (Chua *et al.*, 1995; Presky *et al.*, 1996), and is predominantly expressed on activated Th1 cells and NK cells (Desai *et al.*, 1992). The murine IL-12p40 subunit binds to IL-12R β 1 with high affinity, while murine IL-12p35 binds with very low affinity to IL-12R β 2. Mice deficient in either of the IL-12 chains (IL-12p35 or IL-12p40) or in its receptor components (IL-12R β 1 or IL-12R β 2) have deficient Th1 responses, confirming IL-12 as a critical Th1 polarising cytokine in vivo (Trinchieri *et al.*, 2003; Wu *et al.*, 1997).

1.2.9.2 IL-23

The recently identified IL-12 family member IL-23 is composed of the IL-12p40 subunit in combination with the IL-12p19 subunit (Oppmann *et al.*, 2000). IL-23 was initially found to be similar in certain respects to IL-12p70, such as inducing

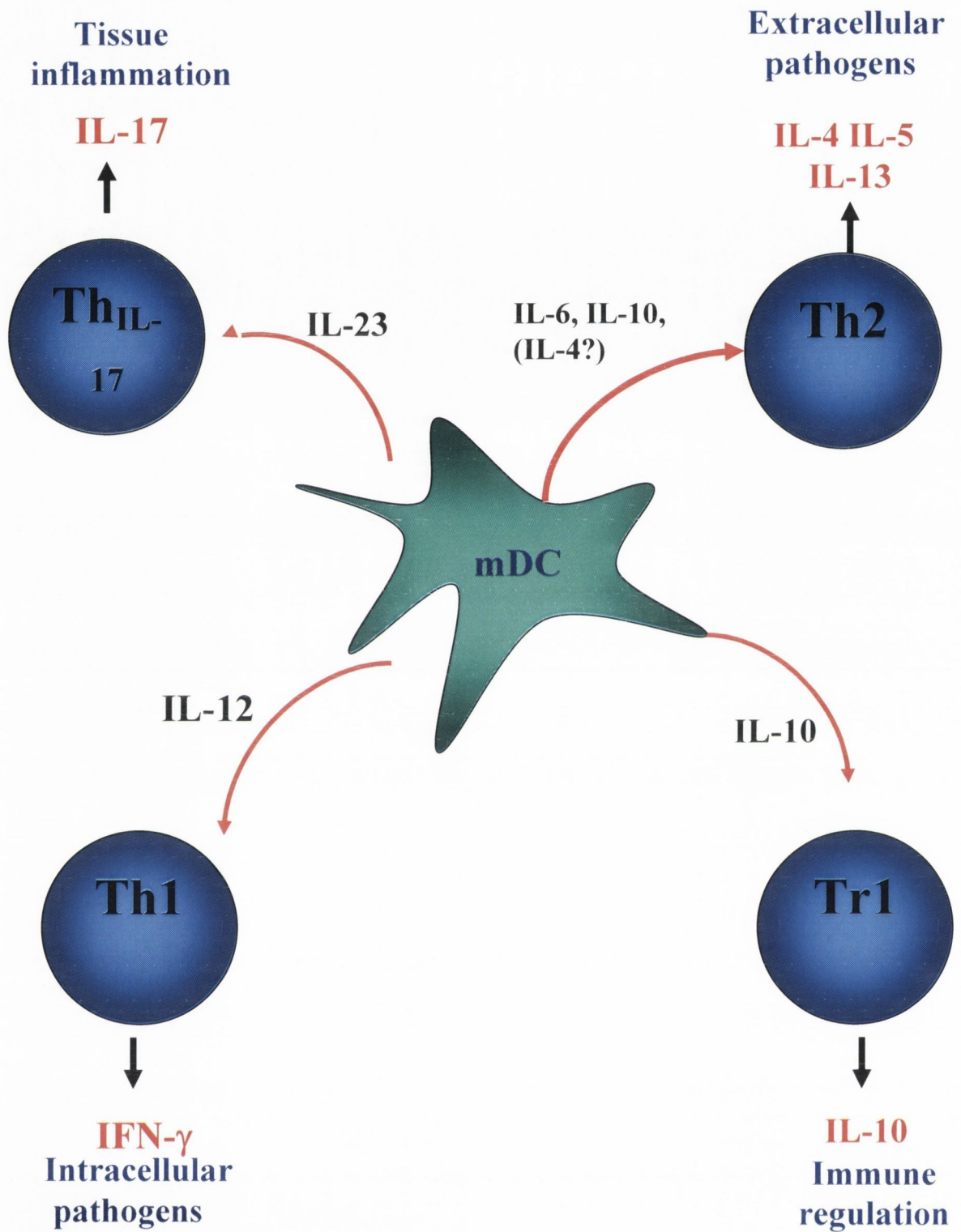


Figure 1.3: Polarisation of naïve T cell responses via DC cytokine production.

IFN- γ from T cells (Belladonna *et al.*, 2002). Unlike IL-12p70, IL-23 does not use IL-12R β 2 as part of its receptor. Instead, the IL-23R, a homologue of IL-12R β 2, forms a complex with IL-12R β 1 to allow for IL-23 signalling. The IL-23R is expressed at low levels on DC, monocytes, NK cells and T cells (Parham *et al.*, 2002).

IL-23 is involved in inflammation; transgenic mice ubiquitously expressing IL-12p19 develop a pathological multi-organ inflammation (Wiekowski *et al.*, 2001). This IL-23-associated inflammation has been attributed to induction of the inflammatory cytokine IL-17 (Aggarwal *et al.*, 2003). Indeed the generation of IL-12p19 knock out mice showed that IL-23 is essential in the development of the autoimmune diseases experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) (Cua *et al.*, 2003; Murphy *et al.*, 2003). The development of T cell-mediated autoimmune diseases had originally been attributed to IL-12p70 and IFN- γ production from Th1 cells, however this assumption was based in part on experiments performed in IL-12p40 knock out mice (Becher *et al.*, 2002). Subsequent experiments with IFN- γ knock out mice demonstrated that IL-23-induced IL-17 producing T cells were responsible for induction of the autoimmune disease and not that Th1 cells (Cua *et al.*, 2003). IL-23, and IL-17, have been further associated with resistance to infection by certain microorganisms, including *Klebsiella pneumoniae*, *Mycobacterium bovis* and *Bordetella pertussis* (Higgins *et al.*, 2006; Umemura *et al.*, 2007; Ye *et al.*, 2001).

1.2.9.3 IL-27

IL-27 is another heterodimeric cytokine of the IL-12 family (Pflanz *et al.*, 2002), which is composed of the Epstein-Barr virus-induced gene 3 (EBI3) and the

novel p28 subunit. The EBI3 subunit of IL-27 signals through WSX1 receptor expressed on NK and T cells, while the p28 subunit signals via the gp130 receptor, which is common to the IL-6R. The p28 subunit of IL-27 is homologous to the p35 subunit of IL-12, whereas EBI3 is related to IL-12p40 and resembles a soluble cytokine receptor (Christ *et al.*, 1998; Devergne *et al.*, 1997). IL-27 is produced by myeloid DC, with the highest concentrations produced by LPS-activated monocytes and monocyte-derived DC. IL-27 preferentially induces proliferation of naïve but not memory T cells and has also been associated with the development of Th1 cells (Pflanz *et al.*, 2002). IL-27 has been found to act both alone and in synergy with IL-17 to promote IFN- γ production and Th1 differentiation.

1.2.9.4 IL-10

IL-10 is a pleuripotent cytokine with potent effects on numerous cell populations, first described in 1989 as a cytokine synthesis-inhibiting factor (CSIF) (Fiorentino *et al.*, 1989). IL-10 exerts potent anti-inflammatory effects, though more recent data has shown IL-10 to have stimulatory effects on certain cell populations. Due to the ‘dual role’ of IL-10, this cytokine is more accurately described as an immunoregulatory cytokine and has also been associated with the generation of CD4⁺ Treg cells (Groux *et al.*, 1997; Levings *et al.*, 2001; Levings *et al.*, 2001; Zeller *et al.*, 1999). Many pathogens induce the production of IL-10 in order to subvert protective immune responses either directly or by inducing Treg cells, which limit Th1 cell development. However, IL-10 is also beneficial to the host during infections, preventing uncontrolled inflammatory responses and immunopathology. Indeed IL-10

deficient mice exhibit a prolonged inflammatory response to acute *Pseudomonas* challenge resulting in neutrophil accumulation in the lung (Chmiel *et al.*, 2002).

Activated innate immune cells, such as DC and M Φ , as well as Treg and Th2 cell populations, produce IL-10. An important target for IL-10 is IFN- γ -producing Th1 cells, on which it acts both directly to inhibit proliferation, and indirectly through inhibition of the Th1 polarising cytokine, IL-12 (Moore *et al.*, 2001). Furthermore, IL-10 suppresses the production of pro-inflammatory cytokines, such as TNF- α , by DC and M Φ (Fiorentino *et al.*, 1991). It has been reported that IL-10 exerts its anti-inflammatory effect in part by altering NF- κ B functions, such as the selective inducing translocation of the p50 subunit, while blocking translocation of the p65/p50 heterodimer (Driessler *et al.*, 2004). However, IL-10 can also act in a stimulatory fashion on NK cells, inducing IFN- γ and TNF- α (Carson *et al.*, 1995).

IL-10-deficient mice develop lethal inflammation of the intestine, which can be prevented by administration of IL-10 (Kuhn *et al.*, 1993). Administration of IL-10 can suppress Th1-mediated inflammatory conditions, such as colitis. However, this was only successful when administered before initiation of colitis, and was found to be ineffective at reversing established inflammation (Barbara *et al.*, 2000; Herfarth *et al.*, 1996; Powrie *et al.*, 1993). IL-10 treatment has also been shown to be beneficial in reducing the clinical signs of EAE (Chen *et al.*, 1994; Rott *et al.*, 1994).

1.2.10 THE ROLE OF CHEMOKINES IN LINKING INNATE AND ADAPTIVE IMMUNITY

Chemokines, owing to their differential capacity to recruit specific leukocyte populations, ultimately determine which cells will regulate and participate in the local

innate and adaptive immune responses. These small proteins, which constitute one of the first innate immune responses mounted following pathogen invasion act as chemoattractants, inducing chemotaxis in nearby responsive cells. Chemokines belong to a supergene family of 8-10 kDa protein mediators, which are sub-classified into two large (CC and CXC) and two small (C and CX3C) groups based on the number and positioning of the most highly conserved cysteines. All chemokines have at least two conserved cysteines, and all but two chemokines, have at least four. In the four cysteine group the first two, which are located in the N-terminus, are either adjacent (CC) or separated by at least one (CXC) or three (CX3C) non-conserved amino acids. The biological activities of chemokines are mediated by two groups of seven transmembrane G-protein coupled receptors, CXCR and CCR (Murphy, 1996).

Chemokines are produced from a variety of activated innate immune cells and allow for the recruitment of leukocytes, such as monocytes and neutrophils, as well as lymphocytes, to areas of infection.

1.2.11 CHEMOKINE CONTROL OF DC MIGRATION

DC migration is an integral component of their sentinel function. Immature DC respond to a number of CC and CXC chemokines including MIP-1 α (CCL3), MIP-1 β (CCL4), MIP-3 α (CCL20), MCP-1 (CCL2) and RANTES (CCL5). However, each immature DC subset appears to respond to different chemokines. For example, Langerhans cells, which express CCR6 and migrate selectively to MIP-3 α and monocyte-derived DC, which express CCR1 and CCR5 migrates to MIP-1 α and MIP-1 β (Yoshie, 2000).

Mature DC in the lymphoid tissue lose their responsiveness to most of these inflammatory chemokines by down regulating appropriate chemokine receptors on their cell surface. These DC subsequently acquire responsiveness to MIP-3 α and SLC/CCL21 through up-regulation of CCR7 on their surface (Caux *et al.*, 2000; Chensue, 2001; Gunn *et al.*, 1998). This change in responsiveness ensures that mature DC home to T cell rich areas in the lymphoid tissue, where MIP-3 α and SLC are exclusively expressed (Willimann *et al.*, 1998). The DC migration pattern orchestrated by chemokines is vital for the induction of an adaptive immune response. Furthermore, chemokines are responsible for the rapid infiltration of DC into inflamed tissue (McWilliam *et al.*, 1996). These recruited DC in turn, participate in production of chemokines necessary for the recruitment and activation of other inflammatory cells (Rescigno *et al.*, 2000).

The nature of the chemokine response can also alter the types of leukocytes that are recruited into the sites of inflammation and ultimately result in the resolution of the inflammatory response (Cook *et al.*, 2000; Zheng *et al.*, 2000). Neutralisation of MIP-1 and CXCR2 (a neutrophil specific chemokine and chemokine receptor) was shown to alleviate pulmonary inflammation by *Propionibacterium acnes* in mice (Standiford and Huffnagle, 1997; Standiford *et al.*, 1997). Neutralisation of SLC exacerbated the pulmonary inflammation due to increased numbers of mature DC, macrophages and neutrophils but decreased CD4⁺ T cells in *P. acnes* infected lungs. The authors concluded that decreased CD4⁺ T cell numbers were due to the fact that DC were retained in the lungs of *P. acnes* infected mice, thus preventing the antigen-specific T cell responses in local lymph nodes (Itakura *et al.*, 2001). This study illustrates the importance of chemokines in bridging the innate and adaptive immune responses.

1.2.12 THE INFLUENCE OF CHEMOKINES ON T CELL RECRUITMENT AND DEVELOPMENT

T cells that are activated in lymph nodes after CCL19/CCL21 mediated encounter with pathogen primed DC are recruited to sites of inflammation by sensing chemokine gradients at these sites. In a Th1-polarised environment, activated T cells up-regulate CXCR3 and CCR5, and are attracted into sites of Th1 inflammation (Sallusto *et al.*, 1998; Sallusto *et al.*, 1998; Sallusto *et al.*, 1998). Tr1 cells have also been shown to express CCR5 (McGuirk *et al.*, 2002), which suggests that these cells are recruited to sites of inflammation, possibly to regulate Th1 responses. In a Th2-polarising environment, T cells down regulate CCR7, up-regulate CCR3, CCR4 and CCR8, and are attracted into sites of Th2 inflammation (Sallusto *et al.*, 1998; Sallusto *et al.*, 1998; Sallusto *et al.*, 1998). The interaction between chemokines and their receptor is therefore an important step in the control of T cell migration into sites of inflammation.

The influence of chemokines on the development of T cell responses was illustrated in a gene transfer study, where the over expression of the Th1-affiliate CXC chemokine IP-10 (CXCL10) in the airways of mice undergoing a dominant Th2-polarised mucosal sensitisation regimen was shown to reduce all parameter of allergic airway inflammation (Wiley *et al.*, 2001). These findings illustrate that localised expression of IP-10 in the lungs promoted a Th1 type response and consequently prevented a Th2 response (Wiley *et al.*, 2001). In addition, MCP-1 (CCL2)-deficient mice had defective Th1 responses (Gu *et al.*, 2000). The manner by which chemokines subvert the development of acquired Th1 or Th2 responses is unknown.

1.3 ADAPTIVE IMMUNITY

The adaptive arm of the immune system provides for long lasting “memory” and tailored immune responses toward specific antigens on invading pathogens. This arm of the immune response requires much longer for full activation, days or weeks, compared with the innate immune response. The adaptive immune response is activated through recognition of pathogen-derived peptides expressed on MHC molecules on mature APC, especially DC. Furthermore, the adaptive immune response can provide for memory of an invading pathogen, thus allowing for rapid adaptive response upon subsequent exposures to the same pathogen.

The cells of the adaptive immune response rely on clonal selection and gene rearrangement, to create an army of immune cells possessing individual receptors. Each receptor responds to a singular antigen, thus allowing for protection against pathogens yet to be encountered.

1.3.1 T HELPER CELLS

These are $CD4^+$ cells, which recognise pathogen-derived peptides displayed on the MHCII molecules on mature APC. T cells use their highly variable T cell receptor (TCR) (section 1.3) to bind the peptide carrying MHCII molecule on the innate cell. Following ligation of TCR by its corresponding peptide-MHCII complex, the naïve $CD4^+$ T cell divides and differentiates.

1.3.2 TH1 AND TH2 CELLS

The discovery of polarised subsets of CD4⁺ T cells that differ in their cytokine secretion pattern has provided a basis for understanding the diversity of T cell dependent immune responses (Mosmann *et al.*, 1986). The differentiation of naïve CD4⁺ T cells into subsets of Th cells is a pivotal process in adaptive immune responses (Abbas *et al.*, 1996). Th1 cells produce IFN- γ and promote cellular immunity, which is critical in the eradication of intracellular pathogens, such as *Mycobacterium tuberculosis* and *B. pertussis* (Mills *et al.*, 1998; North and Jung, 2004). IFN- γ activates M Φ , enhancing their ability to phagocytose and destroy microbes (Duffield, 2003). Th1 cells also promote synthesis of IgG2a antibodies (Abbas *et al.*, 1996; Mahon *et al.*, 1995). In contrast, Th2 cells produce IL-4, IL-5 and IL-13 and promote humoral immunity, allergic reactions to environmental antigens, and resistance to helminth infection (Finkelman *et al.*, 1991; Jarnicki and Fallon, 2003). During Th2 responses, IL-4 and IL-13 induce B cell switching to IgE production, whereas IL-5 is the principle eosinophil activating cytokine (Abbas *et al.*, 1996). In addition to Th1 and Th2 cell types, Th0 populations have also been described that secrete combinations of Th1 and Th2 cytokines (Elson *et al.*, 1995; Firestein *et al.*, 1989). However, it has also been suggested that the Th0 population may represent a transitory precursor subset, which will eventually undergo terminal differentiation into Th1 or Th2 cells (Nakamura *et al.*, 1997).

Th cell differentiation is a central process in defining the adaptive immune response and is regulated at many levels. As well as the critical role of DC-derived cytokine and chemokine production, cytokines produced from Th cells themselves contribute to the polarisation to certain Th subtypes.

1.3.2.1 IFN- γ

IFN- γ is the signature cytokine of Th1 cells, and is important for stabilisation of the Th1 phenotype itself but is also a key cytokine in regulating Th1 differentiation. The interferon family of cytokines act in particular to suppress viral replication and curb other intracellular pathogens. Furthermore, the interferons activate M Φ and up-regulate the expression of MHC molecules on innate immune cells. While differentiated Th1 cells cannot be switched to produce Th2 cytokines, Th1 cells from IFN- γ -deficient mice retain the ability to produce IL-4 if re-stimulated under Th2-polarising conditions (Zhang *et al.*, 2001).

Recently, the mechanistic basis for the actions of IFN- γ has been uncovered. IFN- γ induces the transcription factor, T-box protein expressed in T cells (T-bet) (Afkarian *et al.*, 2002; Lighvani *et al.*, 2001), which promotes the production of IL-12R β 2 and IFN- γ in CD4⁺ T cells (Lighvani *et al.*, 2001). The importance of T-bet in regulating Th1 differentiation was confirmed using T-bet deficient mice, which have a severe defect in Th1 polarised responses (Szabo *et al.*, 2002). In addition, T-bet deficient mice develop a spontaneous reactive airway disease similar to human asthma (Finotto *et al.*, 2002).

1.3.2.1 IL-4

IL-4 is thought to be one of the most important factors to regulate Th2 differentiation. IL-4 is expressed as a 15-19 kDa protein and exists as a dimer (Brown *et al.*, 1988) and is produced by CD4⁺ T cells, mast cells and basophils. IL-4 promotes the differentiation of Th2 cells while suppressing the development of Th1 cells. IL-4

enhances MHCII expression on B cells, and promotes Ig class switching to IgG1 and IgE (Beckmann *et al.*, 1992; Yokota *et al.*, 1988).

IL-4 and the closely related cytokine, IL-13, signal through a shared surface receptor, IL-4 α , which activates the transcription factor STAT-6 (Jung *et al.*, 2002; Takeda and Akira, 2000). STAT-6 deficient mice exhibit a similar phenotype to IL-4 deficient mice (Barnes, 2002).

1.3.3 Th_{IL-17} CELLS

IL-17-producing CD4⁺ T cells (Th_{IL-17}) are a recently described Th cell subset (Park *et al.*, 2005; Yao *et al.*, 1995). These cells are independent of Th1 and Th2 cells (Harrington *et al.*, 2005) and indeed the signature Th1 and Th2 associated cytokines, IFN- γ , IL-4 and IL-13, antagonise Th_{IL-17} development (Kleinschek *et al.*, 2007; Park *et al.*, 2005). Furthermore, Th_{IL-17} cells do not require either the Th1 or Th2 associated transcription factors, T-bet or GATA3, for their differentiation. Instead the transcription factor RAR orphan nuclear receptor (ROR γ t) has been shown to be responsible for Th_{IL-17} development (Ivanov *et al.*, 2006). However, IL-17 production is not limited to CD4⁺ T helper cells, since activated CD8⁺ T cells, as well as NK T cells and $\gamma\delta$ T cells have each been shown to produce IL-17 (Ferretti *et al.*, 2003; Happel *et al.*, 2003; Michel *et al.*, 2007; Shibata *et al.*, 2007; Sutton *et al.*, 2006).

Th_{IL-17} cells and IL-17 production have been associated with the resolution of various bacterial infections, such as infection with *M. bovis*, *Candida albicans*, *Toxoplasma gondii* and *K. pneumoniae* (Happel *et al.*, 2003; Huang *et al.*, 2004; Umemura *et al.*, 2007). Furthermore, IL-17 producing T cells have been linked with the development of autoimmune diseases, such as RA, lupus, and asthma (Aggarwal

and Gurney, 2002; Kolls and Linden, 2004; Moseley *et al.*, 2003). Indeed, IL-17-deficient and ROR γ T-deficient mice are less susceptible to the development of autoimmune diseases, such as RA (Ivanov *et al.*, 2006; Koenders *et al.*, 2005; Koenders *et al.*, 2005).

The IL-12 family member, IL-23, induces IL-17 producing T cells (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005). Furthermore, IL-23 has been associated with autoimmune disease development, and this has been attributed to a decrease in IL-17 production (Cua *et al.*, 2003). However, recently other cytokines have been linked with Th_{IL-17} cell development. These include the Treg cell associated cytokine TGF- β , in combination with either IL-6 or the IL-2 homologue, IL-21 (Bettelli *et al.*, 2006; Korn *et al.*, 2007; Veldhoen *et al.*, 2006), as well as IL-15, which is associated with maintenance of memory T cell subsets (Ferretti *et al.*, 2003). However, it should be noted that IL-2 itself negatively regulates IL-17⁺ T cell differentiation (Kryczek *et al.*, 2007) this may be due to the ability of IL-2, but not IL-21, to induce IFN- γ production.

1.3.3.1 IL-17

There are six members of the IL-17 family, A-F, each of which is in the form of a homodimer. IL-17A and F are inflammatory cytokines, and have been found to be involved in neutrophil recruitment as well as the induction of TNF- α , IL-1, IL-6 from human M ϕ (Ye P and Kolls JK 2001, Jovanovic DV and Pelletier JP 1998), as well as GM-CSF from myofibroblasts (Andoh *et al.*, 2005) and IL-8 (CXCL8) from human bronchial epithelial cells (Laan *et al.*, 1999). IL-8 is a chemokine responsible for the recruitment of neutrophils and therefore represents an important mediator of

IL-17A and IL-17F induced neutrophil activation. However, not all IL-17 family members possess pro-inflammatory abilities. Indeed, IL-17E (IL-25) has been shown to prevent the development of the autoimmune disease EAE, which has been attributed to the negative regulation of Th_{IL-17} cells and production of IL-17A and IL-17F (Kleinschek *et al.*, 2007).

IL-17A and IL-17F signal through the IL-17 receptor (IL-17R), which is a type I transmembrane protein extensively expressed in the lungs, kidneys, liver and spleen as well as in isolated fibroblasts, epithelial cells, mesothelial cells and various myeloid cells (Yao *et al.*, 1995).

1.3.4 REGULATORY T CELLS

In addition to effector T cells that function to eradicate pathogens and control tumours, other populations of T cells can down-regulate immune responses and facilitate the resolution of inflammation. The existence of suppressor T cells and their role in the maintenance of immune homeostasis was first proposed in the early 1970s (Gershon and Kondo, 1971). However, the field eventually floundered because these T cells could not be phenotyped and attempts to clone the putative suppressor cells or factors failed.

Following the rediscovery of suppressor T cells as regulatory T cells in the mid 1990s, it is now fully accepted that distinct subtypes of CD4⁺ T cells, discriminated on the basis of phenotype or cytokine secretion, play a major role in maintenance of self tolerance, largely by their ability to suppress responses mediated by other populations of T cells. Several subsets of Treg cells with distinct phenotypes and distinct mechanisms of action have been described. These include, naturally

occurring CD4⁺CD25⁺Foxp3⁺ Treg cells, type 1 T regulatory (Tr) cells, which secrete high levels of IL-10 but not IL-4 or IFN- γ , and Th3 cells, which secrete TGF- β .

CD4⁺CD25⁺ natural Treg cells mature in the thymus and represent 5-10% of the peripheral CD4⁺ T cell population and have been shown to exhibit potent immunosuppressive activity both in vitro and in vivo (Shevach, 2000). Difficulties with CD25 expression, which is found on all activated T cells, as a marker led to attempts to find alternative phenotypic markers for natural Treg cells. Thus, CD4⁺CD25⁺ Treg cells were further characterised by the constitutive expression of several other activation markers including glucocorticoid-induced TNF-receptor (GITR) (Shimizu *et al.*, 2002), OX40 (CD134) (McHugh *et al.*, 2002), L-selectin (CD62L) (Lepault and Gagnerault, 2000) and CTLA-4 (CD152) (Read *et al.*, 2000). However, the role these markers play in the suppressive abilities of CD4⁺CD25⁺ Treg cells remains poorly defined. Recently the fork head/winged helix transcription factor, Foxp3, has been proposed to be exclusive expressed by natural Treg cells (Ramsdell, 2003). This transcription factor is thought to be critical in the development and function of CD4⁺CD25⁺ Treg cells, as both mice and humans deficient in functional Foxp3 protein suffer from severe autoimmune disease symptoms (Kriegel *et al.*, 2004; Ramsdell, 2003).

Although the mechanism of suppression by natural Treg cells is not fully understood, it appears to be cell contact mediated, involving CTLA-4, but a role for IL-10 and TGF- β has not been excluded (Read *et al.*, 2000; Shevach, 2000). The suppressive capacity of human CD4⁺CD25⁺ Treg cells was blocked by a combination of mAb against CTLA-4 and membrane bound TGF- β in vitro (Nakamura *et al.*, 2001). However, CD4⁺CD25⁺ Treg cells isolated from TGF- β deficient mice do not have compromised suppressive activity. Furthermore, wild-type CD4⁺CD25⁺ Treg

cells suppress CD25⁻ T cells expressing a dominant-negative TGF- β receptor (Piccirillo *et al.*, 2002).

In contrast to naturally occurring CD4⁺CD25⁺ Treg cells, Tr1 and Tr3 cells are thought to acquire their suppressor activity through antigenic activation of CD4⁺CD25⁻ T cells in the periphery. Inducible Tr cells exhibit variable expression of CD25 and other surface markers described for naturally occurring Tr cells (McGuirk and Mills, 2002). The suppressor activity of these Tr cells is cell-cell contact independent and is mediated primarily through the actions of the anti-inflammatory cytokines, IL-10 and TGF- β .

Tr1 cells were first characterised in murine models of inflammatory bowel disease as efficient suppressors of Ag-specific immune responses. T cells from OVA-TCR-transgenic mice, repeatedly stimulated with OVA and IL-10, differentiated into Tr1 cells in vitro (Groux *et al.*, 1997). These Tr1 cells produced IL-10, low to moderate levels of IL-5 and TGF- β , but with little or no IL-2, IL-4 or IFN- γ . Naïve CD4⁺ T cells can be manipulated in vitro to generate homogenous populations of IL-10 producing Tr1 cells (Barrat *et al.*, 2002). The ability of Tr1 cells to suppress proliferative responses has been demonstrated by co-culture of naïve CD4⁺ T cells and Tr1 cells in the presence of allogeneic APC (Groux *et al.*, 1997). Similarly, Tr1 clones specific for filamentous haemagglutinin (FHA) from *B. pertussis* suppress proliferation and cytokine production by a Th1 clone against an unrelated antigen, influenza virus haemagglutinin (HA) (McGuirk *et al.*, 2002).

Th3 cells were originally identified in mice after the generation of oral tolerance to myelin basic protein (MBP). These Th3 cells suppressed MBP-induced experimental autoimmune encephalomyelitis (EAE) in vivo in a TGF- β -dependent manner, as suppression was abrogated by injection of anti-TGF- β Ab (Chen *et al.*,

1994; Fukaura *et al.*, 1996). Th3 cells provide help for IgA production and have suppressive properties against both Th1 and Th2 cells (Weiner, 2001). Furthermore, as TGF- β is broadly expressed and acts on multiple cell types, TGF- β -secreting Th3 cells probably have a major role in many aspects of immune regulation and homeostasis (Gorelik *et al.*, 2002).

Since regulatory Tr1 and Th3 cells arise from naïve or resting CD4⁺ T cells in the periphery, it is highly conceivable that DC activated with an appropriate stimulus can promote their differentiation. Indeed, certain pathogen-derived molecules, including FHA and adenylate cyclase toxin (Cya A) from *B. pertussis*, non-structural protein 4 from hepatitis C virus and cholera toxin (CT) from *Vibrio cholera* have been shown to promote the induction of Tr1 cells, through their interaction with DC or other innate cells, such as M Φ (Brady *et al.*, 2003; Lavelle *et al.*, 2003; McGuirk and Mills, 2002; Ross *et al.*, 2004).

1.3.5 CYTOTOXIC T CELLS

Along with the Th cells the CD8⁺ T cells also play a role in immunity to infectious agents. Unlike the Th cells however, CD8⁺ cytotoxic T cells (Tc) mediate protective immunity through cell-specific cytotoxicity, inducing apoptosis of infected cells by various methods such as Fas-FasL interaction and granzyme B activation of the caspase cascade within the infected cells. Like the Th cells CD8⁺ T cells can be divided into Tc1 and Tc2 type subsets, which produce corresponding cytokines to their CD4⁺ counterparts.

1.3.6 INNATE LIKE LYMPHOCYTES

Innate like lymphocytes (ILL) act as a bridge between innate and adaptive responses. These lymphocytes display only limited diversity in receptors, which are encoded by a few common gene segment rearrangements. Unlike conventional $\alpha\beta$ T cells, such as $CD4^+$ and $CD8^+$ T cells, ILL do not undergo clonal expansion in order to respond to their recognised antigens. The two main cells of this unconventional T cell subset are $\gamma\delta$ T cells and NK T cells.

1.3.7 $\gamma\delta$ T CELLS

The $\gamma\delta$ TCR is composed of a γ and a δ chain as opposed to the α and β chains of the conventional T cells. There are two subsets of $\gamma\delta$ T cells, one of which is located in the lymphoid tissue and possess an array of diversified T cell receptors and a second intraepithelial subset, found in abundance in the skin and at mucosal sites, which possess only limited $\gamma\delta$ TCR diversity.

Unlike $\alpha\beta$ T cells $\gamma\delta$ T cells do not require antigen presentation on MHC molecules. Instead $\gamma\delta$ T cells recognise antigen directly, leading to rapid cytokine production. $\gamma\delta$ T cells do not respond to pathogen-derived peptides alone, $\gamma\delta$ T cells possessing the $V\gamma9/V\delta2$ gene rearrangement are activated by compounds derived from the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, of isoprenoid biosynthesis, which is utilised by many bacteria. One of the primary functions of $\gamma\delta$ T cells is protection against intracellular pathogens, many of which possess the ability to establish chronic and debilitating disease such as tuberculosis and malaria (Goerlich *et al.*, 1991; Kabelitz *et al.*, 1990; van der Heyde *et al.*, 1995). Control of these

microorganisms has been attributed to IFN- γ and TNF- α production by the $\gamma\delta$ T cell. However, it has recently been reported that $\gamma\delta$ T cells provide an early release of IL-17 in response to *E. coli* infection, which is required for resolution of infection via neutrophil recruitment (Shibata *et al.*, 2007).

$\gamma\delta$ T cells have also been linked with development of autoimmune disease (Odyniec *et al.*, 2004; Rajan *et al.*, 1998). However, there is conflicting evidence on the role of these cells in the development of autoimmune diseases. One study showed that mice depleted of $\gamma\delta$ T cells display aggravated symptoms of EAE (Kobayashi *et al.*, 1997), while another reported that $\gamma\delta$ T cells had no effect on the outcome of disease (Clark and Lingenheld, 1998). Although these conflicting results may be due to the stage of disease at which the $\gamma\delta$ T cells are removed, i.e. prior to disease onset as opposed to during the chronic phase of disease, or due to the use of different mAb, one of which targeted only one subset of $\gamma\delta$ T cells.

1.3.8 B CELLS

B cells recognise pathogens via the B cell receptor (BCR). The BCR is an antigen receptor or immunoglobulin molecule, generated by gene rearrangement similar to the TCR. The BCR consists of two heavy (H) chains and two light (L) chains. The H and L chains are composed of a series of domains, each consisting of approximately 110 amino acids. The H chains of the Ig molecules are of several classes (μ , δ , γ , α , ϵ). An assembled Ig molecule, consisting of one or more units of two identical H and L chains and derives its name from the H chain it possesses, therefore generating five immunoglobulins IgM, IgG, IgD, IgA and IgE.

IgM is the most versatile of the antibody classes. In the periphery IgM can be expressed by immature, mature and memory B cells. The presence of IgM on the surface of these cells provides a receptor for B cell activation along with Ig α and Ig β accessory molecules (Ishihara *et al.*, 1992; Stahl and Sibrowski, 2003). While membranes bound IgM is most common, IgM plasma cells secrete polymeric IgM. Polymeric IgM is an important complement activator and thus participates in phagocytosis. IgM is second only to IgA in its contribution to mucosal immunity. In many patients with IgA deficiency, IgM adequately substitutes for IgA for mucosal protection.

The IgG isotype is the most abundant immunoglobulin in the blood. IgG play a part in a wide variety of functions in immunity including the activation of complement and the opsonization of antigen (Sandlie and Michaelsen, 1991). IgG antibodies can be subdivided further into subclasses in mice (IgG1, IgG2a, IgG2b and IgG3). The interaction of CD40 ligand, expressed on the surface of activated T cells with CD40 on the surface of the B cell can stimulate B cell division (Rush *et al.*, 2002). Furthermore, T cell derived cytokines such as IFN- γ and IL-4 can enhance various IgG isotypes from B cells. Th1 derived IFN- γ induces the IgG2a isotype while the Th2 produced IL-4 induces the IgG1 isotype (Snapper *et al.*, 1988; Snapper *et al.*, 1988).

1.4 VACCINATION

The aim of vaccination is to induce an immune response against a pathogen that prevents infection without causing disease. Initial vaccine strategies induced protective immunity did so by deliberately causing mild infection with unmodified

pathogen, which provided long lasting protection against re-infection. However, these vaccines were accompanied by side effects, such as fever and seizures, and in the case of the original smallpox vaccine, full-blown disease in 3% of cases. Side effects also associated with killed bacterial vaccines were due to the plethora of PAMPs present in the whole bacteria preparation, which stimulated innate immune cells and led to inflammatory cytokine release. This led to the development of subunit vaccines, which were composed of antigen portions of the whole bacteria or viruses. These subunit vaccines are devoid of PAMPs and so did not induce inflammation. However, this dearth of immunostimulatory molecules meant that the second-generation vaccines offered far less protection than their whole cell counterpart. However, it is essential that strong adaptive immune responses to protein antigens be elicited in order to develop sufficient vaccination. Therefore, innate immune cell immunostimulators, known as adjuvants, are co-administered with subunit antigen.

1.4.1 ADJUVANTS

Adjuvants are immunostimulants required to polarise and modifying the immune response generated against purified antigen, through activation of innate immune cells. Furthermore, adjuvants allow for a reduction in the antigen load required for the development of protective immunity. Adjuvants are capable of enhancing, accelerating and prolonging the adaptive immune response towards vaccine antigens and are derived from various natural, synthetic or endogenous sources. Some adjuvants are combined within emulsions or other delivery systems, which promote the slow antigen release and protection from rapid elimination.

Traditionally, vaccines have employed a single type of adjuvant, such as aluminium salts or emulsions, although many other molecules have been examined for their potential adjuvant activity. Currently only aluminium hydroxide (alum) and MF59 have been licensed for clinical use in human vaccines. In addition, alum supplemented with killed *B. pertussis* has also been licensed for use as an adjuvant. There is the added bonus for the inclusion of killed microbes as adjuvants as it can generate protection against the bacterium as well as allowing for immunostimulation. For example, the bacterium *B. pertussis*, which is the causal agent of whooping cough, is a source of antigens and adjuvant in the DPT (diphtheria, pertussis, tetanus) vaccine. A number of new adjuvants are undergoing development including emulsions, NOD agonists as well as various TLR agonists, such as the TLR9 agonist CpG, the synthetic TLR4 agonist RC529 and the synthetic TLR7/8 agonist 3M-019. TLR agonists enhance signals 1, 2 and 3 on APC, thus allowing for optimal APC activation and antigen presentation. TLR stimulation has also been shown to be required for the generation of memory CD4⁺ T cells, but not their activation (Pasare and Medzhitov, 2004). Other PRR agonists, such as the NOD2 agonist muramyl dipeptide (MDP), a substructure of bacterial peptidoglycan, has already been used as an adjuvant in veterinary vaccines, such as leishmaniasis, inducing Th1 responses and protection (Lemesre *et al.*, 2005). Furthermore, studies have shown that various PRR agonists can synergise in their immunomodulatory activity, for example, combinations of synthetic TLR2 and TLR4 agonists.

Saponins have also been shown to possess adjuvant ability, and have been tested in both veterinary and human studies. Indeed the saponin QS21 has been shown to drive both a Th1 and antibody response, however the widespread use of saponins has been hampered due to their reactogenicity. Saponins have been incorporated into

ISCOMs, cage like structures containing antigen, cholesterol, phospholipid and saponin, and ISCOM-based vaccines have been shown to promote both antibody and cell mediated responses.

Various other bacterial toxins have also been examined for their adjuvanticity, including cholera toxin (CT) from *V. cholera* and the heat labile enterotoxin (LT) from *E. coli*.

1.4.2 *E. COLI* HEAT LABILE ENTEROTOXIN

LT is the enterotoxigenic agent of *E. coli* and is the major causative agent of traveller's diarrhoea (Spangler, 1992). LT is a member of the AB₅ enterotoxins along with CT, with which it has 82% homology. Both LT and CT are comprised of a single A subunit associated with a hexameric, doughnut shaped, ring of identical B subunits (Rappuoli *et al.*, 1999; Sanchez and Holmgren, 2005; Williams *et al.*, 1999). The B subunits of LT and CT are covalently linked forming a highly stable ring of 55 kDa, which is primarily involved in attachment of the enterotoxins to GM1-ganglioside receptors, on the surface of eukaryotic cells. However, LT has also been shown to bind with other receptors, such as galactoproteins, lactosylceramide and asialo-GM1.

The A subunit is cleaved firstly from the B ring. The A subunit then undergoes further proteolytic reduction to yield an A₁ subunit and a smaller A₂ subunit (Merritt *et al.*, 1994). The cleavage of the A₁ and A₂ subunits renders enzyme activity on the larger A₁ subunit, which possess nicotinamide adenine dinucleotide (NAD)-glycohydrolase and adenosine diphosphate (ADP)-ribosyltransferase activity. The NAD-glycohydrolase mediates the enzymatic cleavage of NAD into free nicotinamide and ADP-ribose. The ADP-ribose is subsequently coupled to the

regulatory guanosine triphosphate (GTP)ase $G_{s\alpha}$, of a membrane bound G protein coupled receptor of the adenylate cyclase system (Cassel and Pfeuffer, 1978; Gill and Richardson, 1980). This coupling maintains the $G_{s\alpha}$ in a permanently activated GTP state, thus stimulating adenylate cyclase to produce increased concentrations of intracellular cyclic adenosine monophosphate (cAMP). This rise in intracellular cAMP concentrations causes protein kinase A (PKA) to phosphorylate and open Cl^- channels, which is coupled with osmotic movement of water into the gut lumen.

LT has been shown to possess potent adjuvant capacity following mucosal immunisation with co-administered antigen (Clements *et al.*, 1988; Hashigucci *et al.*, 1996; Tamura *et al.*, 1994). LT promoted a mixed Th1 and Th2 response and both IgG2a and IgG1 antibody responses to co-administered antigen (Takahashi *et al.*, 1996). Mutant versions of LT, which have single point mutations in the A_1 subunit, such as LTR72 and LTK63 have been shown to retain adjuvant activity. However, LTK63 is completely non-toxic whereas LTR72 retains a small percentage of enzyme activity albeit at a reduced level to that displayed by wild type LT (Douce *et al.*, 1997; Giuliani *et al.*, 1998). The adjuvant effect of LTK63 is reduced when compared with LT and LTR72.

The adjuvant activity of the LT, LTR72 and LTK63 has been attributed to activation of DC. LT and CT have both been shown to up-regulate the expression of the co-stimulatory B7 family members, CD80 and CD86 on DC (Bagley *et al.*, 2002). Furthermore, it was observed that both LT and CT were capable of modulating LPS-induced DC and M Φ cytokine production (Bagley *et al.*, 2002; Cong *et al.*, 2001; Lavelle *et al.*, 2003). It was found that CT and LT increased LPS-induced IL-6, IL-1 β and IL-10 while inhibiting LPS-induced IL-12 and TNF- α production from DC.

1.5 MOUSE MODELS OF HUMAN DISEASES

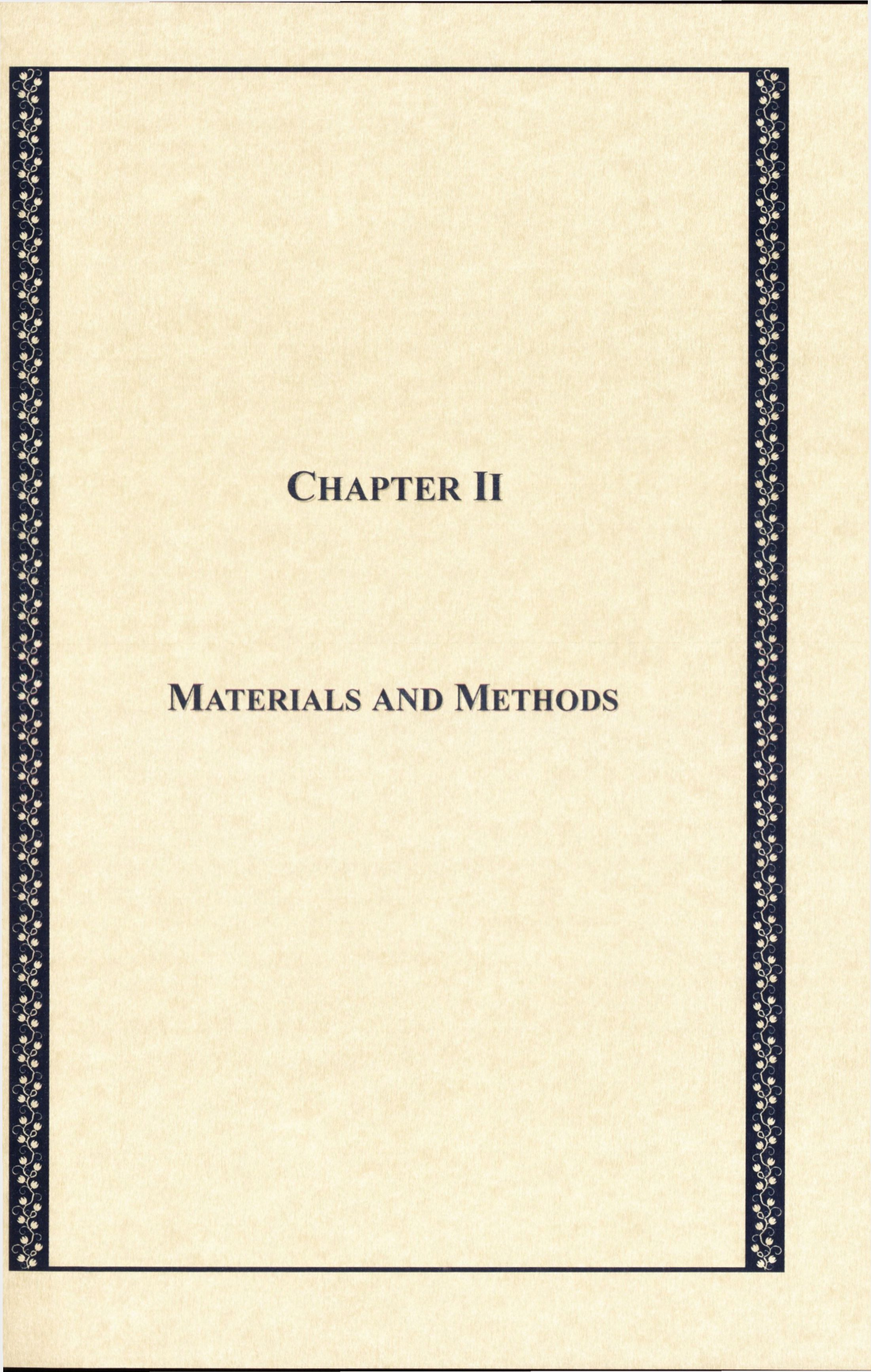
A thorough understanding of the role of the cells and molecules of the immune system in the development of autoimmune and chronic inflammatory diseases is dependant on the use of animal models, as is the development of therapies to treat these diseases. Although not perfect, mouse models have been developed to mimic many of the features of the diseases seen in humans. These models are heavily relied upon by the pharmaceutical industry, for the development and pre-clinical testing of new drugs and have directly led to the generation of safer and more effective therapies for human autoimmune disease. These include inhibitors of TNF- α , Infliximab and Etanercept, as well as the IL-1 antagonist, Anakinra, which are used for the treatment of chronic inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel disease.

However, there are significant limitations in the use of inbred mice for the dissection of human diseases. While the use of inbred mice allows for a greater degree of reproducibility, they can also mask certain differences that occur in out bred populations, which possess randomised gene pools, and therefore do not take into account the natural variation in the human population. Indeed the human population possess at least 3 million single nucleotide polymorphisms (SNPs), in addition to thousands of larger polymorphic alleles. Indeed, inbred mouse strains differ greatly in their susceptibility to certain diseases, such as the observed resistance of BALB/c mice, and susceptibility of C57BL/6 mice strains, to the development of EAE (Hurwitz *et al.*, 2002), thus highlighting the genetic contribution to the development of autoimmune diseases. Furthermore, mouse models do not display the gender-bias observed for human autoimmune diseases, where there is a greater number of females

affected with autoimmune rheumatic diseases compared with males. Therefore, non-human primates, with their greater immunological and biological similarities to humans are in great demand as models for pre-clinical evaluation of highly specific biological therapies. However, for ethical and practical difficulties, experiments with non-human experiments are seriously limited, underlining the need for effective rodent models.

1.6 HYPOTHESIS

The original hypothesis of this project was that the adjuvant activity of LT was linked with its enzymatic activity and was furthermore, dependent upon activation of dendritic cells. However, during the course of the project, Th17 cells were discovered and I found that LT promoted the induction of IL-17 producing cells. In addition I discovered that IL-1 was required for this IL-17 production. Therefore, I addressed the hypothesis that IL-1 was required for promoting the induction of Th17 cells.



CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 AMMONIUM CHLORIDE LYSIS SOLUTION, (0.87%)

4.35 g Ammonium Chloride (NH₄Cl)

Dissolved in 500 mls ddH₂O.

2.1.2 BACTERIAL DERIVED MOLECULES

E. coli LPS (Alexis) and phosphorothioate-stabilized oligodeoxynucleotide-containing CpG motifs (CpG-ODN) (5'GCTAGACGTTAGCGT) were custom synthesized by Sigma-Genosys. Pam3Cys (palmitoyl-3-cysteine-serine-lysine-4) was purchased from InvivoGen (Autogen Bioclear, UK). LT and LTR72 were purchased from Chiron, while LTK63 and LTB were provided by Neil A Williams, University of Bristol, UK.

2.1.3 CELL CULTURE MEDIUM

Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera) was supplemented with 8% heat inactivated (56°C for 60 min) foetal calf serum (FCS), 100 mM L-Glutamine (Gibco), 100 µg/ml streptomycin (Biosera). Complete RPMI was used to culture all dendritic cells as well as cells isolated from murine spleens and lymph nodes.

2.1.4 ELISA STOPPING SOLUTION (1M H₂SO₄)

53.2 mls 18 M sulphuric acid (H₂SO₄)

946.8 mls dH₂O

2.1.5 ELISA WASH BUFFER (10 L)

0.05% Tween 20

500 ml PBS (20X)

Made up in dH₂O

2.1.6 ELUTION BUFFER

1.49 g Na₂HPO₄

4.39 g NaCl

4.25 g imidazole

Dissolved in 250 ml dH₂O, pH 8

2.1.7 FACS BUFFER

2% heat inactivated FCS

0.1% sodium azide

Made up in PBS

2.1.8 FACS BLOCKING BUFFER

50%	FACS buffer
50%	heat inactivated FCS

2.1.9 LURIA BERTANI BROTH (LB)

10 g	tryptone
5 g	yeast extract
10 g	NaCl

Dissolved in 1 L dH₂O, pH 7, autoclaved to sterilise

2.1.10 LB AGAR

15 g	agar
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Dissolved in 1 L LB, autoclaved, plated onto 10 cm Petri dishes.

2.1.11 MINIPREP SOLUTION 1

50 mM	Tris-HCl, pH 8
10 mM	EDTA

2.1.12 MINIPREP SOLUTION 2

0.2 M NaOH

1% SDS

2.1.13 MINIPREP SOLUTION 3

3 M KAc, pH 5.5

2.1.14 NICKLE COLUMN WASH BUFFER

1.49 g NaH₂PO₄

4.39 g NaCl

0.34 g imidazole

Dissolved in 250 ml dH₂O, pH 8

2.1.15 PHOSPHATE-BUFFERED SALINE (PBS) 20X

320 g sodium chloride (NaCl, 1.4 M)

46 g di-sodium hydrogen phosphate (Na₂HPO₄, 0.08 M)

8 g potassium dihydrogen phosphate (KH₂PO₄, 0.01 M)

8 g potassium chloride (KCl, 0.03 M)

Dissolved in 2 L dH₂O, pH 7

2.1.16 PHOSPHATE CITRATE BUFFER

10.2 g citric acid anhydrous ($C_6H_8O_7$)

36.9 g di-sodium hydrogen orthophosphate dodecahydrate
($Na_2HPO_4 \cdot 12H_2O$)

Dissolved in 1 L dH_2O , pH 5, stored at $4^\circ C$

2.1.17 RECOMBINANT PROTEINS FOR IMMUNE CELL STIMULATION

Recombinant mouse IL-23, IL-1 α , IL-1 β , TNF- α , TGF- β were obtained from R&D systems. Recombinant mouse IL-18 was purchased from MBL, recombinant mouse IL-6 was purchased from BD Pharmingen, while IL-33 was a gift from Prof. E. Liew, University of Scotland, Glasgow, Scotland. Recombinant IL-1Ra in the form of Kineret (Anakinra) was purchased from Amgen. The endotoxin content of the recombinant cytokines used was < 1 EU/ μg protein in all cases.

2.1.18 TRIS, BORIC ACID, EDTA (TBE) 10X

107.8 g tris base

50 g boric acid

7.44 g EDTA

Dissolved in 1 L dH_2O , pH 8.3

2.1.19 WASH BUFFER 1

10 mM	Tris HCl, pH 7.5
50 mM	NaCl
25 mM	EDTA
50%	EtOH

Dissolved in 1 L dH₂O

2.2 METHODS

2.2.1 ANIMALS

Specific pathogen-free BALB/c (H-2^d), C3H/HeN (H-2^k), C3H/HeJ (H-2^k) and C57BL/6 (H-2^b) mice were purchased from Harland UK Ltd., Bicester, Olac, UK. $\gamma\delta$ -defective (H-2^b) mice were purchased from The Jackson Laboratory, USA. All mice strains were maintained according to the regulations and guidelines of the Irish Department of Health. IL-10^{-/-} and IL-1R1^{-/-} mice were bred, in house, under pathogen free conditions at Trinity College, Dublin. Experiments were performed under license from the Department of Health and with the approval of the University Ethics Committee. All mice used were female and were 6-12 weeks old at the initiation of the experiments.

2.2.2 CULTURING OF CELL LINES

The GM-CSF expressing cell line, J558, was provided by Dr. Nathalie Winters of the Institute Pasteur, Paris, France. This line had been established by cloning the mouse gene for GM-CSF into a mammalian expression vector, which was finally transfected into the plasmacytoma line V53-Ag (Karasuyama and Melchers, 1988). The first two passages of the J558 cell line were grown in selection medium, which was comprised of complete RPMI (cRPMI; section 2.1.3) supplemented with the antibiotic geneticin (G418 sulphate; Gibco BRL). After the second passage in selection medium, the cells were grown to a 'medium' density, harvested and washed 3 times to ensure all traces of geneticin were removed. The cells were then returned to

culture, in cRPMI alone, for 3-4 days. The supernatant from the cell line was harvested by pelleting the cells by centrifugation at 1200 rpm for 5 min. The cells were re-suspended in cRPMI and cultured for a further 8 passages before being frozen (section 2.2.3). Supernatants collected at each passage were pooled and stored at -20°C until GM-CSF was quantified by enzyme linked immunosorbant assay (ELISA) (section 2.2.15).

2.2.3 CRYOPRESERVATION OF CELLS IN LIQUID NITROGEN

Cells were harvested in the same manner as for sub-culturing and their cell viability assessed. Cells were then centrifuged (1200 rpm for 5 min) and the pellet re-suspended in ice-cold cryopreservation medium, 10% (v/v) dimethylsulphoxide (DMSO) in heat inactivated FCS, to give a final cell concentration of $2-4 \times 10^6$ cells/ml. The cells were then transferred in 1-1.5 ml volumes to cryotubes. Aliquots were then frozen in a 'Mr. Freeze' container at -20°C overnight, to allow for the gradual decrease in temperature ($1^{\circ}\text{C}/\text{min}$), before being transferred to liquid N_2 . To maximise the recovery of cells from liquid N_2 , the cells were thawed quickly in a 37°C water-bath and immediately transferred to ice-cold medium. Cells were washed twice in ice-cold cRPMI to ensure all traces of DMSO were removed from the cell suspension. The final pellet was re-suspended in cRPMI and cell viability assessed.

2.2.4 IMMUNISATIONS

BALB/c, C57BL/6 and IL-1RI^{-/-} mice were immunised by subcutaneous (s.c.) injection into the flank using a total volume of 200 μl (100 μl each side) and mice

boosted 14 days later where stated. Mice were injected with PBS, KLH (10 µg per mouse) or KLH in combination with LT, LTR72, LTK63 (all 1 µg per mouse), LPS, HRF, cHRF or HMGB1 (section 2.2.19) (all 10 µg per mouse) as detailed in figure legends. Each study contained 5 mice per group. Mice were sacrificed by cervical dislocation either a week or 21 days post immunisation, as stated in figure legends, and antigen-specific immune responses assessed.

2.2.5 CELL COUNTS

Cell counts were performed by diluting cells (normally 1:10 or 1:20) in 50% trypan blue (Sigma)/50% PBS (Biosera). A 10 µl volume of the cell suspension was then loaded onto a disposable haemocytometer (Hycor Biomedical, UK). Viable cells, which do not stain and appear white under a light microscope, were then counted.

2.2.6 DETECTION OF MURINE T CELL RESPONSES EX VIVO

Isolated spleen and draining lymph nodes were passed through 70 µm sterile cell strainers (Nunc) to obtain single cell suspensions. Cells were centrifuged at 1200 rpm for 5 min. Spleen cells were re-suspended in 1 ml of 0.87% ammonium chloride solution (section 2.1.1) for 2 min, to lyse contaminating red blood cells, they were then washed and centrifuged at 1200 rpm for a further 5 min. Spleen or lymph node cells were then re-suspended in fresh cRPMI medium. Spleen mononuclear cells from immunised mice (2×10^6 cells/ml), lymph node cells from immunised mice (1×10^6 cells/ml) and spleen cells from naïve mice (5×10^5 cells/ml) were cultured in triplicate wells of 96-well 'U-bottomed' microtitre plates at 37°C and 5% CO₂.

Spleen and lymph node single cell suspensions from immunised mice were cultured with various concentrations of antigen, or with PMA (250 ng/ml: Sigma) and anti-mouse CD3 (1 µg/ml: BD Pharmingen), or with medium alone, as positive and negative controls respectively. Supernatants were removed after 72 hours and the concentration of IL-4, IL-5, IL-10, IL-17 and IFN-γ determined by ELISA (section 2.2.8).

2.2.7 PROLIFERATION ASSAY

Cells obtained from immunised mice were cultured for four days with antigen, as described in section 2.2.6, and pulsed with 0.5 µCi of [³H]-thymidine (Amersham Pharmacia Biotech) in 25 µl of cRPMI. After 6 hours of culture with [³H]-thymidine, cells were harvested onto glass fibre filters (Wallac) with an automatic cell harvester. The filters were dried thoroughly and sealed in plastic sample bags with 5 ml of non-aqueous scintillation fluid (BetaScint, Wallac). The incorporation of [³H]-thymidine in the cells, which is an indirect measure of cell proliferation, was assessed using a Beta-plate scintillation counter (Wallac). Results were then expressed as mean counts per minute (cpm) of [³H]-thymidine incorporation for triplicate cultures of spleen cells or lymph node cells.

2.2.8 T CELL CYTOKINE CONCENTRATIONS BY ELISA

The concentration of IL-4, IL-5, IFN-γ (BD Pharmingen, Table 2.1), IL-17 and IL-10 (R&D, Table 2.1) in test supernatants was measured by ELISA using paired antibodies. High binding certified ELISA plates (Greiner Bio-one) were coated with

50 µl/well of purified rat anti-mouse IL-5, IL-4, IFN- γ (1 µg/ml), IL-17 and IL-10 (2 µg/ml). Plates were then incubated overnight at 4°C after which they were washed 5 times with ELISA wash buffer (section 2.1.5) and incubated with 200 µl/well of blocking solution (IL-4, IL-5, IFN- γ ; 5% w/v dried milk (Marvel); IL-10, IL-17; 1%BSA/5% sucrose w/v; both diluted in PBS) for 2 hours at room temperature (RT) to prevent non-specific binding. Plates were then washed and incubated with 50 µl of test supernatant or a serially diluted recombinant cytokine standard. Test supernatants for IL-4, IL-5 and IFN- γ were diluted 1:2 in PBS. Following overnight incubation, at 4°C, plates were washed and incubated for 1 hour (IL-4, IL-5, IFN- γ) or 2 hours (IL-10, IL-17) at RT, in the dark, with 50 µl/well of biotinylated anti-mouse IL-4, IL-5, IFN- γ (1 µg/ml) or goat anti-mouse IL-10 or IL-17 (400 ng/ml and 200 ng/ml respectively). Horseradish peroxidase (HRP)-conjugated streptavidin (IL-4, IL-5, IFN- γ ; BD Pharmingen, 1:2000 in PBS; IL-10 and IL-17; R&D, 1:200 in 1% BSA) was then added for 20 min at RT, 50 µl/well. Concentrations of cytokine in the test supernatants were determined by washing the plates in wash buffer and incubating them with 50 µl/well o-phenyldiamine (OPD) substrate (0.4 mg/ml; Sigma) dissolved in phosphate citrate buffer with H₂O₂ (7 µl/25 ml phosphate citrate buffer).

The enzyme reaction was quenched by addition of 25 µl/well of stop solution (section 2.1.4) and the absorbance of each well was read at 492 nm using a microtitre plate reader (Molecular Devices, VERSAmax tunable microplate reader). Concentrations of cytokines in the test supernatants were determined with reference to a standard curve prepared using recombinant cytokines of known concentration. The range of each assay was 0-10 ng/ml for IFN- γ , 0-2500 pg/ml for IL-4 and IL-5, 0-1000 pg/ml for IL-17 and 0-2000 pg/ml for IL-10.

2.2.9 ANALYSIS OF KLH-SPECIFIC SERUM IgG SUBCLASS TITRES

Peripheral blood was removed from the thoracic cavity of immunised mice post sacrifice and left overnight at 4°C to clot. Samples were centrifuged at 13,000 rpm for 10 min and sera were removed to fresh tubes and stored at -20°C until ready to be analysed. For analysis, KLH (5 µg/ml in PBS), 50 µl/well, was added to medium binding certified ELISA plates (Greiner Bio-one). Plates were then incubated overnight at 4°C. Excess antigen was removed by washing with wash buffer (section 2.1.5) and non-specific binding sites blocked by incubating plates with 200 µl PBS supplemented with 10% dried milk (Marvel) for 2 hours at RT. Plates were washed again, in wash buffer, and serum samples added. These were serially diluted on the plate in PBS starting at a 1:50 dilution and finishing at a dilution of 1:51,200. Following overnight incubation at 4°C, plates were washed with wash buffer and incubated for 1 hour at RT with 50 µl/well of biotin-conjugated IgG (0.05 µg/ml), IgG1 (0.05 µg/ml) or IgG2a (0.05 µg/ml) from Caltag. Plates were then washed and incubated for 30 min at RT with 50 µl/well of HRP-conjugated streptavidin (R&D; 1:200 in 1% BSA). The plates were washed of excess streptavidin and 50 µl/well of OPD (0.4 mg/ml) dissolved in phosphate citrate buffer with H₂O₂ (7 µl/25 ml phosphate citrate buffer) was added. The enzyme reaction was stopped by adding 25 µl/well stop solution (section 2.1.4) and the absorbance read at 492 nm. Results are expressed as endpoint antibody titres determined by extrapolation of the straight part of the dilution curve to the 492 nm value of the control sera for the naïve mice.

2.2.10 GENERATION OF MURINE BONE MARROW-DERIVED DENDRITIC CELLS

Bone marrow-derived dendritic cells (BMDC) were generated from BALB/c (H-2^d), C3H/HeN (H-2^k), C3H/HeJ (H-2^k), C57BL/6 (H-2^b), IL-10^{-/-} and IL-1RI^{-/-} mice using a method similar to that described by Lutz *et al.* (Lutz *et al.*, 1999). Naïve mice were sacrificed by cervical dislocation and their femurs and tibiae removed and dissected from the surrounding muscle and tissue. The bone marrow was then flushed from both of the bones using a high gauge (>25 G) needle attached to a 20 ml syringe containing cRPMI. A single cell suspension of flushed bone marrow was obtained using a low gauge (<19 G needle) attached to an empty 20 ml syringe. The single cell suspension was centrifuged at 1200 rpm for 5 min and the cells re-suspended in 1 ml of heated 0.87% ammonium chloride (section 2.1.1) for 2 min, to lyse contaminating red blood cells. The cells were washed in fresh cRPMI and pelleted by centrifugation at 1200 rpm for 5 min. The washed mononuclear cells were then re-suspended in 5 ml cRPMI, cell viability and numbers were then determined using the trypan blue counting method (section 2.2.5). The immature BMDC were cultured at 1×10^6 cells/ml, with no more than a total of 50×10^6 cells, in a T175 tissue culture flask (Greiner). BMDC were cultured with 10% FCS and 40 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) in the form of supernatant obtained from the J558 cell line (section 2.2.2). After culture for 3 days, at 37°C and 5% CO₂, 20 mls of fresh medium, containing 40 ng/ml J558 supernatant derived GM-CSF, was added to each flask. On day 6 the flasks were gently removed from the incubator and the supernatant was carefully removed, so as not to dislodge the loosely adherent immature BMDC cells, thus removing contaminating cells such as granulocytes from

the culture. Heated sterile PBS (Biosera), 15 mls, was then added to each flask and the flask gently rolled dislodging the loosely adherent cells. PBS was then collected into sterile 50 ml tubes containing 15 mls fresh medium. A further 15 mls of sterile heated EDTA (0.02%; Sigma) was then added to each flask and flasks incubated for 10 min at 37°C. At this point cells removed into the PBS were pelleted by centrifugation at 1200 rpm for 5 min and re-suspended in 5 ml cRPMI. Fresh medium (15 mls) was added to the flasks, which contained EDTA, and cells were collected after vigorous pipetting. These cells were then centrifuged at 1200 rpm for 5 min and re-suspended in 5 ml fresh medium. Both of the suspensions from the PBS and EDTA steps were pooled and cell viability and numbers assessed using the trypan blue method (section 2.2.5). Cells were re-cultured at 1×10^6 cells/ml, with no more than a total of 50×10^6 cells in a T175 tissue culture flask, in cRPMI supplemented with GM-CSF as before. After a further 4 days at 37°C, day 10, the loosely adherent cells were harvested by gently removing the supernatant and gentle pipetting, viability of BMDC was assessed and the cells were plated out at required concentration in tissue culture plates. The immature DC were allowed to 'rest' overnight at 37°C in medium supplemented with 10-20 ng/ml GM-CSF from the J558 supernatant.

2.2.11 ANALYSIS OF BMDC MATURATION AND CYTOKINE/CHEMOKINE SECRETION

BMDC (5×10^5 cells/ml) were cultured in sterile 96-well round bottomed plates (Nunc) at 37°C for the appropriate time (as indicated in figure legends), supernatants were then removed and frozen at -20°C until analysis was ready to be performed.

For the studies designed to examine the immunomodulatory capacities of LT and its mutants, LTR72 and LTK63 DC were cultured at 37°C with the toxins (1 µg/ml). This incubation was performed for 1, 6 or 12 hours prior to the addition of a second stimulus, such as LPS (50-5000 pg/ml) or CpG (0.8-20 µg/ml). Supernatants were removed 24 hours later. The concentration of IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-23, TNF-α and MIP-1α (CCL3) in test supernatant was quantified by ELISA, using paired antibodies (Table 2.1).

For the studies designed to examine the immunostimulatory properties of endogenous factors, various concentrations of pancreatic elastase (Sigma), high mobility group box 1 (HMGB1) provided by Kevin Tracey, Long Island LiJ, New York, USA, histamine releasing factor (HRF) and cleaved HRF (cHRF), provided by Seamus Martin, Trinity College Dublin, Ireland, were incubated with DC at 37°C for 24 hours before harvesting of the supernatant. Supernatants were analysed for cytokine production. For the studies that aimed at examining the immunomodulatory effect of LT and LTK63 on endogenously-induced cytokines, DC were incubated for 6 hours with LT or LTK63 (1 µg/ml) prior to the addition of CpG (0.8-20 µg/ml), elastase (0.2-5 µg/ml), HMGB1 (1-25 µg/ml), HRF (0.2-5 µg/ml) or cHRF (0.2-5 µg/ml). Supernatants were harvested 24 hours after the addition of the endogenous factors and the concentration of IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, TNF-α and MIP-1α were analysed by specific ELISA using pairs of antibodies (Table 2.1).

For the studies designed to examine the effect of recombinant cytokine on cytokine production from DC, DC were incubated with IL-17 (0.1-10 ng/ml), IL-1β, IL-6 (1-100 ng/ml) or TNF-α (0.5-50 ng/ml) (section 2.1.16). After 24 hours supernatants were harvested and analysed for IL-1β, IL-12p70, IL-23 and IL-6 production by ELISA.

2.2.12 IL-12p40 ELISA

The concentrations of IL-12p40 in the test supernatants were quantified by ELISA, using commercially available paired antibodies (BD Pharmingen, Table 2.1) specific for the IL-12p40 subunit. These antibodies recognise IL-12p40 either as a monomer, dimer, part of the IL-12p70 or IL-23 complexes, with IL-12p19. High binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of purified anti-IL-12p40/p70 at a concentration of 2 µg/ml diluted in PBS. After washing with wash buffer (section 2.1.5), non-specific binding sites were blocked with 200 µl/well 5% dried milk (Marval) w/v in PBS, for 2 hours at RT. Plates were washed again in wash buffer and 50 µl of test supernatant (diluted 1:300 in PBS) and serially diluted recombinant IL-12p40 standard (0-5000 pg/ml) was added to the plates. Following overnight incubation at 4°C, the plates were washed in wash buffer and incubated for 1 hour at RT with 50 µl/well of biotinylated anti-IL-12p40/p70 (2 µg/ml in PBS). Excess detection antibody was then removed by washing followed by the addition of HRP-conjugated streptavidin (PharMingen, 1:2000, diluted in PBS), 50 µl/well and plates were then incubated for 20 min at RT. Plates were washed for a final time in wash buffer and the concentration of IL-12p40 determined by developing plates with OPD substrate (0.4 mg/ml) in phosphate citrate buffer and H₂O₂ (7 µl/25 ml phosphate citrate buffer), 50 µl/well. Once the standard curve had developed sufficiently, the enzyme reaction was then quenched by addition of 25 µl/well stop solution (section 2.1.4). The absorbance at 492 nm was then read using a microtiter plate reader. The cytokine concentrations in the test supernatants were determined with reference to the standard curve for the recombinant murine IL-12p40 of known concentration.

2.2.13 IL-1 β , IL-12p70 and TNF- α ELISA

The concentrations of IL-1 β , IL-12p70 and TNF- α were quantified using commercially available ELISA duo-set kits (R&D systems, Table 2.1). High binding certified 96-well microtitre plates (Greiner Bio-one) were coated overnight at 4°C with 50 μ l/well of rat anti-mouse IL-1 β (4 μ g/ml), IL-12p70 (4 μ g/ml) and TNF- α (0.8 μ g/ml) capture antibody in PBS. Plates were then washed in wash buffer (section 2.1.5) and non-specific binding sites blocked with the addition of 200 μ l of blocking buffer (1% BSA/5% sucrose in PBS), for 2 hours at RT. Plates were washed again in wash buffer and 50 μ l of test supernatant and serially diluted recombinant IL-1 β standard (0-1000 pg/ml), IL-12p70 standard (0-1500 pg/ml) and TNF- α standard (0-2000 pg/ml), all diluted in 1% BSA, added to wells. Supernatants tested for IL-12p70 production were added neat onto plates while those used to detect TNF- α and IL-1 β were diluted 1:2 on their respective plates, in 1% BSA. Following overnight incubation at 4°C, plates were washed in wash buffer and incubated for 2 hours at RT with 50 μ l/well of biotinylated goat anti-mouse IL-1 β (100 ng/ml), IL-12p70 (400 ng/ml) and TNF- α (150 ng/ml) respectively, diluted in 1% BSA. Excess detection antibody was then removed by washing followed by the addition of HRP-conjugated streptavidin (R&D, 1:200 in 1% BSA), 50 μ l/well, and plates were then incubated for 20 min at RT. Plates were washed for a final time in wash buffer and the concentration of mouse IL-1 β , IL-12p70 and TNF- α determined by developing plates with OPD substrate, 50 μ l/well (0.4 mg/ml) in phosphate citrate buffer with H₂O₂ (7 μ l/25 ml phosphate citrate buffer). Once the standard curve had developed sufficiently the enzyme reaction was quenched by the addition of 25 μ l/well stop solution (section

2.1.4). The absorbance at 492 nm was then read using a microtiter plate reader once standard curves for each cytokine had developed sufficiently. The cytokine concentrations in the test samples were determined with reference to the standard curve for the recombinant murine IL-1 β , IL-12p70 and TNF- α of known concentration.

2.2.14 IL-6 ELISA

The concentration of IL-6 in test supernatant was quantified by ELISA, using commercially available paired antibodies (BD Pharmingen, Table 2.1). High binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 μ l/well of purified rat anti-mouse IL-6 (1 μ g/ml) capture antibody in PBS. After washing with wash buffer (section 2.1.5), non-specific binding sites were blocked with 200 μ l/well 3% BSA in PBS, for 2 hours at RT. Plates were washed again in wash buffer and 50 μ l test supernatant (1:2 dilution in PBS) and serially diluted recombinant IL-6 standard (0-5000 pg/ml) was added to plates. Following overnight incubation at 4°C, plates were washed again in wash buffer and plates incubated for 1 hour at RT with 50 μ l/well of biotinylated anti-IL-6 detection antibody (1 μ g/ml in PBS). Excess detection antibody was then removed by washing in wash buffer, followed by the addition of HRP-conjugated streptavidin (BD Pharmingen, 1:2000 in PBS), 50 μ l/well, and plates were then incubated for 20 min at RT. Plates were washed for a final time in wash buffer and the concentration of IL-6 determined by developing plates with 50 μ l/well OPD-substrate (0.4 mg/ml) in phosphate citrate buffer with H₂O₂ (7 μ l/25 ml phosphate citrate buffer). Once the standard curve had developed sufficiently the enzyme reaction was quenched by addition of 25 μ l/well

stop solution (section 2.1.4). The absorbance at 492 nm was then read using a microtitre plate reader. The concentration of cytokine in test samples was then determined with reference to the standard curve for the recombinant murine IL-6 of known concentration.

2.2.15 GM-CSF ELISA

Supernatant obtained from culturing the J558 cell line (section 2.2.2) was measured for GM-CSF using a commercially available ELISA duo-set kit (R&D, Table 2.1). High-binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of rat anti-mouse GM-CSF capture antibody (2 µg/ml) in PBS. After washing with wash buffer (section 2.1.5), non-specific binding sites were blocked by adding 200 µl/well 1% BSA/5% sucrose, in PBS, for 2 hours at RT. Plates were washed again in wash buffer and 50 µl of test supernatant was added either neat or in 5 ten-fold dilutions, between 1:10 and 1:100,000 in 1% BSA, and serially diluted recombinant GM-CSF standard (0-2000 pg/ml) in 1% BSA. Following incubation overnight at 4°C, plates were washed in wash buffer and incubated for 2 hours at RT with 50 µl/well of biotinylated goat anti-mouse GM-CSF (50 ng/ml), diluted in 1% BSA. Excess detection antibody was then removed by washing followed by the addition of HRP-conjugated streptavidin (R&D, 1:200 in 1%BSA), 50 µl/well, and plates were then incubated for 20 min at RT. Plates were washed for a final time in wash buffer and the concentration of GM-CSF determined by developing plates with 50 µl/well OPD substrate (0.4 mg/ml) in phosphate citrate buffer with H₂O₂ (7 µl/25 ml phosphate citrate buffer). Once the standard curve had developed

sufficiently the enzyme reaction was quenched by addition of 25 μ l/well stop solution (section 2.1.4). The absorbance at 492 nm was then read using a microtitre plate reader. The concentration of GM-CSF in test supernatants was determined with reference to the standard curve for the recombinant murine GM-CSF of known concentration.

2.2.16 MIP-1 α ELISA

The concentration of the chemokine MIP-1 α (CCL3) in test supernatants was quantified by ELISA, using commercially available paired antibodies (R&D, Table 2.1). High binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 μ l/well of rat anti-mouse MIP-1 α (0.4 μ g/ml) in PBS. After washing in wash buffer (section 2.1.5), non-specific binding sites were blocked with 200 μ l/well 1% BSA/5% sucrose in PBS, for 2 hours at RT. Plates were washed again in wash buffer and 50 μ l of test supernatant, diluted 1:2 in 1% BSA, and serially diluted recombinant MIP-1 α standard (0-500 pg/ml) was added to each well. Following overnight incubation, at 4°C, plates were washed in wash buffer and incubated for 2 hours at RT with 50 μ l/well of biotinylated goat anti-mouse MIP-1 α (75 ng/ml in 1% BSA). Excess detection antibody was then removed by washing followed by the addition of HRP-conjugated streptavidin (R&D: 1:200 in 1% BSA), 50 μ l/well, and plates were then incubated for 20 min at RT. Plates were washed for a final time in wash buffer and the concentration of MIP-1 α determined by developing plates with 50 μ l/well of OPD substrate (0.4 mg/ml) in phosphate citrate buffer with H₂O₂ (7 μ l/25 ml phosphate citrate buffer). Once the standard curve had developed sufficiently

the enzyme reaction was quenched by addition of 25 μ l/well stop solution (section 2.1.4). The absorbance at 492 nm was then read using a microtitre plate reader. The concentration of chemokine in the test supernatants was determined with reference to the standard curve for the recombinant murine MIP-1 α of known concentration.

2.2.17 IL-23 ELISA

The concentration of IL-23 in test supernatants was quantified by ELISA, using matched pair monoclonal antibodies (eBioscience, Table 2.1). High binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C, with 50 μ l/well, purified rat anti-mouse IL-23 (1:250) capture antibody in PBS. After washing with wash buffer (section 2.1.5), non-specific binding sites were blocked with 200 μ l/well blocking buffer (1X reagent diluent), for 2 hours at RT. Plates were washed again in wash buffer and 50 μ l/well of test supernatant (diluted 1:2 in 1X reagent diluent) and serially diluted recombinant IL-23 standard (0-4000 pg/ml) diluted in 1X reagent diluent, were added. Following overnight incubation, at 4°C, plates were washed in wash buffer and incubated for 1 hour at RT with 50 μ l/well of biotinylated anti-IL-23 detection antibody (1:500 in 1X reagent diluent). Excess detection antibody was then removed by washing followed by the addition of HRP-conjugated streptavidin (eBioscience; 1:2000 dilution in 1X reagent diluent), 50 μ l/well, and plates incubated for 20 min at RT. Plates were washed for a final time in wash buffer and the concentration of IL-23 determined by developing plates with 50 μ l/well 3,3',5,5'-tetramethylbenzidine (TMB) substrate (eBioscience). Once the standard curve had developed sufficiently the enzyme reaction was quenched by addition of 25 μ l/well stop solution (section 2.1.4). The absorbance at 450 nm was read using a

microtitre plate reader. The cytokine concentration in the test supernatants was then determined with reference to the standard for the recombinant murine IL-23 of known concentration.

2.2.18 FLOW CYTOMETRY ANALYSIS

DC were stimulated with various modulators as described in section 2.2.11 and after 24 hours were transferred from wells, into 1 ml FACS buffer (section 2.1.6) at a concentration of 1×10^6 cells/ml. The cells were then centrifuged at 1200 rpm for 5 min. Cells (3×10^6) were then re-suspended in 1 ml FACS block buffer (section 2.1.7) and placed on ice for 20 min, followed by washed twice in FACS buffer (10 mls/ 3×10^6 cells), by centrifugation at 1200 rpm for 5 min. Cells were next transferred to FACS tubes (Falcon) in a volume of 100-200 μ l of FACS buffer and incubated with the appropriate conjugated antibody or isotype control antibodies for 20 min at RT, in the dark. Excess antibodies were removed from cells by washing twice in FACS buffer (3 mls/tube) with centrifugation at 1200 rpm for 5 min between each wash. Surface marker expression on DC was analysed by flow cytometry using a FACS CaliberTM flow cytometer (Becton-Dickinson, San Jose, CA, USA.). The FACS machine was calibrated using the Autocomp software in conjunction with commercially prepared fluorescent beads (Calibrate beads, Becton-Dickinson). Immunofluorescence analysis was then performed and the results analysed using CELLQuestTM software. 50,000 events were analysed per sample, unless otherwise stated.

2.2.19 PREPARATION OF RECOMBINANT MURINE HMGB1

2.2.19.1 PCR AMPLIFICATION OF MURINE HMGB1 DNA

A fragment of the murine HMGB1 gene, corresponding to nucleotides 1-648 was amplified by polymerase chain reaction (PCR). PCR is a standard molecular cloning method for amplifying fragments of DNA using two oligonucleotide primers in combination with DNA polymerase. Taq DNA polymerase from *Thermus aquaticus* was used due to retention of DNA polymerase activity at high temperatures up to 80°C, although typically it is used at 72°C. The murine HMGB1 gene was amplified using the conditions listed below using primers purchased from Biotech.

Primers: Forward 5'CGTGGTACCGGCAAAGGAGATCCTAAG3'

KpnI

Reverse 5'GTACTCGAGTTATTCATCATCATCTTC3'

XhoI

PCR program: 5 min @ 94°C

1 min @ 94°C

1 min @ 54°C

45 sec @ 72°C

10 min @ 72°C

2.2.19.2 VISUALISATION OF DNA BY GEL ELECTROPHORESIS

DNA fragments were visualised by electrophoresis on an agarose gel. The PCR amplified HMGB1 DNA was separated on a 1.2 % w/v agarose (Sigma) gel in tris, boric acid, EDTA solution (TBE; section 2.1.18), for 45 min at 100 V. Samples were run along side an appropriate molecular weight marker of known size to aid identification of the DNA fragments. The gel was prepared with 30 µg/ml ethidium bromide, which binds to the DNA fragments in the gel and allows them to be visualised under a ultra violet (UV) lamp. Following identification by reference to the molecular weight marker, the murine HMGB1 fragment (~650bp) was excised from the gel.

2.2.19.3 PURIFICATION OF DNA FROM AGAROSE GEL

The fragment was released from the agarose by solublising in NaI (300 µl/100 µg gel) and incubated at 50⁰C for 10 min. The liquid agarose gel mixture was then incubated with silica solution in 3 M NaCl for 15 min at RT. The silica solution binds to DNA contained within the agarose gel fragment. The silica was then pelleted by centrifugation at 22,000 rpm for 1 min and the pellet washed twice by re-suspending in 500 µl wash buffer 1 (section 2.1.19) and mixed for 15 sec, followed by centrifugation at 22,000 rpm for 1 min. The DNA was eluted from the silica pellet by the addition of 20 µl of DNAase/RNAase free water, which was mixed for 20 sec before centrifugation at 22,000 rpm for 1 min. Murine HMGB1 DNA was contained within the supernatant.

2.2.19.4 LIGATION OF DNA INTO CLONING AND EXPRESSION PLASMIDS

Purified murine HMGB1 DNA fragments were ligated into the pET-45b plasmid using the Kpn1 and Xho1 restriction sites. The murine HMGB1 DNA fragments was added to the plasmid at a ratio of 3:1 for optimal ligation and successful transformation recovery. This ratio was determined by comparing the intensity of the linearised vector and insert on a 1.2% agarose gel, and factoring this by the molecular size of the DNA fragments. The DNA was incubated at 45°C for 5 min to melt cohesive ends then placed immediately on ice. T4 DNA ligase buffer (1 µl) then added, containing 1 mM ATP, followed by T4 DNA ligase (0.1 Weiss units; Gibco BRL, NY, USA). The reaction was then incubated at 16°C, overnight. This protocol generated the murine HMGB1.pET-45b plasmid, which was transformed into competent DH5α *E. coli*.

2.2.19.5 GROWTH OF *E. COLI*

DH5α *E. coli* were used as the host strain for the murine HMGB1.pET-45b plasmid and for recombinant protein expression. The *E. coli* were grown from frozen glycerol stocks (section 2.2.19.6) by streaking a small amount of frozen bacteria with an inoculating loop on a Luria Bertani broth (LB) agar plate (section 2.1.10) containing the antibiotic ampicillin (AMP) (150 µg/ml LB). This was incubated overnight at 37°C and single colonies then recovered. Single colonies were grown separately in starter cultures with 3 ml LB (section 2.1.9) containing AMP. Following overnight incubation of the starter culture at 37°C (260-280 rpm), 100 µl of starter

culture was added to 50 ml LB containing AMP. This was again incubated overnight at 37°C (260-280 rpm) to generate a midi culture.

2.2.19.6 GLYCEROL STOCKS OF *E. COLI*

Glycerol stocks of *E. coli* were prepared and stored at -80°C. The *E. coli* was grown overnight in a 4 ml culture, which was pelleted by centrifugation at 13,000 rpm for 2 min. The pellet was re-suspended in an equal volume of fresh LB and pelleted once more by centrifugation at 13,000 rpm for 2 min. Each culture was re-suspended in a total volume of 1 ml LB to which 1 ml of 10% v/v glycerol in dH₂O was added. This *E. coli* glycerol mixture was then aliquoted into cryotubes and stored at -80°C.

E. coli was cultured from glycerol stocks by scraping a small amount of frozen bacteria from the stock tube using an inoculation loop. This inoculation loop containing bacteria was then streaked onto an LB agar plate containing the appropriate antibiotic, and incubated overnight at 37°C.

2.2.19.7 HEAT SHOCK TRANSFORMATION OF *E. COLI*

DH5α *E. coli* were thawed on ice (50 µl/tube) and 1 µg of murine HMGB1.pET.45b plasmid was added to each tube and incubated on ice for 30 min, to allow for the plasmid to coat the bacterial cells. The *E. coli* were heat shocked for 40 sec at 42°C to induce uptake of plasmid, and then allowed to recover on ice for 2 min. LB (450 µl/tube) was then added and bacteria incubated at 37°C rotating at 220 rpm for 1 hour. The transformed *E. coli* were then plated 10 µl or 100 µl, on LB agar plate containing AMP, and incubated overnight at 37°C. Single colonies were recovered

and grown in liquid LB (2.2.19.5). The presence of the murine HMGB1.pET45b plasmid was confirmed by miniprep (2.2.19.8). Once the presence of murine HMGB1.pET45b was confirmed within the transformed bacteria, larger scale cultures were grown, which were subsequently frozen in glycerol (section 2.2.19.6).

2.2.19.8 MINIPREP OF PLASMID DNA

A single colony from a LB agar plate inoculated with transformed *E. coli* was grown in a starter culture of 3 ml LB containing AMP (2.2.19.5). Following overnight incubation at 37°C (260-280 rpm), 1 ml of bacterial culture was removed to a fresh tube and centrifuged at 13,000 rpm for 2 min, to pellet bacteria. The supernatant was removed and the pellet re-suspended in 100 µl miniprep solution 1 (section 2.1.11). Miniprep solution 2 (100 µl; section 2.1.12) was then added and mixture inverted 10 times, followed by incubation at RT for 2 min. Miniprep solution 3 (100 µl; section 2.1.13) was then added to the tube and this was mixed by inversion 10 times, followed by incubation at RT for 2 min. The tube was then centrifuged at 13,000 rpm for 10 min, at RT. The supernatant was removed to a fresh tube and 1 ml ice-cold 100% ethanol added, followed by centrifugation at 13,000 rpm for 10 min. Supernatant was removed and the pellet incubated with 700 µl 70% ethanol and centrifuged at 13,000 rpm for 5 min. All supernatant was removed and the pellet left to air dry at RT for approx. 30 min. The pellet was re-suspended in 50 µl DNAase free water. Isolated plasmid was run on a 1.2% agarose gel, by electrophoresis, to confirm that the murine HMGB1.pET45b plasmid had transfected into the DH5α *E. coli*.

2.2.19.9 BACTERIAL EXPRESSION OF MURINE HMGB1 RECOMBINANT PROTEIN

A preliminary small-scale induction of recombinant protein was performed to determine optimal expression conditions. Once these conditions had been determined a larger-scale expression of recombinant protein was performed. For the larger-scale expression, a starter culture, generated from glycerol stocks of transformed *E. coli* (section 2.2.19.6) was set up in LB containing AMP. The starter culture was grown overnight in at 37°C rotated at 260-280 rpm. The following day the starter cultures were used to inoculate 50 ml of LB containing AMP, thus producing medium cultures. The medium cultures were incubated overnight at 37°C (260-280 rpm) after which the culture was diluted to an OD₆₀₀ of 0.1-0.2. The culture was then incubated at 37°C until it had and grown to an OD₆₀₀ of 0.4. These cultures were then incubated with 1mM IPTG (C₉H₁₈O₅S), as per the optimisation protocol obtained from the small-scale induction, in order to induce the expression of the recombinant protein. After 3 hr incubation at 37°C rotating at 260-280 rpm, the bacteria were pelleted by centrifugation at 1200 rpm for 5 min followed by the removal of supernatant and storage of pellets at -20°C.

2.2.19.10 PURIFICATION OF RECOMBINANT MURINE HMGB1 PROTEIN

Frozen pellets (section 2.2.19.9) were thawed on ice for 15 min, and then incubated with BugBuster protein extraction reagent (Novagen) 25 ml/500 ml original culture volume, by rotation at RT for 20 min. The bacteria cell debris were then centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was collected and

loaded onto a nickel column (25 ml/column), which had previously been reconstituted with nickel column wash buffer (section 2.1.14). When the supernatant had drained from the column, the nickel column was washed with nickel column wash buffer (8 ml) and the flow through collected into a fresh collection tube. Bound recombinant protein was then eluted from the column with 5 ml elution buffer (section 2.1.6) collected in 500 µl fractions in fresh tubes. The concentration of protein in each aliquot was determined using the Bradford method and samples were then tested to ensure only a single protein was returned.

2.2.20 INDUCTION AND ASSESSMENT OF EAE

C57BL/6 and IL-1RI^{-/-} mice were immunised s.c. with 150 µg of myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (Cambridge BioSciences) emulsified in complete Freund's adjuvant (CFA) supplemented with 5 mg/ml killed *Mycobacterium tuberculosis* (Chondrex) in 100 µl. Mice were injected i.p. with 500 ng of PT (Sigma) on day 0 and day 2 post immunisation. From day 6, mice were observed for signs of clinical disease daily. Disease severity was recorded as follows: grade 0, normal; grade 1, limp tail; grade 2, wobbly gait; grade 3, hind limb weakness; grade 4, hind limb paralysis; grade 5, tetraparalysis/death.

2.2.21 DETERMINATION OF MOG-SPECIFIC IMMUNE RESPONSES

Spleen cells were isolated from C57BL/6 and IL-1RI^{-/-} mice, as described in section 2.2.6, 21 days post induction of EAE. Cells (2×10^6 cells/ml) were cultured with MOG (4, 20 µg/ml), PMA (1 ng/ml; Sigma) and anti-CD3 (1 µg/ml; BD

Pharmingen) or medium only. Supernatants were collected after 72 hours and cytokine concentrations were determined by two site ELISA using antibody pairs specific for IL-4, IL-5, IFN- γ , IL-17 and IL-10 (section 2.2.8). One day following the removal of supernatants for cytokine estimation, [3 H] thymidine (1 μ Ci/well; Amersham Pharmacia) was added and the cells were cultured for a further 6 hours, after which cells were harvested and proliferation was assessed by [3 H] thymidine incorporation (section 2.2.7).

2.2.22 T CELL PURIFICATION

Mouse T cell enrichment columns, CD4 and CD8 subset column kits (R&D) were used to purify CD3 $^+$, CD4 $^+$ and CD8 $^+$ T cells from spleens of C57BL/6 mice. T cell purity was routinely >95%. T cells and spleen cells from C57BL/6, IL-1RI $^{-/-}$ and $\gamma\delta^{-/-}$ mice were stimulated, either alone or in combination with recombinant cytokines (section 2.1.17), as detailed in figure legends. Where indicated, cells were co-stimulated with anti-CD3 (1 μ g/ml) and anti-CD28 (10 μ g/ml) (BD Pharmingen). For experiments with CD4 $^+$ and CD8 $^+$ T cells, IL-2 (Hoffman-La Roche Inc) was also included.

2.2.23 ISOLATION OF $\gamma\delta^+$ T CELLS AND $\gamma\delta$ T CELL DEPLETED CELLS

Spleen cells or purified CD3 $^+$ T cells from C57BL/6 mice (50×10^6 cells/ml) were incubated with mouse $\gamma\delta$ -FITC antibody (1.5 μ l/ 10^6 ; BD Pharmingen) for 20 min at RT, in the dark. Cells were then washed in cRPMI and centrifuged at 1200 rpm

for 5 min. $\gamma\delta^+$ ($\gamma\delta$ T cells) and $\gamma\delta^-$ ($\gamma\delta$ depleted cells) cells were then sorted using a Dako MoFlo High speed cell sorter by positive and negative selection respectively.

2.2.24 FACS ANALYSIS FOR INTRACELLULAR PROTEINS

Spleen cells from C57BL/6 mice (1×10^6 cells/ml) were stimulated with IL-1 β , IL-23, TGF- β , IL-6 (section 2.1.17) and anti-mouse IFN γ (BD Pharmingen) either alone or in combination, for 3 days. Where stated, cells were re-stimulated for the final 6 hours prior to harvesting with PMA (10 ng/ml; Sigma) and ionomycin (1 μ g/ml; Sigma). Cells were cultured for the final 4 hours in the presence of brefeldin A (10 μ g/ml; Sigma). This was followed by harvesting and blocked of cells with Fc γ blocker (1 μ g/ml; BD Pharmingen) for 15 min at RT. Cells were then placed in FACS tubes (Falcon) and incubated with antibodies specific for mouse CD3, CD4, CD8 α , $\gamma\delta$ TCR, NKG2D or isotype controls (BD Pharmingen), as detailed in figure legends. Cells were incubated for 15 min in the dark at RT before being twice washed with FACS buffer (section 2.1.7) (3 mls/tube) and centrifuged at 1200 rpm for 5 min. Samples were re-suspended in 50 μ l buffer A (Fix & Perm Cell Permeabilization kit; Caltag) and incubated for 15 min at RT, in the dark. The cells were then washed twice in FACS buffer (3 ml/tube) and after the final wash were re-suspended in 50 μ l buffer B containing antibodies specific for mouse IL-17, IFN- γ or isotype control antibody (1:10; Caltag). Cells were incubated in the dark for a further 15 min at RT followed by two washes in FACS buffer (3 mls/tube). Cells were re-suspended in 100-200 μ l of FACS buffer and flow cytometric analysis performed using a Dako Cyan ADP flow cytometer.

2.2.25 REAL TIME PCR

CD3⁺ T cells (3.5×10^6 cells/ml), FACS sorted $\gamma\delta$ T cells (0.5×10^5 cells/ml) and FACS sorted $\gamma\delta$ T cell depleted cells (0.5×10^5 cells/ml), were stimulated with IL-1 (10 ng/ml), IL-23 (10 ng/ml) or IL-1 and IL-23, in the presence of 1 μ g/ml anti-CD3, for 72 hours. RNA was harvested from cells 48 hours later using an RNEasy isolation kit (CD3⁺ T cells; Qiagen) or TRIZOL reagent ($\gamma\delta$ T cells and $\gamma\delta$ T cell depleted cells; Sigma). This was followed by reverse transcription using a QuantiTect Reverse transcription kit (Qiagen). Real-time PCR for the detection of IL-17 mRNA was performed using a sense and antisense primers for IL-17A (Mm00439619_m1; Applied Biosystems) or ROR γ T (Mm012610022_m1; Applied Biosystems) and a FAM-labelled MGB Taqman probe. 18S ribosomal RNA (Applied Biosystems) was used as an endogenous control. Samples were assayed on an Applied Biosystems 7300 PCR machine.

2.2.26 STATISTICAL ANALYSIS

Statistical analyses were performed using the computer-based mathematical package InStat 3. Statistical differences in mean antibody, cytokine and chemokine values between experimental groups were determined by one-way ANOVA using Tukey multiple comparisons test. P values of less than 0.05 are considered significant.

Table 2.1 Origin and specificities of antibodies for ELISA

Mouse IFN-γ	R4-6A2	cIFN- γ	Purified	BD Pharmingen
Mouse IFN-γ	XMG1.2	dIFN- γ	Biotin	“
Mouse IL-4	11B11	cIL-4	Purified	“
Mouse IL-4	BVD6-24G2	dIL-4	Biotin	“
Mouse IL-5	TRFK5	cIL-5	Purified	“
Mouse IL-5	TRFK4	dIL-5	Biotin	“
Mouse IL-6	MP5-20F3	cIL-6	Purified	“
Mouse IL-6	MP5-32C11	dIL-6	Biotin	“
Mouse IL-12p40	C15.6	cIL-12p40	Purified	“
Mouse IL-12p40	C17.8	dIL-12p40	Biotin	“
Mouse IL-12p70	-	cIL-12p70	Purified	R&D
Mouse IL-12p70	-	dIL-12p70	Biotin	“
Mouse IL-10	-	cIL-10	Purified	“
Mouse IL-10	-	dIL-10	Biotin	“
Mouse IL-1β	-	cIL-1 β	Purified	“
Mouse IL-1β	-	dIL-1 β	Biotin	“
Mouse TNF-α	-	cTNF- α	Purified	“
Mouse TNF-α	-	dTNF- α	Biotin	“
Mouse MIP-1α	-	cMIP-1 α	Purified	“
Mouse MIP-1α	-	dMIP-1 α	Biotin	“
Mouse GM-CSF	-	cGM-CSF	Purified	“
Mouse GM-CSF	-	dGM-CSF	Biotin	“

Mouse IL-23	-	cIL-23	Purified	eBioscience
Mouse IL-23	-	dIL-23	Biotin	“
Mouse IgG	-	dIgG	Biotin	Caltag
Mouse IgG1	A85-1	dIgG1	Biotin	“
Mouse IgG2a	R19-15	dIgG2a	Biotin	“

^a c = coating,

d = detection

Table 2.2 **Origin of recombinant cytokines used as ELISA standards**

CYTOKINE	SOURCE
Murine IFN- γ	BD Pharmingen
Murine IL-4	BD Pharmingen
Murine IL-5	BD Pharmingen
Murine IL-6	BD Pharmingen
Murine IL-12	BD Pharmingen
Murine IL-12p70	R&D Systems
Murine IL-1 β	R&D Systems
Murine IL-10	R&D Systems
Murine MIP-1 α	R&D Systems
Murine TNF- α	R&D Systems
Murine IL-17	R&D Systems
Murine IL-23	eBiosciences

Table 2.2 FACS antibodies and relevant isotype controls

SURFACE MARKER	FLUORESCENT LABEL	ISOTYPE	SUPPLIER
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CD3	APC	Hamster IgG1	BD Pharmingen
CD40	FITC	Hamster IgG	BD Pharmingen
CD86	FITC	Rat IgG2a	BD Pharmingen
ICAM-1	FITC	Hamster IgG	BD Pharmingen
γδ TCR	FITC	Hamster IgG2	BD Pharmingen
CD4	FITC	Rat IgG2a	BD Pharmingen
I-A/I-E (MHCII)	PE	Rat IgG2a	BD Pharmingen
CD80	PE	Rat IgG2a	BD Pharmingen
B7-H1	PE	Rat IgG2a	BD Pharmingen
IL-1RI	PE	Rat IgG1	BD Pharmingen
CD11c	Tri	Hamster IgG	Caltag
CD8α	Tri	Rat IgG2a	BD Pharmingen

INTRACELLULAR CYTOKINES	FLUORESCENT LABEL	ISOTYPE	SUPPLIER
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IL-17	PE	Rat IgG1	Caltag
IFN-γ	FITC	Rat IgG1	Caltag

CHAPTER III

MODULATION OF INNATE AND ADAPTIVE

IMMUNE RESPONSES BY *E. COLI* HEAT

LABILE ENTEROTOXIN

3.1 INTRODUCTION

In general, immunisation with purified protein antigens does not induce strong T cell or B cell responses. This imposes a significant constraint on the use of highly purified recombinant proteins as subunit vaccines. However, the immunogenicity of these proteins can be enhanced with the addition of adjuvants, which activate the innate immune system and subsequently promote strong adaptive immune responses. The enterotoxins *E. coli* heat-labile enterotoxin (LT) and cholera toxin (CT) from *Vibrio cholera* both possess potent adjuvant properties when administered parenterally or by mucosal routes (Douce *et al.*, 1999; Rappuoli *et al.*, 1999; Snider, 1995; Williams *et al.*, 1999). Indeed LT is one of the most potent mucosal adjuvants identified to date. Both CT and LT are structurally very similar, with approximately 80% homology between the two structures (Rappuoli *et al.*, 1999). Their cytotoxicity is mediated by the enzymatically active A subunit, which contains a ADP ribosylating enzyme, attached to a pentameric B subunit, responsible for binding of the holotoxin to the cell (Rappuoli *et al.*, 1999; Williams *et al.*, 1999). Both CT and LT B subunits bind primarily to GM1 ganglioside receptors on the cell surface, although the B subunit of LT differs from that of CT in that it can also bind to a broad range of other ganglioside and non-ganglioside receptors (Angstrom *et al.*, 1994), perhaps explaining the difference in T cell and B cell responses induced by CT and LT when administered as adjuvants with by-stander antigen. Following receptor ligation the toxins are taken into eukaryotic cells via receptor mediated endocytosis. Subsequently, the A and B subunits are separated and the A subunit translocated across the cell membrane, where the enzymatic portion of the A subunit activates

Gs α , a GTP-binding protein. This G-protein activation by the A subunit stimulates adenylyl cyclase, leading to the release of intracellular cAMP.

Studies employing LT as adjuvant have shown that it promotes the induction of mixed Th1 and Th2 responses to co-administered antigens, accompanied by serum antibody IgG1 and IgG2a production (Clements *et al.*, 1988; Hashigucci *et al.*, 1996; Tamura *et al.*, 1994). Furthermore, when administered via the nasal or oral routes, LT strongly promotes secretory IgA. In order to generate toxins with adjuvanticity without toxicity, site-directed mutants of the toxin have been generated. These contain single point mutations, which render their enzyme inactive or only partially active (Rappuoli *et al.*, 1999; Williams *et al.*, 1999). Some of these molecules retained adjuvant properties similar to the wild type toxin, but have much reduced toxicity, thus making them much better candidates for use as adjuvants in vaccine formulations (Douce *et al.*, 1999; McCluskie *et al.*, 2001; Rappuoli *et al.*, 1999; Williams *et al.*, 1999).

Two mutants of LT that have been extensively investigated and shown to retain adjuvant activity include LTR72, containing an Ala-Arg mutation at position 72, outside of the active region of the enzyme, which retains some enzyme activity, and LTK63, mutated at an essential amino acid (Ser-Lys) at position 63 within the active portion of the A subunit, rendering it enzymatically inactive. The mutants have reduced (LTR72) or no (LTK63) toxicity, as displayed using an in vivo rabbit ileal loop assay (Rappuoli *et al.*, 1999). In vivo studies with the LT mutants LTR72 and LTK63 as adjuvants have shown that they retain immunomodulatory properties (Douce *et al.*, 1999; Giuliani *et al.*, 1998; Peppoloni *et al.*, 2003; Rappuoli *et al.*, 1999; Williams *et al.*, 1999), indicating that the adjuvant properties associated with wild type LT are not entirely mediated by enzyme activity. However, the T and B cell

responses induced differ in some respects from those induced by the wild type toxin. The mutant LTR72 induces T and B cell responses comparable with the wild type toxin, including the enhancement of Th1 and Th2 responses as well as IgG1 and IgG2a antibody production (Giuliani *et al.*, 1998; Rappuoli *et al.*, 1999; Williams *et al.*, 1999). In contrast, the enzymatically inactive mutant LTK63 promotes a predominantly Th2 response with higher IgG1 than IgG2a antibody production. In general LTK63 is a less potent adjuvant than LTR72 or the wild type toxin (Rappuoli *et al.*, 1999; Williams *et al.*, 1999). However, these effects are dose dependent and LTK63 is comparable to LTR72 when used at higher concentrations. The mechanism whereby LTK63 activates immune responses remains unclear, since the adjuvanticity of LT has traditionally been attributed to cAMP induction. Therefore the aim of this study was to determine the roles of enzyme activity and binding components of LT in the induction of innate and adaptive immunity. LTK63 and LTB provide useful tools to dissect the roles of different compounds of toxin in directing immune responses.

3.2 RESULTS

3.2.1 Wild type LT and LTR72 promote a mixed Th1/Th2 response, whereas LTK63 promotes a polarised Th2 response to a co-administered antigen

In order to assess their adjuvant activity, identical concentrations of wild type LT, LTR72 or LTK63 were injected subcutaneously (s.c.) into the footpad of BALB/c mice with KLH as antigen. Mice were boosted with the same formulation 14 days later. Seven days after the boost, spleen cells, draining lymph nodes (LN) and blood were harvested from immunised mice. Antigen-specific cytokine production and serum antibody responses were measured and compared with that observed in mice immunised with KLH alone. Spleen and LN cells from mice immunised with KLH alone produced small concentrations of IL-10, IL-4, but no IFN- γ or IL-5 (Fig. 3.1). Spleen and lymph node cells from mice immunised with KLH in the presence of either LT or LTR72 produced significantly higher concentrations of IL-4, IL-5, IL-10 and IFN- γ , when compared with that produced by spleen cells from mice immunised with KLH alone (Fig. 3.1, Fig. 3.2). This indicated that mixed Th1 and Th2 responses were induced after immunisation with antigen and either LT or LTR72. Immunisation with the mutant LTR72 induced greater concentrations of IFN- γ than wild type LT. In contrast LTK63 enhanced the induction of KLH-specific IL-4, IL-5 and IL-10, but not IFN- γ , above that produced after immunisation with KLH alone. The data indicate that LTK63 promotes a Th2 response when given as an adjuvant for immunisation with KLH.

3.2.2 LT or LTR72 promotes mixed IgG1/IgG2a antibody production, whereas LTK63 generates a polarised IgG2a antibody response to a co-administered antigen

In addition to the observed adjuvant effect, which LT and the LT mutants exerted on T cell responses, the enterotoxins also induced B cell activation and associated antibody production, which is an important facet of adjuvant activity. The ratio of IgG1 to IgG2a antibodies provides indirect evidence of an adjuvant promoting a predominantly Th1 or Th2-type response *in vivo*. A high production of IgG1 over IgG2a is consistent with a predominantly Th2 response, while a high production of IgG2a over IgG1 indicates a polarised Th1 response. LT, LTR72 and LTK63 all enhanced KLH-specific serum IgG production (Fig. 3.3) as well as significant titres of both IgG1 and IgG2a when compared with that produced by mice immunised with KLH alone. However, the ratio of IgG1:IgG2a antibody production after immunisation with LTK63 was higher (13:1), when compared with that induced after immunisation with LT or LTR72 (4.3:1 and 4.6:1 respectively) (Fig. 3.3). These are consistent with the mixed Th1/Th2 response observed with both the wild type toxin and LTR72 and the polarised Th2 response induced with LTK63.

3.2.3 LT induces the maturation of BMDC

The data in figures 3.1 and 3.2 demonstrated that LT, LTR72 and LTK63 could each enhance adaptive immune responses. This indicated that the immunomodulatory activity of LT was not entirely dependent upon the presence of enzyme activity, but that the presence of an active enzyme response may contribute to

the type of T cell response induced. Given that innate immune cells are important regulators in the priming of T cells and dictate T cell responses, differential activation of innate cells may explain the differences observed in T cell cytokine production and antibody production by the wild type toxin and its mutants. DC are critical for priming of naïve T cells, but must first be activated to undergo maturation. Maturation of the DC results in surface expression of major histocompatibility complex class II (MHCII), as well as co-stimulatory molecules, such as CD80 and CD86. This DC maturation can be stimulated by TLR agonists, such as LPS, and by other pathogen derived molecules. Indeed this study found that incubation of BMDC with LPS enhanced expression of CD80, CD86, B7-H1, ICAM-1, MHCII and CD40, indicative of DC maturation (Fig. 3.4). Stimulation of BMDC with LT also enhanced cell surface expression of the co-stimulatory molecules CD80, CD86 and B7-H1, but in contrast to LPS, LT inhibited expression of ICAM-1 and CD40 on DC (Fig. 3.4, Fig. 3.5). Furthermore, LT modulated LPS-induced co-stimulatory molecule expression, inhibiting LPS-induced expression of CD86, ICAM-1 and CD40, but enhancing CD80 (Fig. 3.4).

3.2.4 Maturation of DC by LT is not dependent on cAMP production

The data shown in figures 3.4 and 3.5 demonstrated that LT was capable of altering surface marker expression on BMDC and could also modulate LPS-induced DC maturation. Given that the adjuvancy of LT was at least in part independent of its enzyme activity (Fig. 3.1, Fig. 3.2, Fig. 3.3) this study examined the role of enzyme activity in DC surface marker expression. Both LTR72 and LTK63 increased the expression of CD80, CD86 and B7-H1 on BMDC while inhibiting expression of

CD40 and ICAM-1. Demonstrating that the modulation of BMDC surface markers by LT was not cAMP dependent. However, the regulatory effect of LTK63 was much reduced compared with that induced by wild type LT (Fig. 3.5). Modulation of LPS-induced surface marker expression was also found not to be dependent on the enzyme activity of LT, although LT and LTR72 were capable of mediating the observed responses much faster than LTK63 (Fig. 3.6, Fig. 3.7). LT also modulated LPS-induced co-stimulatory molecule expression, inhibiting LPS-induced expression of CD86, CD40 and ICAM-1 and enhancing CD80, following 1 hour pre-treatment of the DC prior to the addition of LPS (Fig. 3.6). The effect of LT on surface marker expression decreased over time; the up-regulation of CD80 and inhibition of CD40 and ICAM-1 was more pronounced following a 1 hour pre-treatment when compared with 24 hour pre-treatment. Conversely, the strongest modulation of the surface markers by LTK63, including increased CD80 and inhibited CD40 and ICAM-1, was observed after 24 hour pre-treatment of DC prior to addition of LPS (Fig. 3.7). Indeed, 1 hour pre-treatment with LTK63 prior to addition of LPS had little effect on LPS-induced co-stimulatory molecule expression.

3.2.5 LT does not induce DC cytokine production but can modulate TLR ligand-induced cytokine responses

The previous experiments demonstrated that LT could alter surface marker expression on DC and was capable of modulating LPS-induced DC maturation. The ability of LT to induce or modulate DC cytokine production was therefore examined. Incubation of immature DC with LT did not induce the production of IL-1 β , IL-10, IL-12p40, IL-12p70, IL-6, TNF- α (Fig. 3.8) or IL-23 (Fig. 3.9). In contrast incubation

of DC with LPS stimulated the production of the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α , IL-12p40 and IL-12p70 as well as the anti-inflammatory cytokine IL-10 (Fig. 3.8). However, LT was capable of modulating LPS-induced cytokine production. LT significantly enhanced of LPS-induced production of IL-1 β and IL-10. Notably, LT could significantly increase the production of IL-1 β from DC stimulated with as low as 50 pg/ml LPS, at which concentration LPS was unable to induce IL-1 β production by itself. However, synergistic enhancement of LPS-induced IL-10 by LT appears to require higher concentrations of the TLR4 agonist. Incubation of LT with 50 pg/ml LPS did not significantly enhance IL-10 production from the DC, however high concentrations of IL-10 were secreted from DC stimulated with LT in combination with either 500 or 5000 pg/ml LPS (Fig. 3.8). In addition to the enhancing effect on IL-1 β and IL-10 production, LT inhibited the production of LPS-induced IL-12 and TNF- α from DC. LPS (500 and 5000 pg/ml) induced production of IL-12p40 and IL-12p70 from DC and this was almost totally inhibited when DC were pre-incubated with LT for 6 hours (Fig. 3.8). Similarly, DC stimulated with doses of LPS as low as 50 pg/ml produced the pro-inflammatory cytokine TNF- α and this was significantly reduced when DC were pre-incubated with LT. It was also found that the TLR9 agonist CpG was capable of inducing the T_{IL-17} polarising cytokine IL-23 from DC and this was significantly increased following co-incubation with LT (Fig. 3.9).

3.2.6 LT modulation of DC cytokine production is not dependent on enzyme activity of the toxin

To address the role of the enzyme activity of LT in its immunomodulatory effects, the partially enzyme active (LTR72) and totally inactive (LTK63) LT mutants

were tested for their effects on the production of IL-10, IL-1 β , IL-12 and TNF- α from LPS-stimulated DC. The data shows that LTR72 and LTK63 were both capable of significantly inhibiting LPS-induced IL-12p40, IL-12p70 and TNF- α , while enhancing LPS-induced IL-10 (Fig. 3.10). Like LT, LTR72 inhibited LPS-induced IL-12p70 and increase LPS-induced IL-10 following a 1 hour pre-treatment. However, the modulatory effect of LTR72 on LPS-induced IL-12p40 and TNF- α required at least 6 hours pre-treatment. The enzymatically inactive mutant LTK63 was capable of significantly modulating LPS-induced IL-10 and IL-12p70 after a 6 hour pre-treatment before the addition of LPS, but could only inhibit LPS-induced IL-12p40 and TNF- α if incubated with DC for 12 hours prior to the addition of LPS. In contrast to the time-dependence of the LTK63 mediated effects on LPS-induced IL-10, IL-12 and TNF- α production, the mutant significantly enhanced IL-1 expression when incubated with DC for 1 hour prior to the addition of LPS, consistent with that shown by wild type LT and LTR72 (Fig. 3.11).

3.2.7 IL-10 is not responsible for the inhibition of LPS-induced CD40, ICAM-1, IL-12 or TNF- α by LT

Given that IL-10 plays a potent regulatory role in innate and adaptive immune responses and since LT synergises with LPS for the production of this cytokine from DC, the role of IL-10 in the negative regulation of surface marker and cytokine expression in LPS stimulated DC was examined. Experiments on DC from wild type C57BL/6 and IL-10^{-/-} mice demonstrated that LT inhibited CD40 and ICAM-1 expression on DC from IL-10^{-/-} and wild type mice, suggesting that the ability of LT to inhibit LPS-induced CD40 and ICAM-1 was not dependent upon IL-10 (Fig. 3.12).

Similarly LT inhibited LPS-induced production of IL-12 and TNF- α by DC from both C57BL/6 and IL-10^{-/-} mice, demonstrating that the ability of the toxin to inhibit these cytokines was not dependent upon IL-10 production (Fig. 3.13). LT was, however, unable to enhance LPS-induced IL-1 β from IL-10^{-/-} DC as demonstrated by wild type DC.

3.2.8 LT mediated modulation of LPS-induced cytokine production is not due to a soluble factor

The above experiments demonstrated that the modulation of DC cytokine and surface marker expression by LT was not mediated by IL-10. A study was therefore carried out to determine whether any soluble factor induced by the toxin was responsible for the observed modulation of cytokine production. DC were incubated with LTK63 for 1, 6 or 12 hours after which time either LPS was added directly to the culture or supernatants were removed and transferred to naïve DC and these were then stimulated with LPS. If the increase in IL-10 or the suppression of IL-12 were due to a soluble factor then this factor would be transferred with the supernatant and would be capable of modulating the LPS-induced responses of the naïve DC. Wild type LT has been shown to be capable of modulating LPS-induced cytokine responses after only short pre-treatment with DC. Therefore, wild type LT was not used in this experiment since there would be potential transfer of toxin with the transferred supernatant, which could modulate LPS-induced cytokine responses directly, masking any potential responses mediated by the soluble factor. Therefore the LT mutant, LTK63, which required longer incubation in order to modulate cytokine production

from DC, was used in the study, since LTK63 did not induce measurable cytokine production by DC after 1 hour pre-treatment.

As previously demonstrated LTK63 modulated LPS-induced IL-10 and IL-12 produced from DC (Fig. 3.14). However, transfer of supernatants from LTK63-treated DC to naïve DC did not transfer the ability to synergistically enhance IL-10 or inhibit IL-12p40 or IL-12p70 production. These results rule out a role for a soluble factor in the modulation of the LPS-induced cytokine production by LT.

3.2.9 IL-1RI plays a role in the adjuvant property of LT and LTK63

IL-1 has previously been shown to have adjuvant activity (Staats and Ennis, 1999) and has been implicated as a co-factor in activation of Th2 cells (Helmby and Grencis, 2004). Given that both enzymatically active LT and its inactive mutant, LTK63, significantly increased LPS-induced IL-1 production the role of toxin-induced IL-1 in the adjuvanticity of LT was examined using IL-1RI defective mice. Wild type C57BL/6 and IL-1RI^{-/-} mice were immunised with KLH alone or with LT or LTK63. Assessment of antigen-specific T cell responses showed that IL-1RI plays a role in the antigen-specific T cell proliferation induced by immunisation with LTK63 but not with LT (Fig. 3.15). Immunisation of C57BL/6 and IL-1RI^{-/-} mice, with PBS alone, did not promote proliferative T cell responses. However, C57BL/6 mice immunised with KLH induced antigen-specific proliferative responses in spleen cells. Immunisation of C57BL/6 mice with KLH and LT or KLH and LTK63 was found to significantly increase the proliferation of antigen-stimulated spleen cells above that produced by immunisation with KLH alone. KLH and KLH and LTK63 induced proliferation was significantly lower in spleen cells from IL-1RI^{-/-} compared

with wild type mice. However the proliferative responses of IL-1RI^{-/-} mice immunised with LT and KLH were not reduced when compared with wild type mice (Fig. 3.15). Antigen-specific cytokine production induced following immunisation with KLH in the presence of LT and LTK63, were also significantly lower in spleen cells from IL-1RI^{-/-} mice when compared with spleen cells from C57BL/6 mice (Fig. 3.16). Furthermore, antigen re-stimulated spleen cells from C57BL/6 mice immunised with KLH alone produced IL-10, IL-5 and small concentrations of IL-4 but no IFN- γ , and this antigen-specific cytokine production was significantly lower in spleen cells from IL-1RI^{-/-} mice compared with spleen cells from C57BL/6 mice. Immunisation with KLH in the presence of LT or LTK63 produced high concentrations of IL-10, IL-5, IL-4 and IFN- γ , but this antigen-specific cytokine production was significantly reduced in spleen cells from IL-1RI^{-/-} mice compared with spleen cells from wild type mice (Fig. 3.16). Similarly spleen cells from C57BL/6 mice immunised with KLH and LTK63, but not KLH alone, secreted antigen-specific IL-17 and this was significantly reduced in spleen cells from IL-1RI^{-/-} mice (Fig. 3.17) Furthermore, stimulation of spleen cells from C57BL/6 mice immunised with PBS, KLH, or KLH and LTK63 with PMA and anti-CD3 induced production of IL-17. This polyclonally activated IL-17 was, however, significantly reduced in all spleen cell populations from IL-1RI^{-/-} mice.

3.2.10 Induction of IL-1 β is not responsible for the activation of DC by LT

Given the link between IL-1 and the adjuvant activity of LT and LTK63, demonstrated by the data in Fig. 3.1, 3.2 and 3.3, experiments were carried out to address the role of toxin-modulated, LPS-induced IL-1 on DC maturation. LT was

found to be capable of enhancing surface expression of CD80 and CD86, while inhibiting ICAM-1 and CD40 expression on DC from wild type and IL-1RI^{-/-} mice (Fig. 3.18), suggesting that IL-1 is not involved with the toxins ability to induce DC maturation. Furthermore, the modulatory effect of LT on enhancement of LPS-induced IL-1 and IL-10 and inhibition of LPS-induced IL-12 and TNF- α was comparable in DC from IL-1RI^{-/-} and C57BL/6 mice (Fig. 3.19). Indeed LPS-induced IL-1 β production by DC from IL-1RI^{-/-} mice was increased when compared with DC from wild type mice (Fig. 3.19), probably due to loss of IL-1 β from the culture, through binding to IL-1RI on DC from wild type mice. These experiments indicate that the role of IL-1 in adjuvant effects of LT may not be through modulation of DC maturation and regulatory cytokine production.

3.3 DISCUSSION

Vaccination is regarded as one of the most important medical advances ever made, providing protection against diseases, which would otherwise have resulted in the loss of millions of lives worldwide. However, certain traditional vaccines, especially those utilising heat killed bacteria, such as the whole cell pertussis vaccine, have been found to be unsafe for clinical practice due to associated side effects, such as fever and seizures. In order to circumvent this problem new, second generation vaccines have been developed, which utilise purified or recombinant antigens from the bacteria. These antigenic subunits are unfortunately not strongly immunogenic and therefore do not induce potent immune responses by themselves. To overcome this problem, adjuvants, capable of enhancing and polarising immune responses are required to increase and prolong the antigen-specific immune response.

One of the most powerful vaccine adjuvants studied to date is the heat labile enterotoxin of *E.coli* (LT). LT is a member of the AB₅ enterotoxins and is responsible for symptoms associated with Traveller's diarrhoea. These symptoms are due increased cAMP in cells of the intestine mediated by the enzymatic A subunit of the toxin. However, the adjuvant properties associated with LT cannot be fully attributed to enzyme activity and increased intracellular cAMP, since mutant versions of LT which have either partial or no enzyme activity retain adjuvant ability (Gagliardi *et al.*, 2000; Richards *et al.*, 2001; Ryan *et al.*, 1999). This study confirmed this finding with immunisation experiments involving LT and the mutants LTR72, which retains some enzyme activity and LTK63, which is devoid of enzyme activity. It was found that parenteral immunisation with KLH in the presence of LT and LTR72 generated a mixed Th1/Th2 response, in combination with IgG1 and IgG2a antibody production.

Indeed LTR72 appeared to be a better inducer of Th1 responses than wild type LT. In contrast, immunisation with antigen in the presence of the enzymatically inactive LTK63 induced polarised Th2 cytokine production, in combination with high ratio of IgG1 to IgG2a antibody production. This data is in broad agreement with previously published studies, which linked enzymatically inactive mutant enterotoxins and the induction of Th2 responses (Douce *et al.*, 1999; Yamamoto *et al.*, 1997). The data indicates that the adjuvant property associated with the enterotoxins is not totally dependent upon enzyme activity and therefore cAMP production, however an active or partially active toxin may be required to induce more polarised Th1 type responses. A further finding of this study was the demonstration that LT, LTR72 and LTK63 were strong adjuvants when administered via a parenteral route and not just by mucosal delivery as previously reported (Fraser *et al.*, 2003; Nashar *et al.*, 1993; Richards *et al.*, 2001). This is an important observation, since intranasal immunisation with an influenza vaccine, containing wild type LT as the adjuvant, has been associated with the development of Bell's palsy in a small number of vaccines and the subsequently withdrawal of the nasal vaccine for human use (Mutsch *et al.*, 2004). Indeed, studies with the related enterotoxin of *Vibrio cholera* (CT), CT can track to the brain and induce inflammation following delivery by the intranasal but not parenteral route (Armstrong *et al.*, 2005).

This study aimed to discriminate between the adjuvant and immunomodulation activity associated with enzyme activity and the non-toxic AB complex. Since adaptive responses are promoted and directed by innate immune activation, the differential ability of LT, LTR72 and LTK63 to activate DC may explain the differences in the adaptive immune responses generated by the different toxins. Previous studies have shown that the enterotoxin CT, a homologue of LT,

modulated DC co-stimulatory marker expression and cytokine production (Bagley *et al.*, 2002; Lavelle *et al.*, 2004), enhancing LPS-induced DC and macrophage IL-10. The present study demonstrated that LT was also capable of modulating LPS-induced cytokine production and furthermore that LT alone could partially mature DC, as shown by up-regulation of surface marker expression. Stimulation of DC with LT enhances the expression of the co-stimulatory molecules, CD80, CD86 and B7-H1. However, LT decreased expression of ICAM-1 and CD40 on DC. LT could also modulate LPS-induced DC surface marker expression enhancing LPS induced CD80 and B7-H1. However, LT decreased expression of LPS-induced CD86, ICAM-1 and CD40 on DC, but did not affect LPS-induced expression of major histocompatibility complex class II (MHCII). The effect of LT alone or in the presence of LPS will provide the DC with the first two of the three signals, which the DC requires for T cell activation, up-regulated expression MHCII and co-stimulatory molecules. The third signal required for T cell activation, cytokine production, could not be induced by stimulation of DC with LT alone. However LT was capable of modulating TLR-induced cytokine production, enhancing IL-10, IL-23 and IL-1 β production and suppressing LPS-induced IL-12 and TNF- α production. However, as observed with the adjuvant properties of LT, modulation of LPS induced DC maturation was not dependent upon enzyme activity of the toxin.

This study has provided new information to show that both LTR72 and LTK63 modulate endogenous and LPS-induced co-stimulatory molecule expression and cytokine production by DC, which is not comparable with wild type LT stimulation as it may occur with differential kinetics. The enzymatically inactive LTK63 was capable of inducing expression of the co-stimulatory molecules, CD80 and CD86, as well as increasing LPS-induced CD80 and IL-10 and decreasing LPS-

induced CD86, CD40, ICAM-1, IL-12 and TNF- α production from DC. However, LTK63 required at least 12 hour pre-treatment with DC prior to the addition of LPS in order to modulate responses. In contrast LT could modulate these responses with only a 1 hour pre-treatment prior to the addition of LPS. However up-regulation of LPS-induced IL-1 β by both LTR72 and LTK63 was as potent as that observed following stimulation with wild type LT. Preliminary results from the laboratory have found a non-enzyme associated increase in cAMP, 12 hours post stimulation of DC with enterotoxin (C Brereton, unpublished observation). This result provides a possible explanation for the delayed kinetics in modulation of DC maturation by LTK63.

The regulation of LPS-induced IL-10 was comparable between LT, LTR72 and LTK63. IL-10 is a known regulatory cytokine, capable of controlling the expression of the Th1 polarising cytokine IL-12 (Rahim *et al.*, 2005). Therefore, the role of LPS-induced, LT enhanced, IL-10 in the modulation of DC activation was examined. It was found that IL-10, which was enhanced by LT, was not responsible for its modulatory effects, since LT inhibited LPS-induced IL-12, TNF- α , CD40 and ICAM-1 by DC from IL-10^{-/-} and C57BL/6 mice. However, LT did not enhance LPS-induced IL-1 β production by DC from IL-10^{-/-} mice. This suggests that LPS-induced IL-10 plays a role in IL-1 production in DC from wild type mice. DC from IL-10^{-/-} mice also produced higher concentrations of IL-12 in response to LPS stimulation, which suggests that IL-10 may also play a role in the suppression of LPS-induced IL-12 in wild type DC. These experiments demonstrate that the modulation of DC maturation by LT was not IL-10 dependent. Indeed examination of the mechanism for regulation of DC responses by LT, LTR72 and LTK63 demonstrated that certain of the modulatory effects on cytokine production and surface marker expression may be due to modulation of cell signalling pathways within the DC rather

than release of an extracellular factor, such as a cytokine. This agrees with data published demonstrating that ERK phosphorylation is required for TLR-agonist induced IL-10 production in DC (Pulendran *et al.*, 2004). Indeed unpublished results from the laboratory using inhibitors of ERK phosphorylation show that ERK is, at least partially, involved in the synergistic enhancement of LPS-induced IL-10 by LT, LTR72 and LTK63, although other signalling pathways also appear to be involved.

LT, LTR72 and LTK63 had comparable modulatory effects on LPS-induced IL-1 β . IL-1 β has previously been shown to possess adjuvant properties (Staats and Ennis, 1999). This study demonstrates that antigen-specific T cell cytokine production and proliferation were reduced in spleen cells from IL-1RI^{-/-} mice suggesting that IL-1 plays a role in the adjuvanticity of LT and its mutants. Indeed IL-1RI appears to have a crucial role in the development of IL-17 producing T cells with both antigen-specific and PMA and anti-CD3 activated IL-17 production significantly reduced in spleen cells from IL-1RI^{-/-} mice. However, the role of IL-1 in the adjuvancy of LT and its mutants is not due to IL-1RI on the surface of DC given that DC from C57BL/6 and IL-1RI^{-/-} mice displayed comparable regulation of CD80, CD86, ICAM-1 and CD40 expression. It was also found that LT modulation of LPS-induced DC activation was not dependent on IL-1.

It is possible that activation and modulation of DC may not be the only method by which enterotoxins promote their adjuvanticity. This study and others have shown that LT inhibits LPS-induced IL-12 production from DC, the key cytokine involved in promoting a Th1 type response, from DC (Cong *et al.*, 2001; Lavelle *et al.*, 2003). However, this study clearly demonstrated that immunisation with LT and LTR72 generates a population of IFN- γ producing Th1 cells. This finding suggests that DC alone cannot explain the adjuvancy associated with LT and that other cells of

the immune system may respond to stimulation with LT to promote Th1 type responses.

This study clearly shows that functional enzyme activity is not wholly responsible for the adjuvant properties of LT. Furthermore the enzyme activity of LT was found not to be required for LT to induce maturation of DC or modulate LPS-induced maturation of DC. However, the enzyme activity did have a role as DC were activated more quickly with LT and LTR72 than LTK63. This project also found that the adjuvant activity of LT was at least partially IL-1RI dependent and furthermore that IL-1RI is required for the development of IL-17 producing T cells.

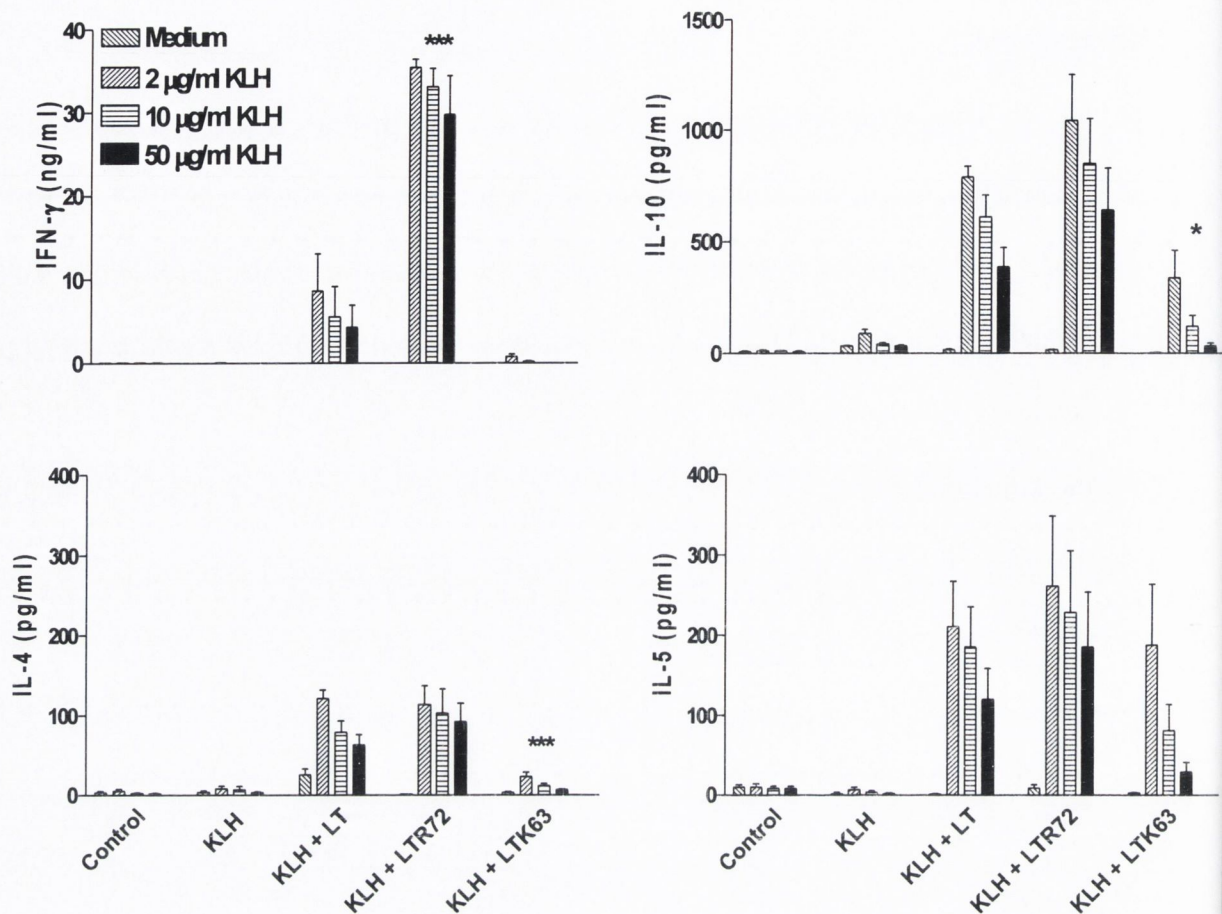


Figure 3.1: LT enhances splenic Th1 and Th2 responses to KLH, while LTK63 promotes a Th2-biased response. BALB/c mice ($n = 5$) were immunised s.c. with PBS, KLH (10 μ g), KLH and LT (1 μ g), KLH and LTR72 (1 μ g) or KLH and LTK63 (1 μ g) and boosted 14 d later. Mice were sacrificed 7 d after the boost and spleen cells re-stimulated ex vivo with KLH (2, 10 or 50 μ g/ml). Supernatants were removed after 72 h and tested by ELISA for IL-4, IL-5, IL-10 and IFN- γ production. LT + KLH versus LTR72 + KLH or LTK63 + KLH: *, $P < 0.05$; ***, $P < 0.001$.

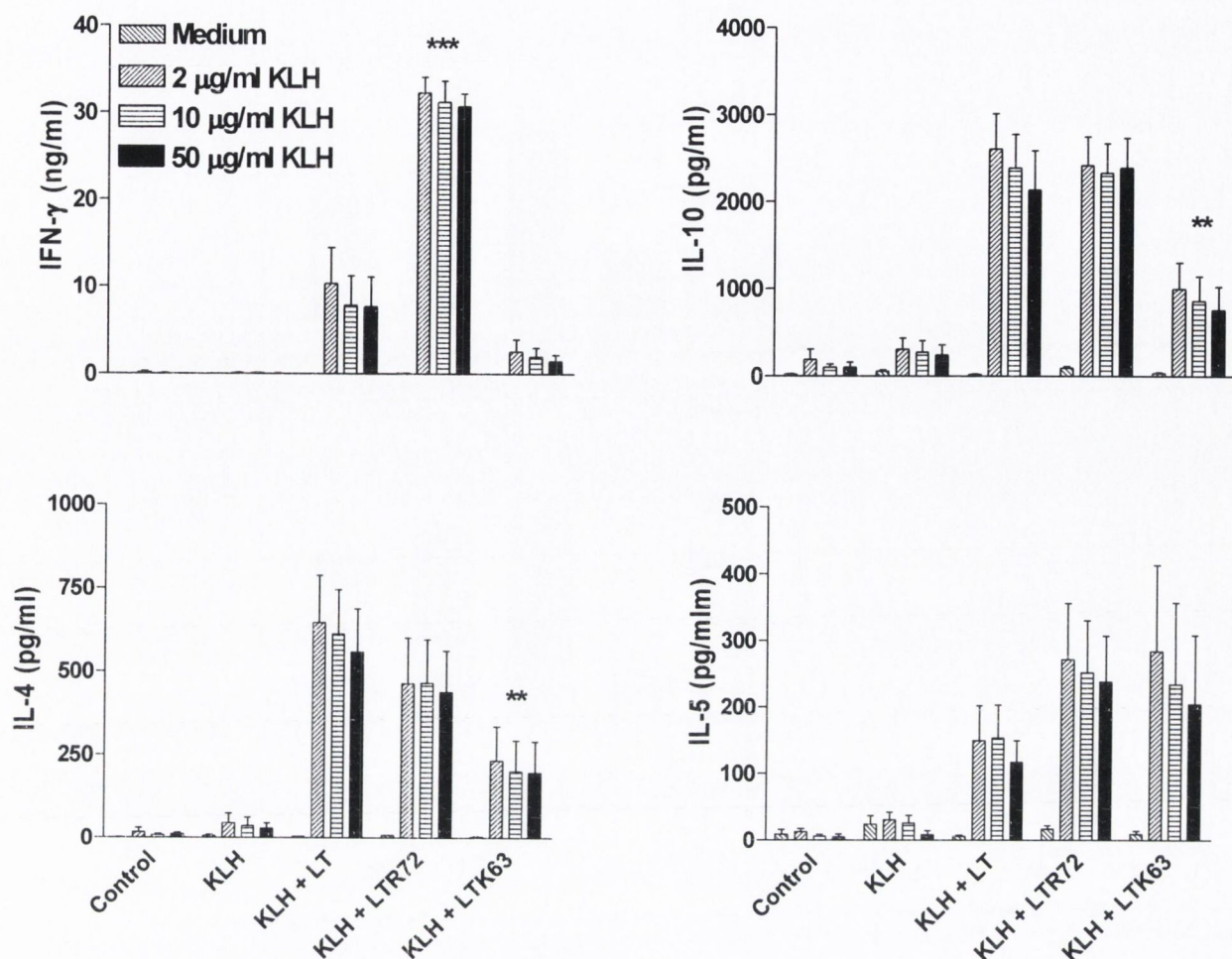


Figure 3.2: LT enhances lymph node Th1 and Th2 responses to KLH, while LTK63 induces a Th2-biased response. BALB/c mice (n = 5) were immunised s.c. with PBS, KLH (10 μg), KLH and LT (1 μg), KLH and LTR72 (1 μg) or KLH and LTK63 (1 μg) and boosted 14 d later. Mice were sacrificed 7 d after boost and popliteal lymph node cells harvested and re-stimulated ex vivo with KLH (2, 10 or 50 μg/ml). Supernatants were removed after 72 h and tested by ELISA for IL-4, IL-5, IL-10 and IFN-γ production. LT + KLH versus LTR72 + KLH or LTK63 + KLH: **, P < 0.01; ***, P < 0.001.

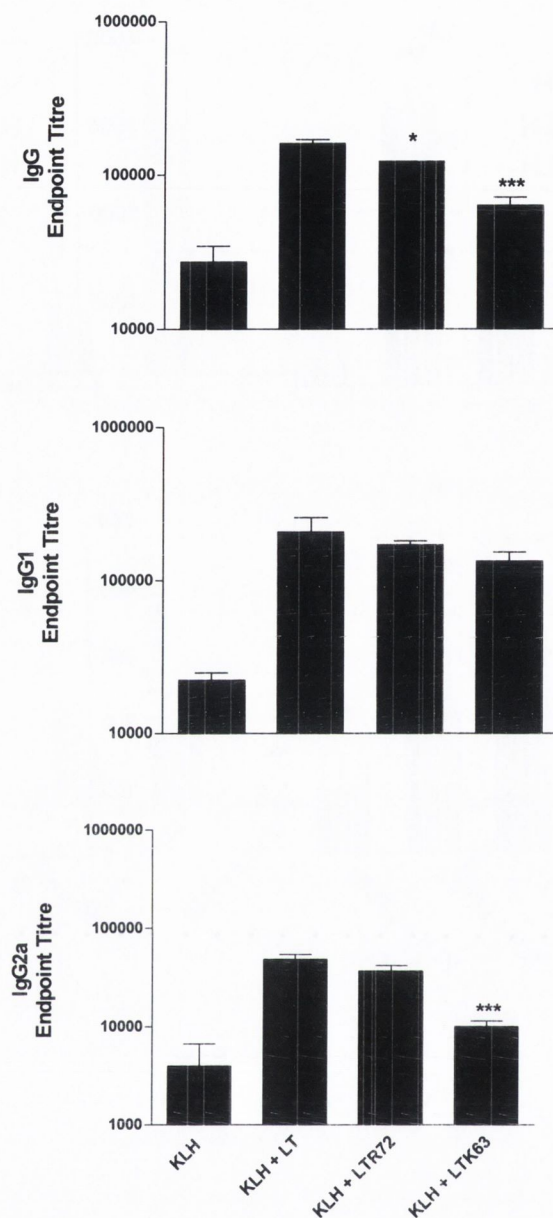


Figure 3.3: LT and LTR72 both enhance IgG2a and IgG1, whereas LTK63 predominantly enhances IgG1 responses. BALB/c mice were immunised s.c. with PBS, KLH (10 µg), KLH and LT (1 µg), KLH and LTR72 (1 µg) or KLH and LTK63 (1 µg) and boosted 14 d later. Mice were sacrificed 7 d after the boost and blood taken assayed for KLH-specific serum antibody total IgG, IgG1 and IgG2a by ELISA. LT + KLH versus LTR72 + KLH or LTK63 + KLH: *, $P < 0.05$; ***, $P < 0.001$.

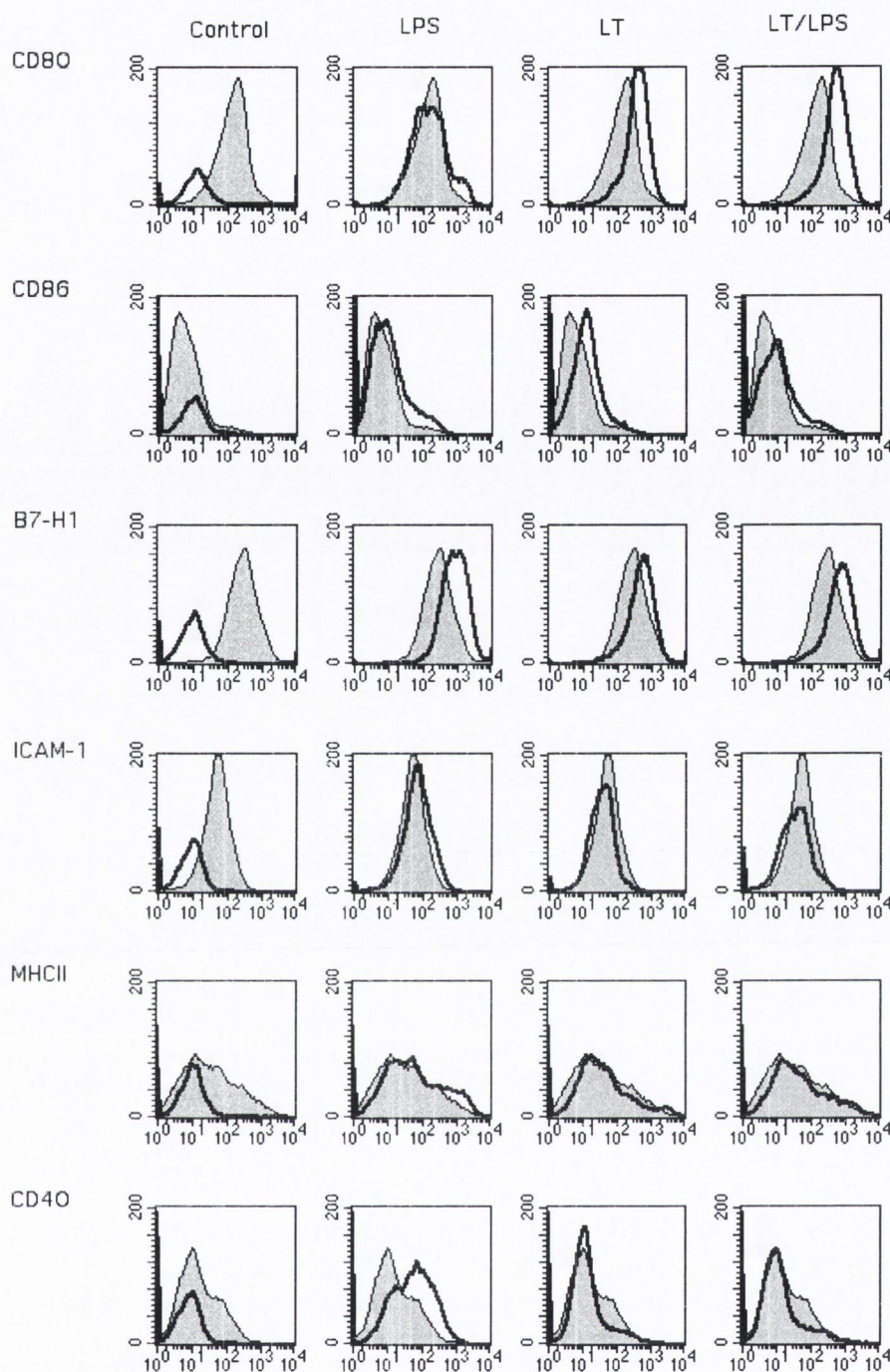


Figure 3.4: Wild type LT can mature DC and selectively modulate LPS-induced DC maturation. BMDC from BALB/c mice (1×10^6 cells/ml) were stimulated with LT ($1 \mu\text{g/ml}$), LPS (10 ng/ml), LT followed 6 h later with LPS or with medium alone. Cells were harvested 24 h later and stained with antibodies specific for CD80, CD86, B7-H1, ICAM-1, MHCII, CD40 or with isotype matched controls (black line control column). Results from flow cytometric immunofluorescence analysis are shown for treated (black line) compared to untreated (grey histogram) DC, gated on CD11c^+ cells.

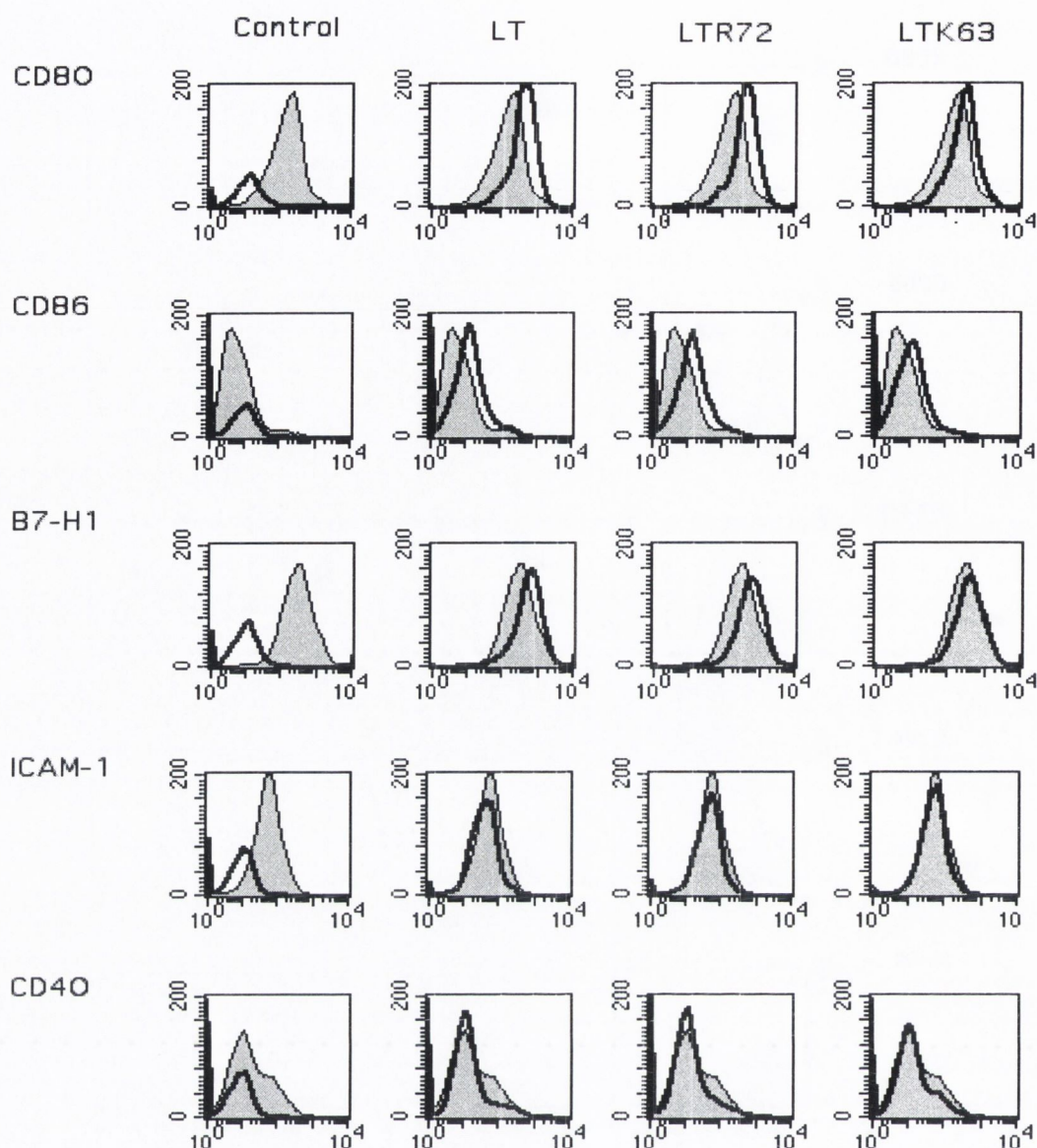


Figure 3.5: The ability of LT to increase expression of CD80, CD86 and B7-H1 is not dependent on the enzyme activity of the toxin. BALB/c BMDC (1×10^6 cells/ml) were incubated with LT, LTR72, LTK63 ($1 \mu\text{g/ml}$) or with medium alone for 24 h. Cells were then washed and stained with antibodies specific for CD80, CD86, B7-H1, ICAM-1, CD40 or with isotype matched controls (black line, control column). Results from flow cytometric immunofluorescence analysis are shown for treated (black line) compared to untreated (grey histogram) DC, gated on CD11c^+ cells.

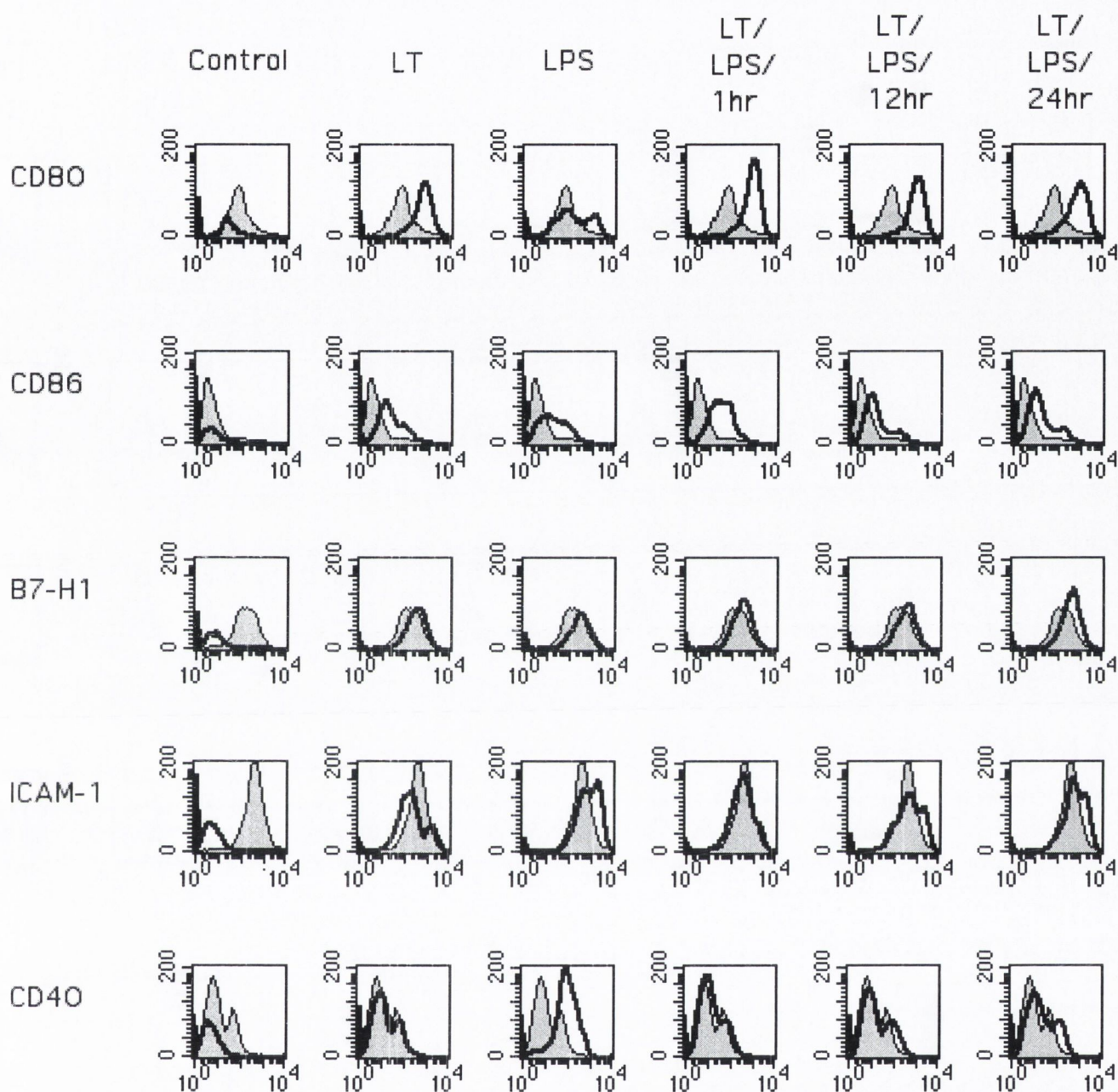


Figure 3.6: LT is capable of modulating LPS induced DC surface marker expression after short pre-treatment. BMDC from C57BL/6 mice (1×10^6 cells/ml) were incubated with LT (1 μ g/ml), LPS (10 ng/ml), LT for 1, 12 or 24 h prior to LPS or with medium alone. Cells were cultured for a further 24 h and then washed and stained with antibodies specific for CD80, CD86, B7-H1, ICAM-1 and CD40 or with isotype matched controls (black line, control column). Results from flow cytometric immunofluorescence analysis shown for treated (black line) compared with untreated (grey histogram) DC, gated on CD11c⁺ cells.

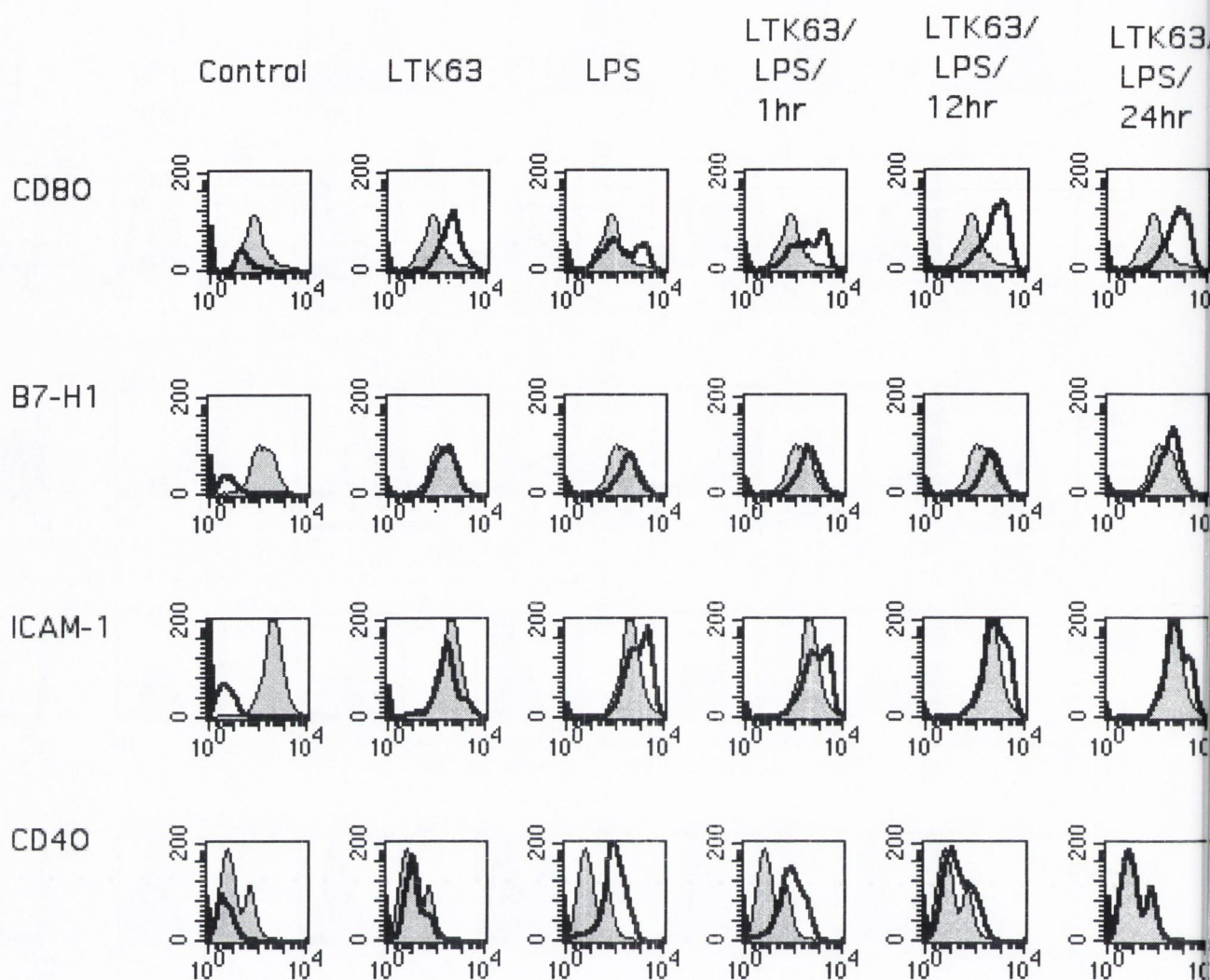


Figure 3.7: An enzymatically inactive LT mutant, LTK63, can inhibit LPS induced CD40 and ICAM-1 but only if added to cells at least 12 h prior to the addition of LPS. BMDC from C57BL/6 mice (1×10^6 cells/ml) were incubated with LTK63 (1 μ g/ml), LPS (10 ng/ml), LTK63 for 1, 12 or 24 h prior to LPS or with medium alone. Cells were cultured for a further 24 h then washed and stained with antibodies specific for CD80, B7-H1, ICAM-1 and CD40 or with isotype matched controls (black line, control column). Results from flow cytometric immunofluorescence analysis shown for treated (black line) compared with untreated (grey histogram) DC, gated on CD11c⁺ cells.

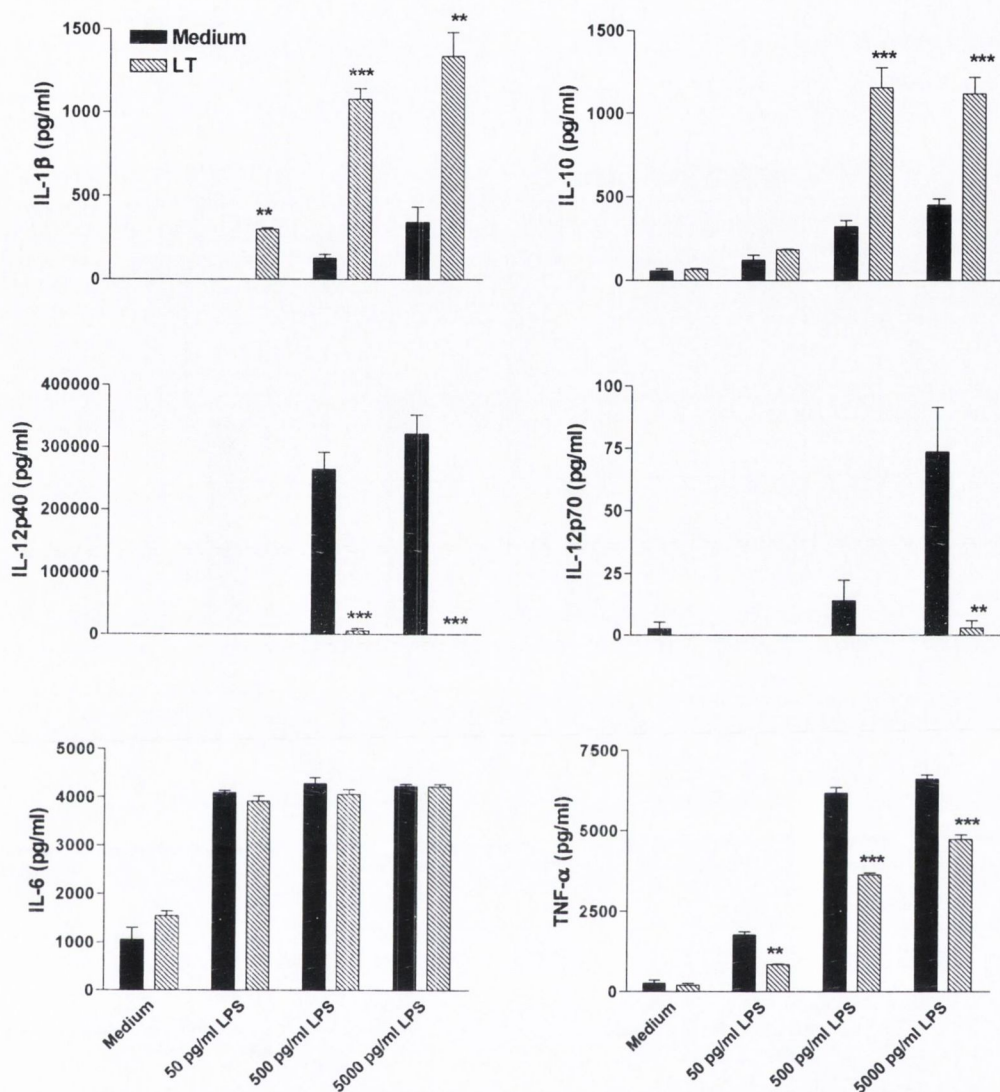


Figure 3.8: LT synergises with LPS for the production of IL-10 and IL-1 β , but inhibits LPS induced IL-12 and TNF- α from DC. BMDC from BALB/c mice (5×10^5 cells/ml) were stimulated with LT (1 μ g/ml), LPS (50, 500 or 5000 pg/ml), LT for 6 h prior to the addition of LPS or with medium alone. Supernatants were removed after 24 h and tested by ELISA for IL-1 β , IL-10, IL-12p40, IL-12p70, TNF- α and IL-6 production. LT + LPS versus LPS alone: **, P < 0.01; ***, P < 0.001.

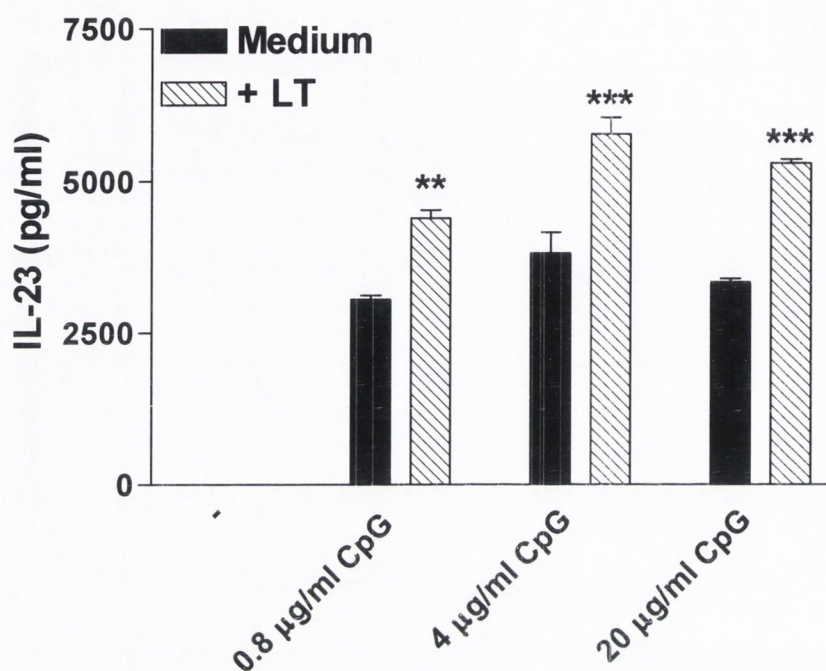


Figure 3.9: LT synergises with CpG for the production of IL-23 from DC. BMDC from C3H/HeJ mice (5×10^5 cells/ml) were stimulated with CpG (0.8, 4 or 20 $\mu\text{g/ml}$), LT (1 $\mu\text{g/ml}$), LT for 6 h prior to the addition of CpG or with medium alone. Supernatants were removed after 24 h and tested by ELISA for IL-23 production. LT + CpG versus CpG alone: **, $P < 0.01$; ***, $P < 0.001$.

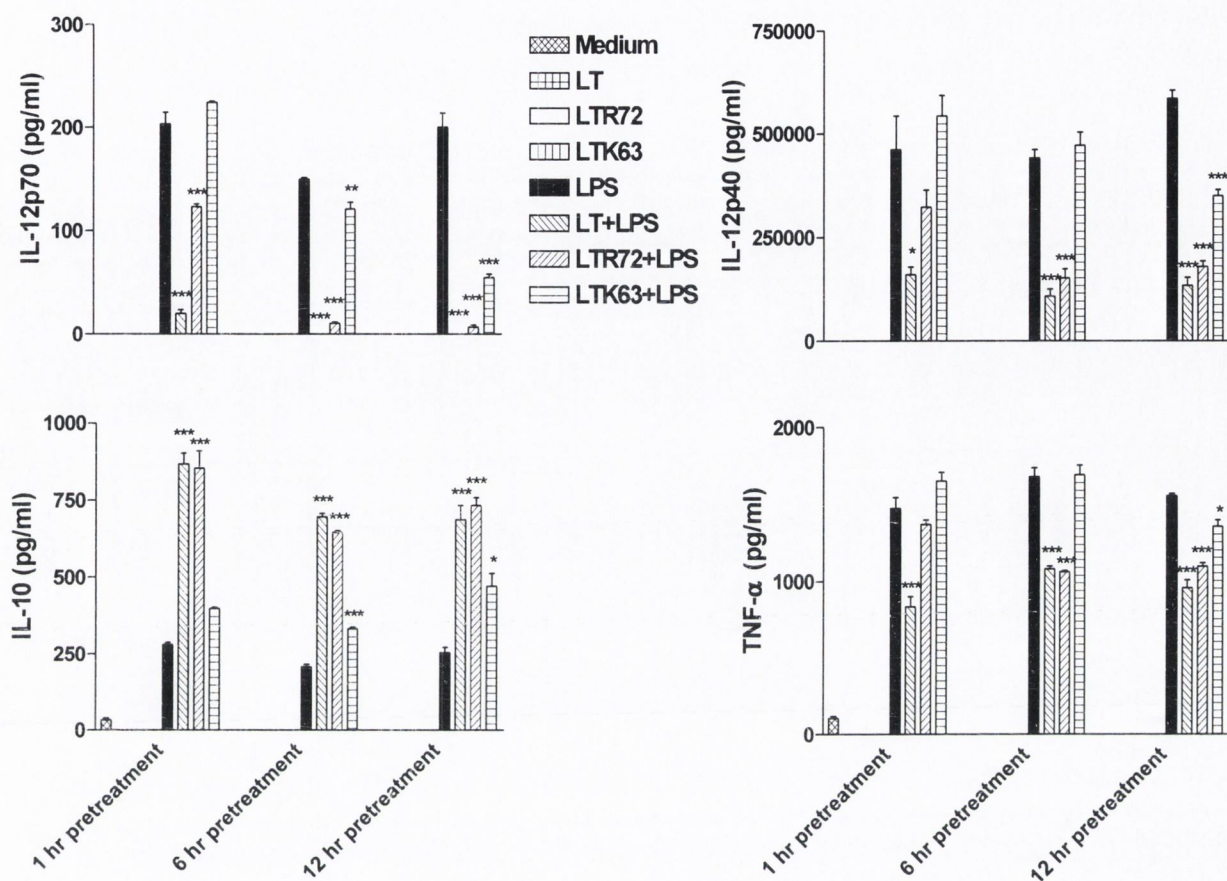


Figure 3.10: LTK63 can modulate LPS induced cytokines following 6-12 h pretreatment. BMDC from BALB/c mice (5×10^5 cells/ml) were incubated with LT, LTR72 or LTK63 (1 μ g/ml), LPS (5 ng/ml), LT, LTR72 or LTK63 for 1, 6 or 12 h prior to addition of LPS or with medium alone. Supernatants were removed after 24 h and tested by ELISA for IL-12p40, IL-12p70, IL-10 and TNF- α production. Treatment + LPS versus LPS alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

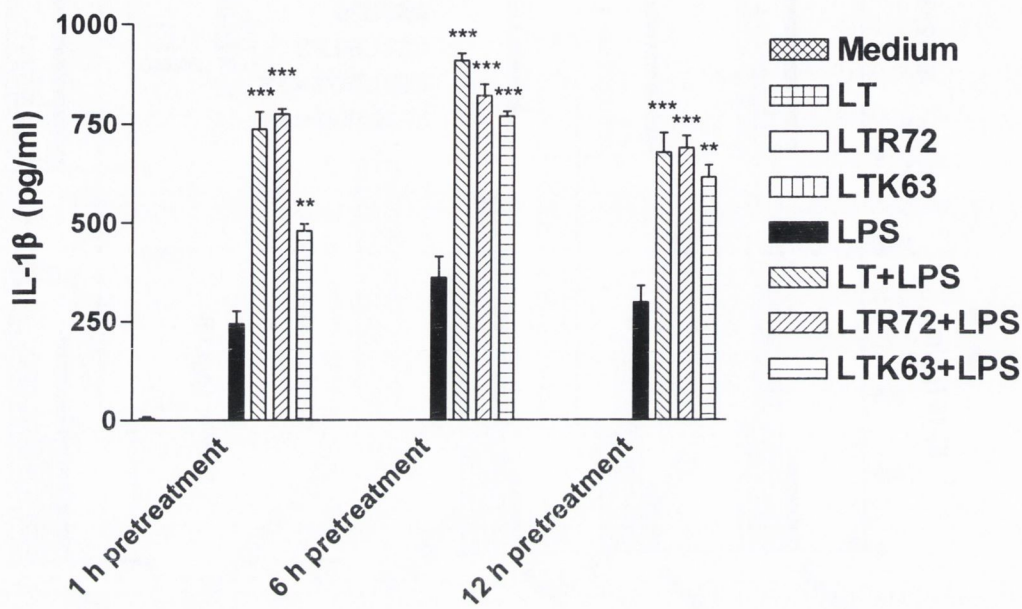


Figure 3.11: The ability of LT to enhance LPS-induced production of IL-1 β does not require an enzymatically active toxin or pre-treatment of the cells prior to addition of LPS. BMDC from BALB/c mice (5×10^5 cells/ml) were incubated with LPS (5 ng/ml), LT, LTR72 or LTK63 (all 1 μ g/ml), LT, LTR72 or LTK63 1, 6 or 12 h prior to the addition of LPS or with medium alone. Supernatants were removed after 24 h and tested by ELISA for IL-1 β production. Treatment + LPS versus LPS alone: **, $P < 0.01$; ***, $P < 0.001$.

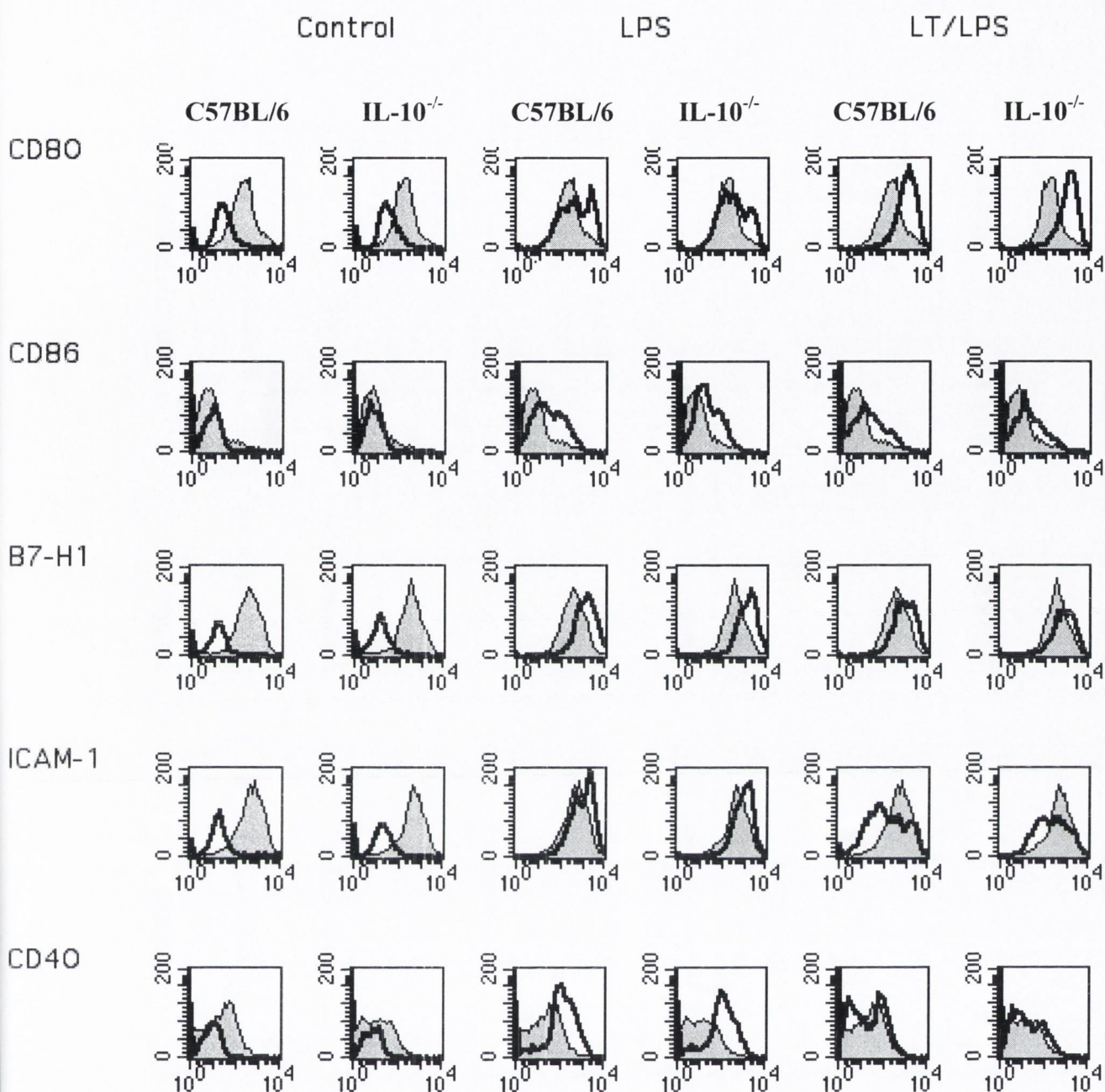


Figure 3.12: LT can inhibit LPS induced up-regulation of CD40 and ICAM-1 on DC in the absence of IL-10. BMDC from C57BL/6 or IL-10^{-/-} mice (1×10^6 cells/ml) were incubated with LPS (10 ng/ml), LT (1 μ g/ml), LT 6 h prior to LPS or medium alone for 24 h. Cells were then washed and stained with antibodies specific for CD80, CD86, ICAM-1, B7-H1, CD40 or with isotype-matched controls (black line, control column). Results from flow cytometric immunofluorescence analysis are shown for treated (black line) compared to untreated (grey histogram) DC, gated on CD11c⁺ cells

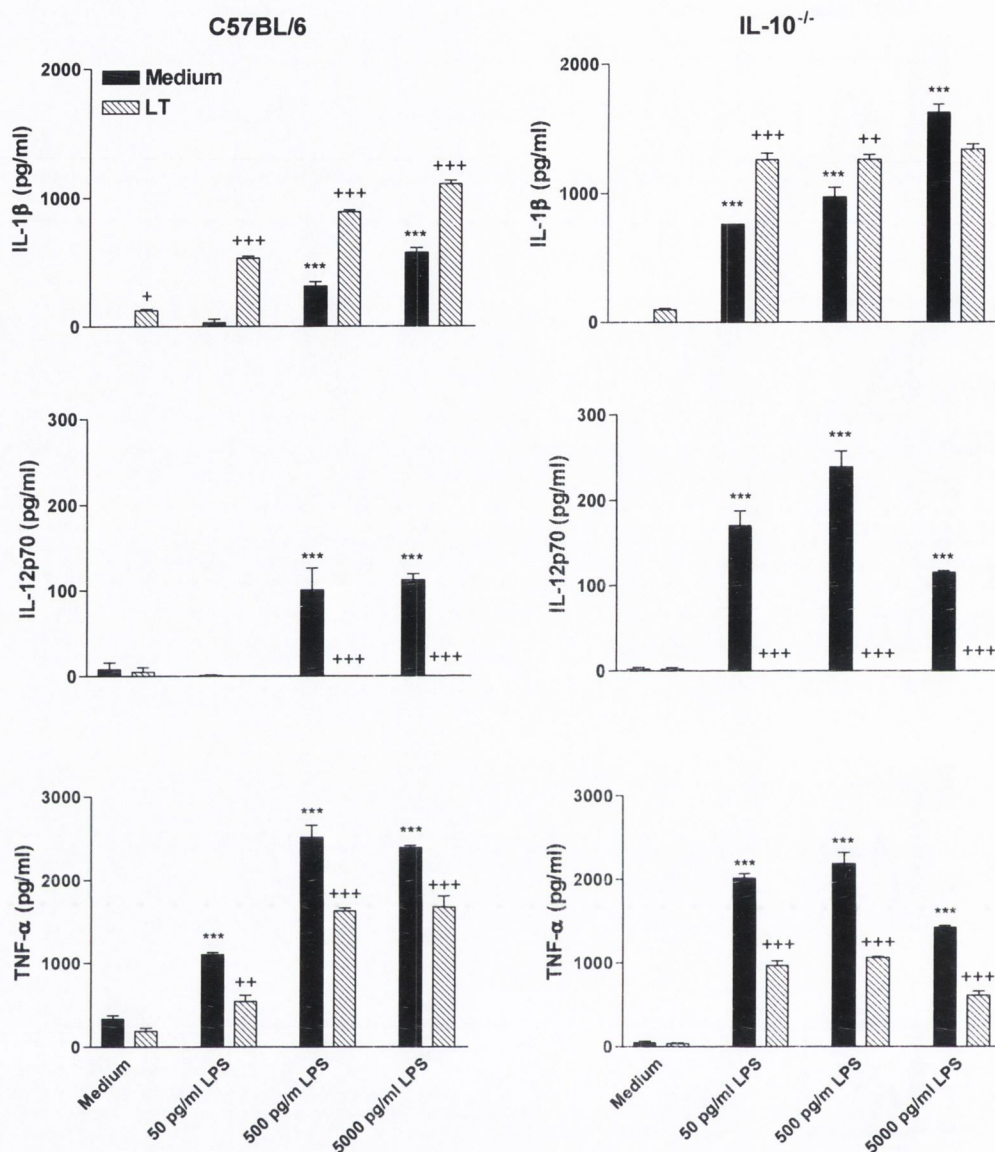


Figure 3.13: Inhibition of LPS-induced IL-12p70 and TNF- α by LT is not mediated by IL-10. BMDC from C57BL/6 and IL-10^{-/-} mice (5×10^5 cells/ml) were incubated with LPS (50, 500 or 5000 pg/ml), LT (1 μ g/ml), LT 6 h prior to addition of LPS or with medium alone. Supernatants were removed after 24 h and tested for IL-1 β , IL-12p70 and TNF- α production by ELISA. LPS versus medium alone: ***, $P < 0.001$. Treatment + LPS versus LPS alone: ++, $P < 0.01$; +++, $P < 0.001$.

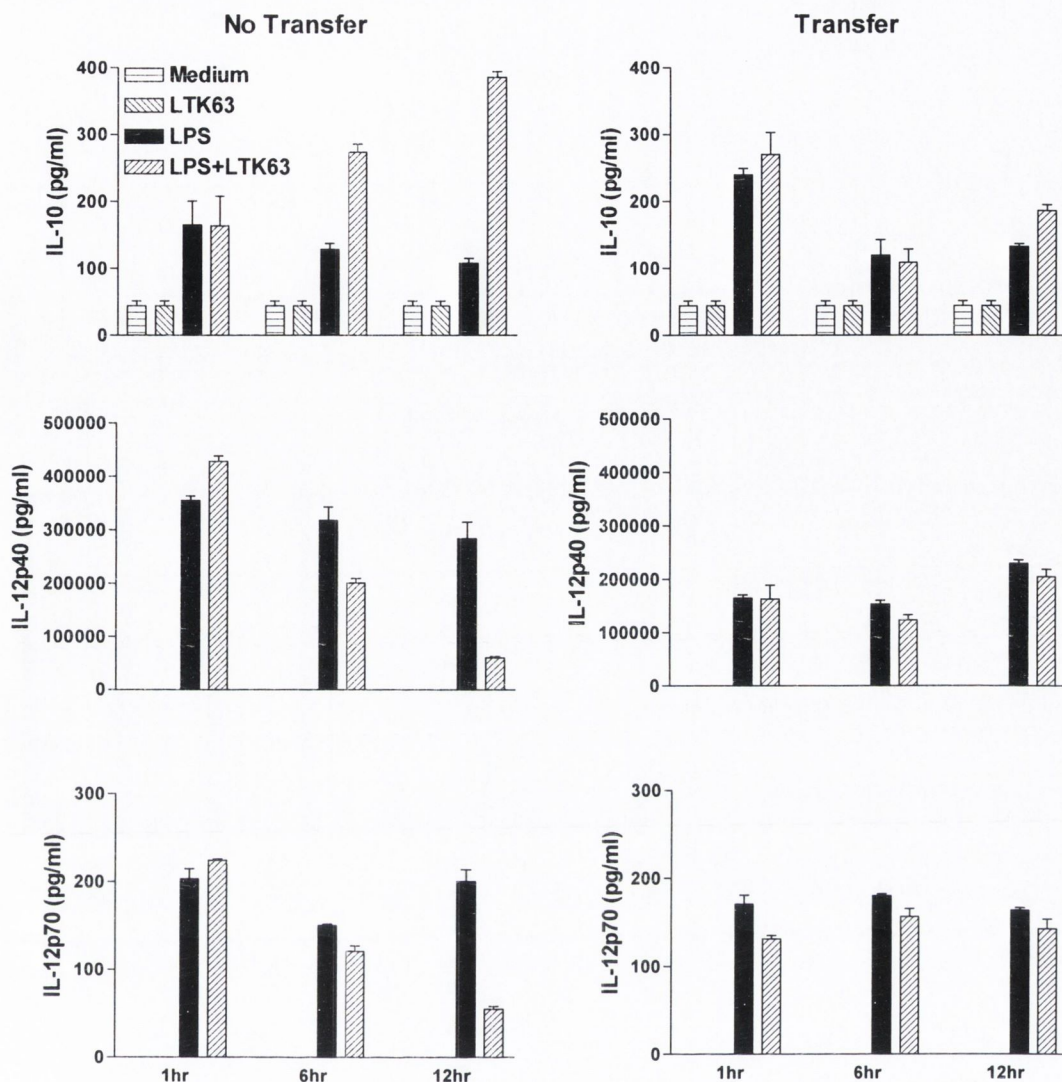


Figure 3.14: Enhancement of LPS induced IL-10 and inhibition of IL-12 from DC by LTK63 is not due to a soluble extracellular factor. BMDC from BALB/c mice (1×10^6 cells/ml) were stimulated either with LPS (5 ng/ml), LTK63 (1 μ g), LTK63 1, 6 or 12 h prior to LPS or with medium alone (**No Transfer**), or with LTK63 for 1, 6 or 12 h followed by the removal and transfer of supernatant onto fresh immature DC which were then stimulated with LPS (**Transfer**). Supernatants were removed after 24 h following LPS addition and tested by ELISA for IL-10, IL-12p70 and IL-12p40 production.

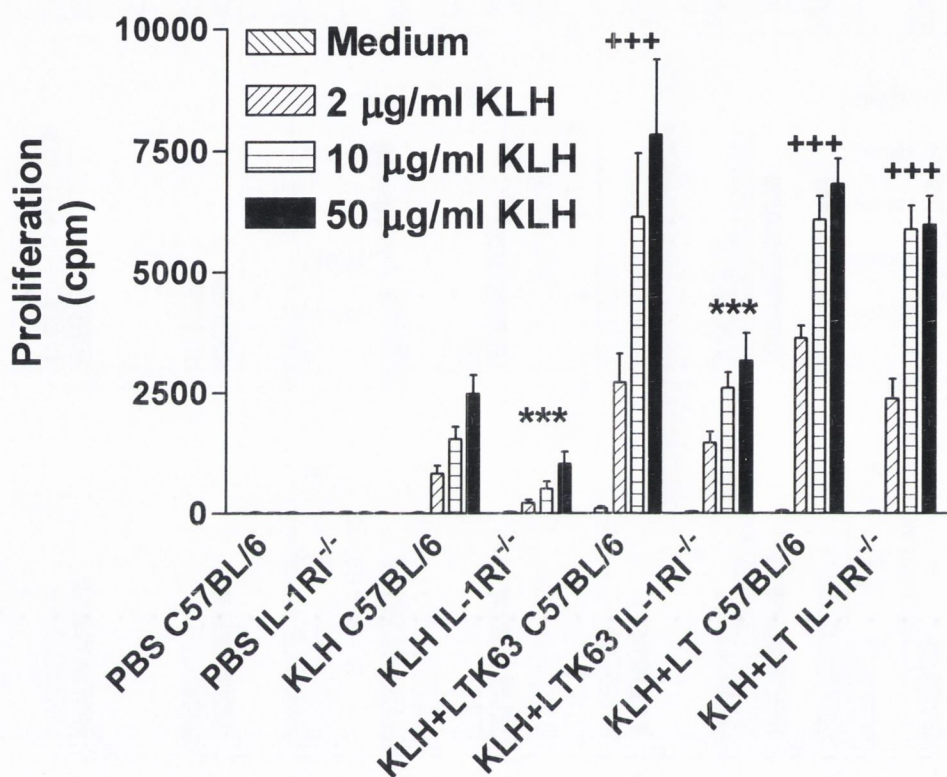


Figure 3.15: The adjuvant effect of LTK63 is dependent on IL-1RI. C57BL/6 or IL-1RI^{-/-} mice (n = 5) were immunised s.c. with PBS, KLH (10 µg), KLH with LT (1 µg) or KLH with LTK63 (1 µg). Mice were sacrificed after 7 d and spleen cells re-stimulated ex vivo with KLH (2, 10 or 50 µg/ml). After 72 h stimulation cells were incubated for a further 6 h in the presence of thymidine, [³H]. Data represents proliferation response as [³H] incorporation by KLH-stimulated spleen cells. C57BL/6 versus IL-1RI^{-/-}: ***, P < 0.001. Adjuvant + KLH versus KLH alone: +++, P < 0.001.

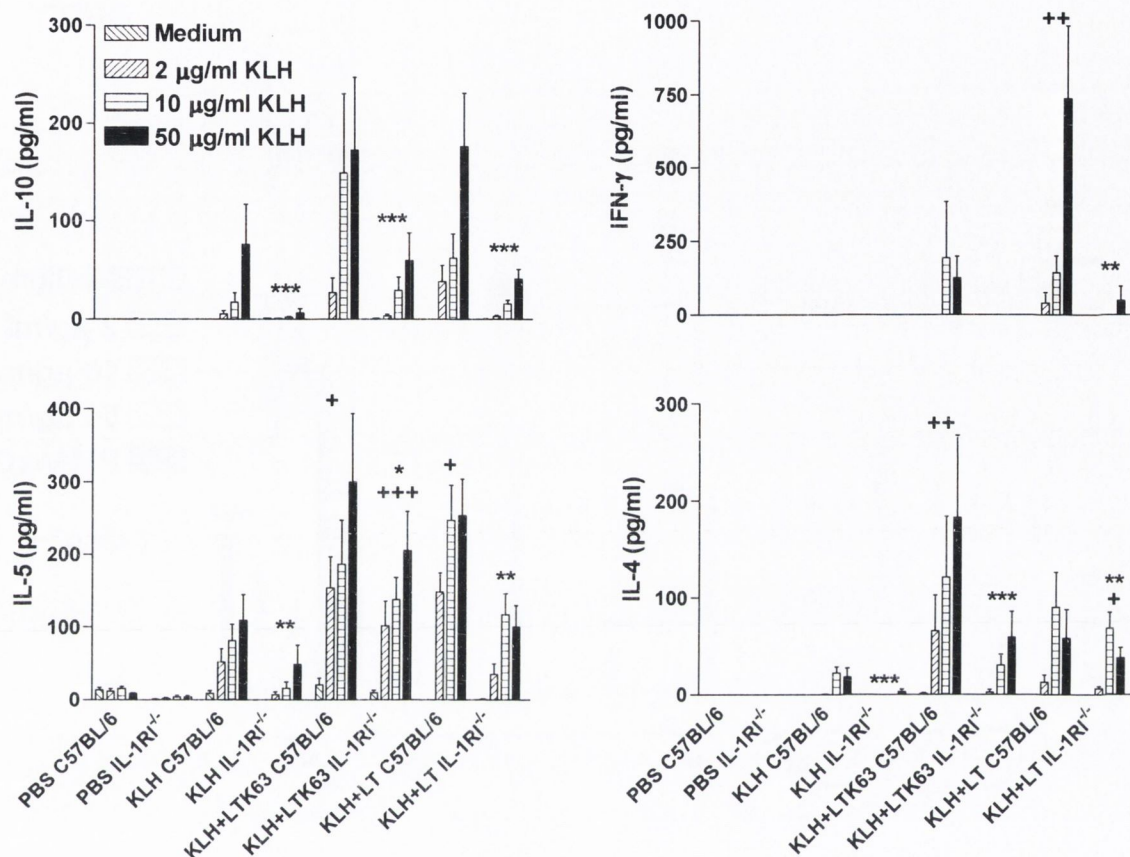


Figure 3.16: The ability of LT and LTK63 to enhance antigen-specific T cell cytokine production is partially dependent on IL-1RI. C57BL/6 or IL-1RI^{-/-} mice (n = 5) were immunised s.c. with PBS, KLH (10 μg), KLH with LT (1 μg) or KLH with LTK63 (1 μg). Mice were sacrificed after 7 d and spleen cells re-stimulated ex vivo with KLH (2, 10 or 50 μg/ml). Supernatants were removed after 72 h and tested for IL-4, IL-5, IL-10 and IFN-γ production by ELISA. C57BL/6 versus IL-1RI^{-/-}: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Adjuvant + KLH versus KLH alone: +, P < 0.05; ++, P < 0.01; +++, P < 0.001.

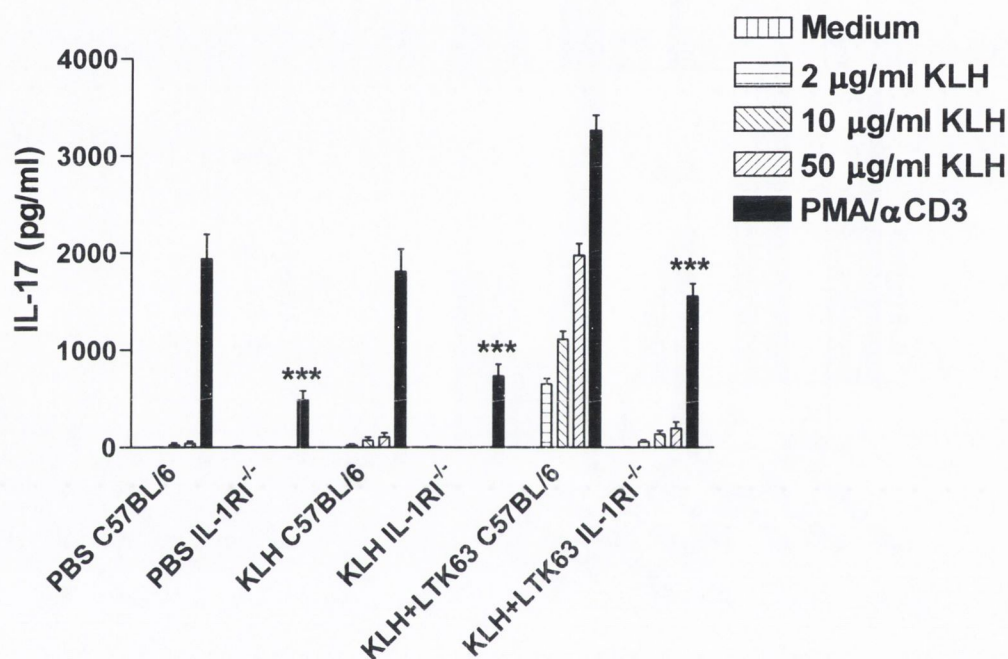


Figure 3.17: LTK63 promotes the induction of IL-17 producing T cells in an IL-1RI dependent manner. C57BL/6 and IL-1RI^{-/-} mice were immunised s.c with PBS, KLH (10 µg) or KLH and LTK63 (10 µg). Mice were sacrificed after 7 d and spleen cells re-stimulated ex vivo with KLH (2, 10 or 50 µg/ml). Supernatants were removed after 72 h and tested by ELISA for IL-17 production. PMA/αCD3 C57BL/6 versus PMA/αCD3 IL-1RI^{-/-}: ***, $P < 0.001$.

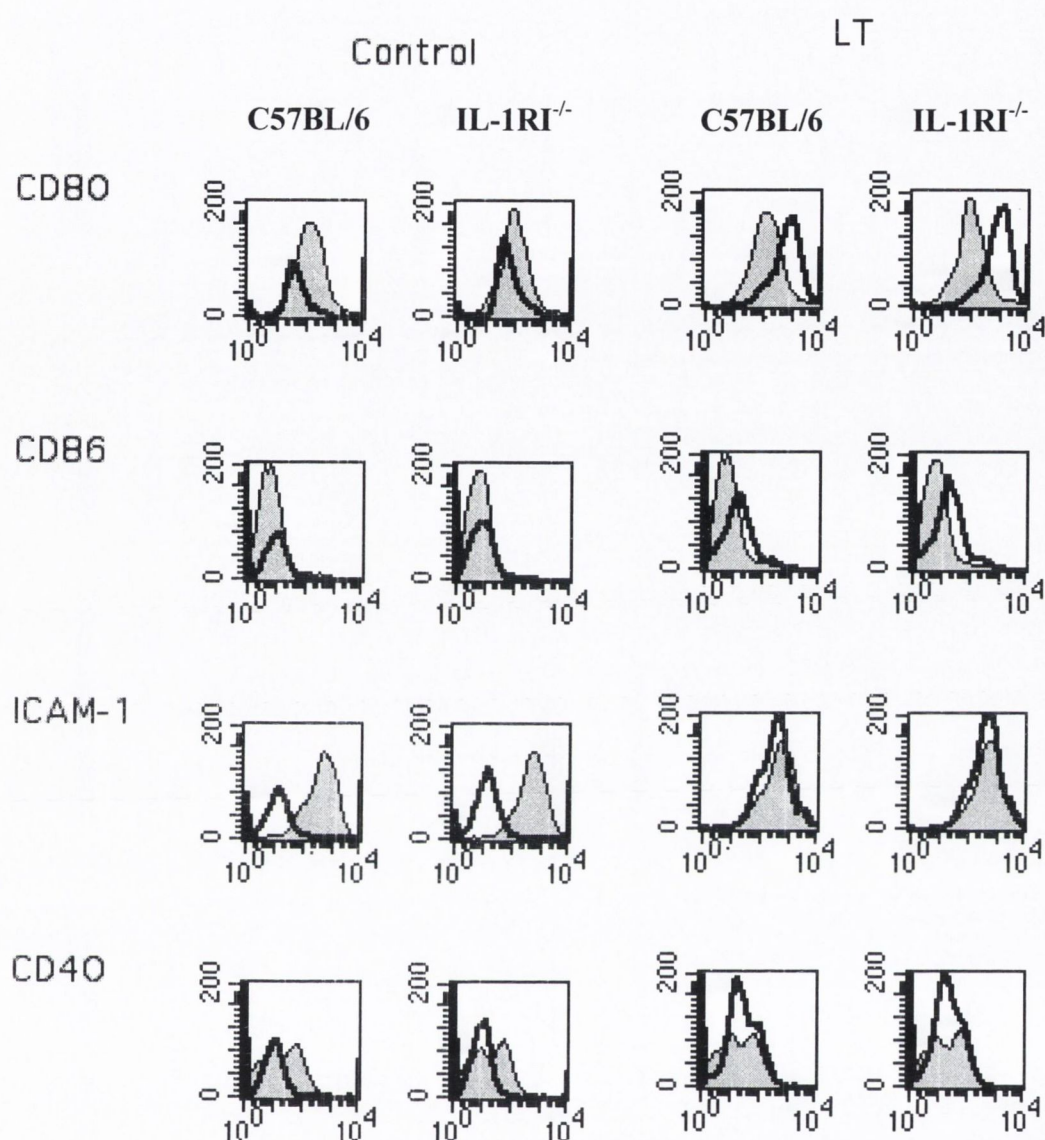


Figure 3.18: The ability of LT to mature DC does not require IL-1. BMDC from C57BL/6 and IL-1RI^{-/-} mice (1×10^6 cells/ml) were incubated with LT (1 μ g/ml) or medium alone for 24 h. Cells were then washed and stained with antibodies specific for CD80, CD86, ICAM-1, CD40 or with isotype matched controls (black line, control column). Results from flow cytometric immunofluorescence analysis are shown for treated (black line) compared to untreated (grey histogram) DC, gated on CD11c⁺ cells.

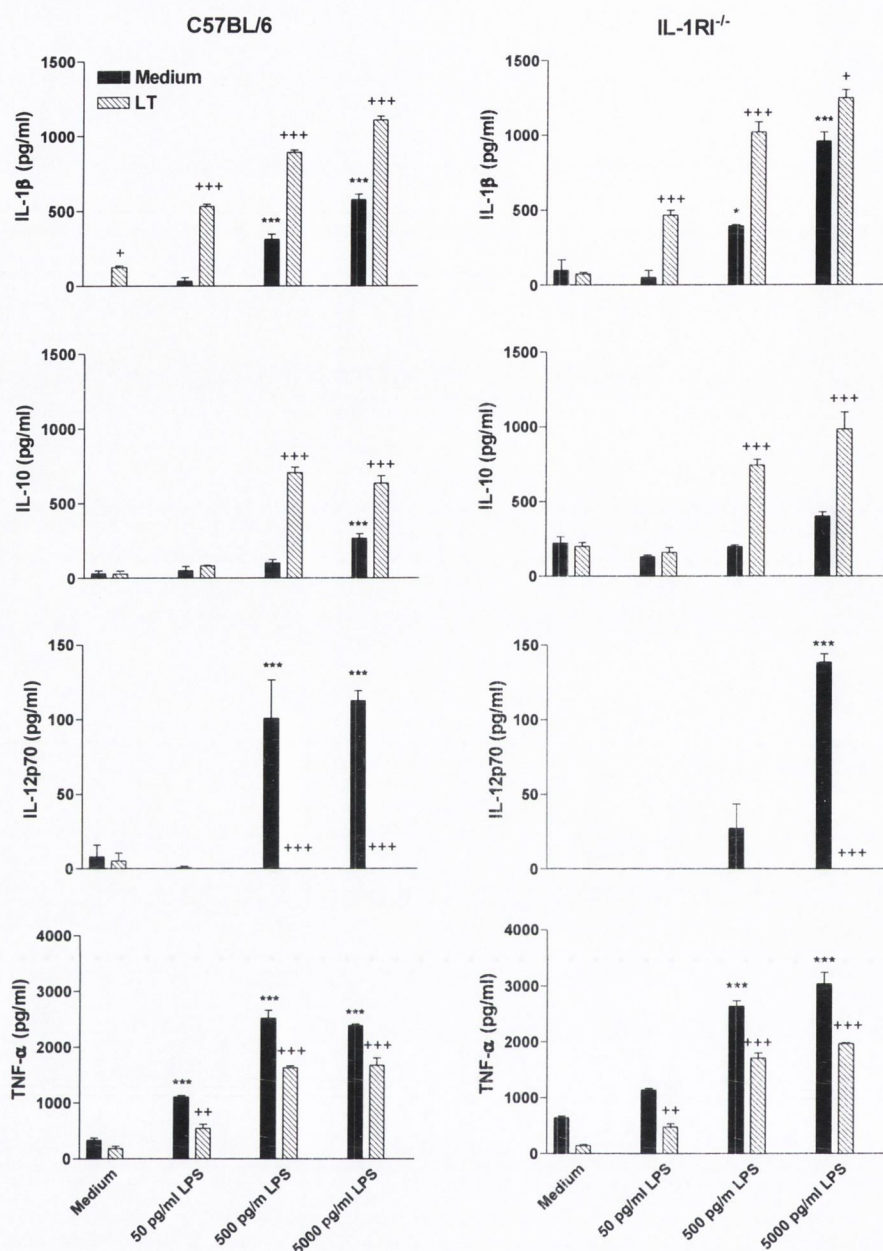


Figure 3.19: The ability of LT to enhance IL-1 β and IL-10 and inhibit IL-12 and TNF- α production by DC is not dependent on IL-1RI. BMDC from C57BL/6 and IL-1RI^{-/-} mice (5×10^5 cells/ml) were stimulated with LPS (50, 500 or 5000 pg/ml), LT (1 μ g/ml), LT 6 h prior to the addition of LPS or with medium alone. Supernatants were removed after 24 h and tested by ELISA for IL-1 β , IL-10, IL-12p70 and TNF- α production. LPS versus medium alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Treatment + LPS versus LPS alone: +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$.

CHAPTER IV

A CRUCIAL ROLE FOR IL-1 IN THE DEVELOPMENT OF IL-17 PRODUCING T CELLS

4.1 INTRODUCTION

In higher eukaryotic organisms, the immune response to pathogens is manifested as a two-pronged attack, initially through the innate immune response and subsequently through adaptive immunity. The innate response senses danger and acts as the first line of defence against the invading organism, utilising soluble factors, including complement (Morgan *et al.*, 2005), in conjunction with innate immune cells, macrophages and dendritic cells (DC), to detect and mount a non-specific response against invading pathogens (Medzhitov, 2001). The innate immune cells produce a cascade of cytokines in response to pathogen stimulation. These include pro-inflammatory cytokines, such as IL-1 and IL-6, as well as T helper cell polarising cytokines, such as IL-10, IL-12p70 and IL-23, which generate regulatory T cells (Treg), T helper type 1 cells (Th1) and T cells which produce IL-17 (T_{IL-17}) respectively. Regulatory cytokine production is one of three signals provided by the activated innate cell in response to the invading organism, which leads to the priming of the adaptive immune response. The first signal provided by the innate immune response is the presentation of processed antigens loaded onto major histocompatibility complex (MHC) molecules on the surfaces of antigen presenting cells (APC). These MHC molecules interact with T cell receptors (TCR) expressed on the surface of T cells, an interaction which is strengthened by the second signal, provided through up-regulated expression of co-stimulatory makers, such as CD80 and CD86 on the innate cell, which interact with CD28 on the T cell surface, thus generating a specific immune response tailored toward the invading pathogen (Banchereau and Steinman, 1998).

There are two main categories of T cells, CD4⁺ T helper (Th) cells, which produce cytokines, promote differentiation and expansion of B and activate macrophages, and CD8⁺ cytotoxic T cells, which utilise cytotoxic pathways such as FAS-FAS ligand interactions, to induce apoptosis in infected cells (Ridge *et al.*, 1998).

T helper cells can be subdivided into specific subsets, Th1 cells, which produce IFN- γ and are generated primarily to respond to intracellular pathogens, and type 2 (Th2) cells, which produce IL-4, IL-5 and IL-13 and function to protect against extracellular pathogens. A further subset of T cells, termed Treg cells, act as regulators of other T cell responses. This subset includes natural and inducible Tregs, which utilise the suppressive cytokines IL-10, TGF- β or cell-to-cell contact mechanisms to regulate innate and adaptive immune responses (McGuirk and Mills, 2002; Taylor *et al.*, 2006). A fourth subset of T cells requires the IL-12 family member, IL-23, for development and produces the inflammatory cytokine, IL-17 (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005). This subset has proven difficult to define given that the expression of IL-17 is not limited to either of the conventional $\alpha\beta$ T cell subsets as unconventional $\gamma\delta$ T cells also produce IL-17 (Shibata *et al.*, 2007). Indeed various titles have been appropriated to these IL-17 producing cells, Th₁₇ (Mangan *et al.*, 2006), Th_{IL-17} (Iwakura and Ishigame, 2006) and T_{IL-17} (He *et al.*, 2006). Here, these cells will be referred to as T_{IL-17}, given that this title does not indicate that these IL-17 producing cells are solely helper T cells or the seventeenth set of T cells discovered. However, where CD4 T cells are specifically referred to in relation to IL-17 production, the cells are designated as Th_{IL-17} cells.

IL-23 is comprised of the common IL-12 family member, IL-12p40, in combination with a novel IL-12p19 subunit. Similarly the IL-23 receptor is comprised

of IL-12R β 1, common to most of the IL-12 family member receptors, in combination with IL-23R (Figure 1). T_{IL-17} cells have inflammatory properties mediated through production of IL-17, which recruits neutrophils to the site of infection and promotes production of chemokines and inflammatory cytokines including IL-1 and TNF- α . Th_{IL-17} cells are a distinct T cell lineage, independent of Th1 and Th2 cell subsets, and they do not share a common precursor with other T helper subtypes (Harrington *et al.*, 2005; Park *et al.*, 2005). Differentiation of these cells has been shown to depend upon the transcription factor Rar-associated orphan receptor C (RORC) expression, also referred to as ROR γ T (Ivanov *et al.*, 2006), while Th1 and Th2 cell differentiation is controlled by the transcription factors, T-bet and GATA3 respectively (Tato and O'Shea, 2006). Indeed the differentiation of T_{IL-17} cells is inhibited by the signature Th1 and Th2 cytokines, IFN- γ and IL-4 (Harrington *et al.*, 2005).

T_{IL-17} cells play a major role in the development of T cell mediated autoimmune diseases, where deregulation of the immune responses to self antigens leads to destruction of host tissues. Traditionally it had been believed that autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (MS), were mediated by Th1 cells, through the production of IFN- γ . However, studies on the autoimmune disease, experimental autoimmune encephalomyelitis (EAE), the mouse model for MS, showed that IFN- γ ^{-/-} mice had an increased disease susceptibility and severity (Krakowski and Owens, 1996). Furthermore, disease was exacerbated in mice deficient in the Th1-polarising cytokine, IL-12 (Cua *et al.*, 2003). T_{IL-17} cells also function in immunity to infection where it has been linked with the control and abatement of mycobacterial infections such as TB (Umemura *et al.*, 2007) and *Bordetella pertussis* infection (Higgins *et al.*, 2006).

Many of the experiments, which linked Th1 cells to the development of autoimmune disease, were performed in IL-12p40^{-/-} mice, prior to the discovery of IL-23. Subsequent experiments with IL-23^{-/-} (IL-12p19^{-/-}) mice demonstrated that it was this cytokine and not IL-12 that was responsible for the development of autoimmune disease (Cua *et al.*, 2003). Recent studies have also implicated the Treg polarising cytokine, TGF- β , in combination with the pro-inflammatory cytokine IL-6, and not IL-23, in the generation of T_{IL-17} cells (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006). However, these papers, which suggest that IL-23 is merely a co-factor in T_{IL-17} development, do not explain why IL-23^{-/-} mice do not develop autoimmune diseases.

T_{IL-17} cells may function in autoimmunity by promoting the induction of pro-inflammatory chemokines and cytokines, such as IL-1 β and TNF- α . It has been shown that IL-1 and TNF- α can mediate inflammatory pathology in many autoimmune diseases, and antibodies or receptor antagonists targeting these inflammatory cytokines have been used as therapeutics against autoimmune diseases (Badovinac *et al.*, 1998; Feldmann, 2002; Feldmann and Maini, 2002; Yang *et al.*, 2005). In the autoimmune disease, RA, IL-17-mediated induction of IL-1 has also been associated with the promotion of joint destruction (Koenders *et al.*, 2005). Furthermore, IL-1RI^{-/-} mice have a reduced incidence of EAE, demonstrating a role for IL-1 in the induction of inflammatory response in EAE (Schiffenbauer *et al.*, 2000). In addition, IL-1 β has been detected in MS lesions and persons with high ratios of IL-1 β to IL-1 receptor antagonist (IL-1Ra) are at a greater risk of having a relapse with relapse-onset MS than those with a low ratio (de Jong *et al.*, 2002). Furthermore, mutations in the IL-1 gene, which result in increased production of IL-1, have been linked with the development of early onset systemic juvenile idiopathic arthritis (JIA)

(Cimaz *et al.*, 2007; Pascual *et al.*, 2005), in which IL-1Ra has been used as a successful therapy.

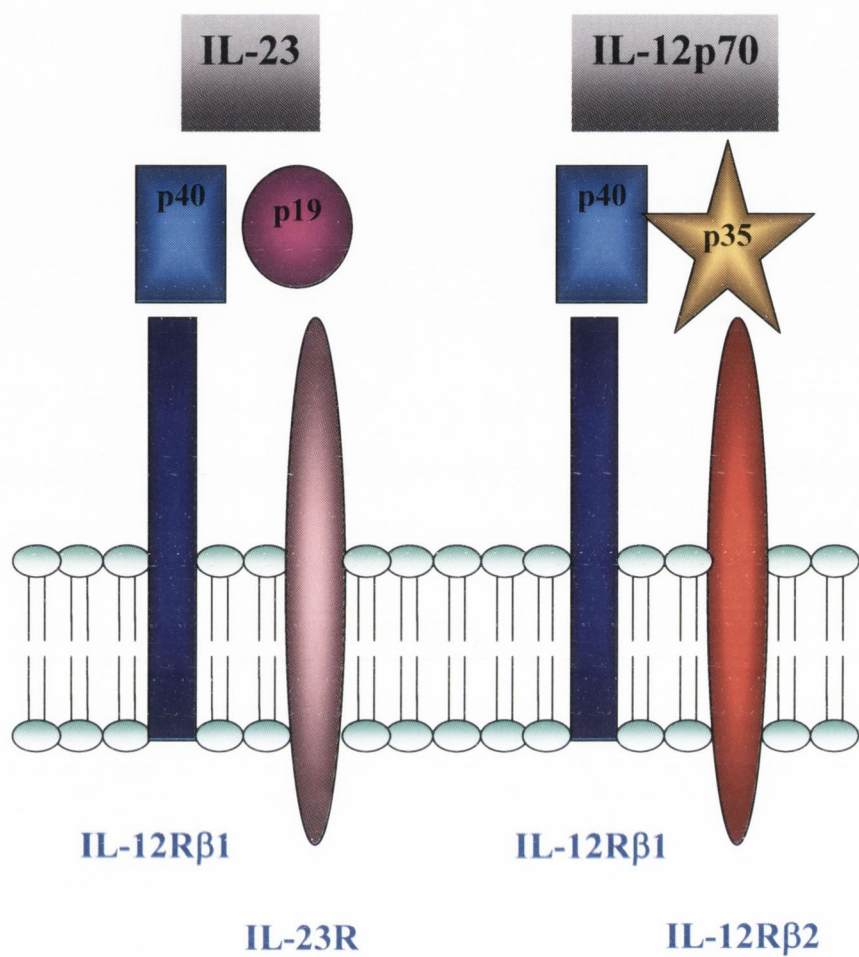


Diagram 4.1: IL-12 family members, IL-23 and IL-12p70, and their receptors

Adapted from Holscher C., Med Microbiol Immunol (2004).

4.2 RESULTS

4.2.1 IL-1RI is required for the induction of antigen-specific T_{IL-17} cells

During the course of experiments designed to examine the role of IL-1 in the efficacy of vaccine adjuvants, C57BL/6 and IL-1RI^{-/-} mice were immunised subcutaneously with KLH either alone or in combination with LPS. It was observed that draining lymph node cells from IL-1RI^{-/-} mice secreted significantly less IL-17 when compared with cells from C57BL/6 wild type mice after antigen re-stimulation in vitro (Fig. 4.1). This defect in cytokine production in IL-1RI^{-/-} mice was restricted to IL-17 production, as KLH-specific IFN- γ and IL-10 from mice immunised with KLH and LPS was similar in wild type and knock out mice. It should be noted, however, that IL-17 production was not completely absent in IL-1RI^{-/-} mice, as there was a low concentration of KLH-specific IL-17 induced by cells from IL-1RI^{-/-} mice immunised with KLH and LPS.

In addition to examining the role of IL-1RI in the induction of antigen-specific T_{IL-17} cells, lymph node cells from this experiment were also stimulated with PMA and anti-CD3 as a positive control. Consistent with the antigen-specific cytokine production, PMA and anti-CD3 stimulated IL-17 production was significantly reduced in lymph node cells from IL-1RI^{-/-} mice (Fig. 4.2). Although IFN- γ and IL-10 concentrations were also reduced, the effect on these cytokines was not as dramatic as that observed for IL-17 production (Fig. 4.2). PMA and anti-CD3 induced IL-10 in IL-1RI^{-/-} cells was reduced by only 20% compared with wild type cells. In contrast, IL-17 production by IL-1RI^{-/-} mice was reduced by > 95% compared with wild type mice.

4.2.2 IL-1 synergises with the T_{IL-17} polarising cytokine IL-23 to promote IL-17 production from T cells

The data shown in Fig. 1 indicate a role for IL-1RI, and therefore the cytokine IL-1, in the development of IL-17 producing T cells. However, previously it has been shown that T cells, which produce IL-17, are promoted by the IL-12 family member, IL-23 (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005). Therefore, we examined the role of IL-23 in IL-1-mediated IL-17 production. Spleen cells from C57BL/6 wild type and IL-1RI^{-/-} mice were stimulated with IL-1 α , IL-23, IL-1 α and IL-23 or with PMA and anti-CD3 and IL-17 production was quantified in supernatants after 3 days. IL-23 alone induced low concentrations of IL-17 from wild type spleen cells. However, in combination with IL-1 α the concentration of IL-17 produced was significantly enhanced. In contrast, IL-23 in combination with IL-1 α failed to stimulate IL-17 production by spleen cells from IL-1RI^{-/-} mice (Fig. 4.3). The synergy between IL-1 α and IL-23 for IL-17 production was further confirmed with the addition of IL-1Ra, an antagonist for IL-1 signalling. IL-1Ra abolished IL-17 production by C57BL/6 spleen cells (Fig. 4.4) when cells were co-stimulated with IL-1 and IL-23.

PMA and anti-CD3 induced IL-17 production by spleen cells from wild type mice, but failed to stimulate IL-17 production by spleen cells from IL-1RI^{-/-} mice (Fig. 4.3). IFN- γ was not induced by IL-1 α or IL-23 either alone or in combination. However, both C57BL/6 and IL-1RI^{-/-} spleen cells secreted IFN- γ in response to PMA and anti-CD3, although the concentrations were 20% lower in spleen cells from IL-1RI^{-/-} mice (Fig. 4.3).

Intracellular staining with anti-IL-17 and FACS analysis confirmed these results and demonstrated that stimulation of spleen cells with IL-1 and IL-23 alone

induced low frequencies of IL-17-producing CD3⁺ T cells but in combination synergised to promote relatively high frequencies (6.6%) of CD3⁺ T cells secreting IL-17, compared with 0.5% for cells stimulated with medium only (Fig. 4.5). The number of IL-17 secreting CD3⁺ T cells, induced by IL-1 and IL-23, was increased, by 25%, in the presence of anti-IFN- γ , which is in agreement with published reports that IFN- γ inhibits the induction of T_{IL-17} cells (Cua *et al.*, 2003; Murphy *et al.*, 2003). Purified CD3⁺ T cells from C57BL/6 mice also produced IL-17 in response to stimulation with IL-23 and IL-1 β (Fig. 4.6) in the presence of anti-CD3. However, neither IL-1 β nor IL-23 alone induced IL-17 production from these cells.

4.2.3 Kinetics of IL-17 production from CD3⁺ T cells

The previous experiments, demonstrating IL-1 and IL-23 synergy in promoting IL-17 production from spleen cells (Fig. 4.3, Fig. 4.4) and CD3⁺ T cells (Fig. 4.5, Fig. 4.6) had been carried out using 3-day stimulation. In order to examine the kinetics of IL-17 production, CD3⁺ cells from C57BL/6 spleens were cultured with IL-1 β and IL-23 for 12, 24, 48 and 72 hours. The results demonstrated that significant concentrations, of IL-17 were induced by IL-1 β and IL-23 stimulated CD3⁺ cells only at 48 and 72 hours post-activation (Fig. 4.7). Small quantities of IL-17 were detected at 24 hours post-stimulation, however the protein was undetectable in supernatants removed after 12 hours. CD3⁺ cells treated with medium alone did not induce IL-17 following 12, 24, 48 or 72 hours of culture (Fig. 4.7). It was also observed that IL-1 β and IL-23 could induce low but significant concentrations of IFN- γ from stimulated CD3⁺ cells at 72 h, while T cells stimulated with medium alone did not produce this cytokine (Fig. 4.7).

4.2.4 Both IL-1 α and IL-1 β are capable of synergising with IL-23 to induce IL-17 production from CD4⁺ and CD8⁺ T cells

There are many members of the IL-1 family of pro-inflammatory cytokines (Boraschi *et al.*, 1996), including the closely related members, IL-1 α and IL-1 β , both of who signal via IL-1RI. The previous experiments focused on either IL-1 α or IL-1 β to activate IL-17-producing T cells. However, given their mutual ability to signal via the IL-1RI and the observed role for IL-1RI in T_{IL-17} development (Fig. 4.1, Fig. 4.2) the roles of IL-1 α and IL-1 β were compared within the same experiment. CD4⁺ and CD8⁺ T cells from C57BL/6 mice were stimulated with either IL-1 α or IL-1 β alone or in combination with IL-23, and IL-17 production examined. Recent publications have reported that CD4⁺ cells are the primary source of IL-17 production in response to infection (Park *et al.*, 2005). Therefore, this experiment also aimed to examine the role of CD4⁺ and CD8⁺ subpopulations of CD3⁺ T cells for their ability to produce IL-17 in response to IL-1 and IL-23. It should be noted that IL-2 was also added to cultures in this experiment but subsequent experiments, which did not include this cytokine, found its presence to be unnecessary for IL-17 production following stimulation with IL-1 and IL-23. The results in Fig. 4.8 show that both IL-1 α and IL-1 β induced dose dependent production of IL-17 but only in combination with IL-23, with the highest dose of IL-1 α and IL-1 β analysed (1 ng/ml) resulting in the strongest synergy with IL-23 for IL-17 production. Furthermore, both CD4⁺ and CD8⁺ T cells were capable of responding to stimulation with IL-23 in combination with IL-1 α or IL-1 β for IL-17 production, although higher concentrations of IL-17 were detected in supernatants of CD4⁺ T cells following stimulation with IL-1 α and IL-23.

4.2.5 IL-23 alone induces the transcription of IL-17 and the IL-17 associated transcription factor ROR γ T mRNA

Previous studies have demonstrated that IL-17 production, induced by TGF- β and IL-6 (Ivanov *et al.*, 2006) was associated with activation of the transcriptional factor ROR γ T. Therefore the effect of IL-1 and IL-23 on ROR γ T expression was examined, together with IL-17 mRNA expression. CD3⁺ T cells from C57BL/6 mice were stimulated for 2 d with IL-1 and IL-23 alone and in combination, after which ROR γ T and IL-17 mRNA expression was quantified by real time PCR on RNA isolated from stimulated cells. IL-23, but not IL-1 stimulation of CD3⁺ T cells enhanced expression of IL-17 mRNA and the IL-17 associated transcription factor, ROR γ T (Fig. 4.9, Fig. 4.10). Co-incubation of IL-23 with IL-1 did not further enhance IL-23-induced IL-17 or ROR γ T mRNA expression.

4.2.6 IL-1 and IL-23 do not require anti-CD3 stimulation to induce IL-17 production

Previous studies on TGF- β and IL-6 induced IL-17 showed that co-stimulation with anti-CD3 and anti-CD28 was necessary for IL-17 production by CD4⁺ T cells (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). Therefore, the role of anti-CD3 in IL-1 and IL-23 induced IL-17 production was examined. CD3⁺ T cells from C57BL/6 mice were cultured with IL-1 and IL-23 in the presence and absence of anti-CD3. It was observed that in both the presence and absence of anti-CD3, IL-1 and IL-23 synergised to induce significant concentrations of IL-17 (Fig. 4.11). However, the concentration of IL-17 produced in the absence of anti-CD3 was significantly lower

than that induced in the presence of co-stimulation. Neither IL-1 nor IL-23 alone induced IL-17 production in either the presence or absence of anti-CD3 stimulation (Fig. 4.11).

4.2.7 IL-18, TNF- α and IL-6, but not IL-33, synergised with IL-23 to promote IL-17 production in the absence of anti-CD3 stimulation

IL-18 is a member of the IL-1 family and has been reported to have certain overlapping functions to those displayed by IL-1 α and IL-1 β (Debets *et al.*, 2000). Therefore, IL-18 was examined for its ability to promote IL-17 production in the presence of IL-23 stimulation.

Spleen cells from C57BL/6 and IL-1RI^{-/-} mice were cultured for 3 d in the presence of IL-18 with and without IL-23 and also with IL-1 and IL-23 as a positive control. Stimulation of spleen cells with IL-18 alone failed to induce IL-17 production. In contrast co-stimulation with IL-18 and IL-23 induced significant concentrations of IL-17 by spleen cells from wild type mice. However this synergy was only observed with the highest concentration of IL-18 tested (10 ng/ml) (Fig. 4.12). Small concentrations of IL-17 were also observed when C57BL/6 cells were stimulated with IL-23 in the presence of a lower concentration of IL-18 (1 ng/ml) although this was found to be not significantly above that produced by medium alone. IL-18 and IL-23-induced IL-17 production was partially reduced in spleen cells from IL-1RI^{-/-} mice although this concentration was not significantly different to that produced by spleen cells from C57BL/6 mice. In contrast, IL-17 induced by IL-1 in synergy with IL-23 was completely abrogated in spleen cells from IL-1RI^{-/-} mice (Fig. 4.11). Furthermore spleen cells from IL-1RI^{-/-} mice failed to secrete IL-17 in response

to PMA and anti-CD3, whereas spleen cells from wild type mice secreted significant concentrations of IL-17.

A more distant member of the IL-1 family, IL-33, which signals via ST2, a member of the IL-1R family, was also examined for its ability to induce IL-17 production in combination with IL-23. In the absence of anti-CD3 stimulation IL-33 failed to synergise with IL-23 for IL-17 production by spleen cells from either wild type or IL-1RI^{-/-} mice (Fig. 4.13). However, as previously observed IL-1 and IL-23 synergised to induce IL-17 production by spleen cells from C57BL/6 mice in the absence of anti-CD3 stimulation and this synergy was absent in spleen cells from IL-1RI^{-/-} mice. Stimulation of C57BL/6 and IL-1RI^{-/-} spleen cells with PMA and anti-CD3 induced IL-17 production, however the levels of IL-17 detected were higher in spleen cell supernatants from the wild type mice. In the presence of anti-CD3 co-stimulation, IL-33 alone induced significant concentrations of IL-17, as did IL-23, although this IL-17 production was not detected in spleen cells from IL-1RI^{-/-} mice (Fig. 4.14). Co-stimulation with IL-33 and IL-23 increased IL-17 production marginally and this IL-17 production was absent in spleen cells from IL-1RI^{-/-} mice. Addition of anti-CD3 enhanced IL-1 and IL-23-induced IL-17 production by spleen cells from C57BL/6 mice and this production was also abrogated in spleen cells from IL-1RI^{-/-} mice.

In the previous experiments it was shown that various IL-1 family members, in combination with IL-23, could promote IL-17 production. Therefore it was decided to examine the effect of other pro-inflammatory cytokines, such as TNF- α and IL-6 on IL-23-induced IL-17 production. Spleen cells from C57BL/6 and IL-1RI^{-/-} mice were cultured for three days in the presence of IL-23 alone or in combination with increasing concentrations of TNF- α , IL-6 or with PMA and anti-CD3. Spleen cells

stimulated with IL-23 or the highest dose of TNF- α examined, 1 ng/ml, alone produced small concentrations of IL-17 although these concentrations were not significantly above those produced by medium alone (Fig. 4.15). However, co-incubation of spleen cells from wild type mice with IL-23 and increasing concentrations of TNF- α enhanced IL-17 production, which at concentrations of 0.1 or 1 ng/ml TNF- α was significantly greater than that observed with medium alone. In contrast, the combination of IL-23 and TNF- α failed to induce IL-17 production in spleen cells from IL-1RI^{-/-} mice (Fig. 4.15). As previously demonstrated, stimulation of spleen cells from wild type C57BL/6 mice with PMA and anti-CD3 induced IL-17 production, which was significantly reduced in spleen cells from IL-1RI^{-/-} mice.

IL-6, alone, was unable to induce IL-17 production by spleen cells from C57BL/6 or IL-1RI^{-/-} mice. However, the combination of IL-23 and IL-6 did synergise to promote IL-17 production from wild type spleen cells, although only at the highest dose of IL-6 examined (10 ng/ml) (Fig. 4.16). In contrast, spleen cells from IL-1RI^{-/-} mice did not produce IL-17 when stimulated with IL-6 and IL-23. Similarly the combinations of IL-1 with IL-23 and PMA with anti-CD3 failed to induce IL-17 production by spleen cells from IL-1RI^{-/-} mice, while wild type cells stimulated in the same manner produced significant concentrations of IL-17 (Fig. 4.16).

4.2.8 LPS and Pam3Cys synergise with IL-23 to promote IL-17 production from spleen cells in an IL-1RI-dependent manner

TLR agonists have previously been shown to be potent stimulators of pro-inflammatory cytokines, inducing IL-1, IL-6 and TNF- α production from dendritic

cells (DC) and macrophages (O'Neill, 2004; Wan and Bramson, 2001). Having already demonstrated that IL-1, TNF- α and IL-6 can synergise with IL-23 to induce IL-17 production, it was decided to examine the effect of TLR agonists on IL-17 production. Spleen cells from wild type and IL-1RI^{-/-} mice were incubated with the TLR 4, 2 and 9 agonists, LPS, Pam3Cys and CpG respectively, alone or in the presence of IL-23. None of the TLR ligands alone or IL-23 or IL-1 alone, were capable of inducing IL-17 production from spleen cells. However, the TLR4 and TLR2 agonists, LPS and Pam3Cys, like IL-1 were capable of synergising with IL-23 to promote concentrations of IL-17 significantly above those induced by IL-23 alone from spleen cells (Fig. 4.17). Indeed the synergy between LPS and IL-23 was comparable with that displayed by IL-1 and IL-23. Interestingly, CpG, which binds to TLR9, was unable to promote IL-17 in the presence of IL-23. The synergy between IL-23 and LPS, Pam3Cys and IL-1 for IL-17 induction was completely absent in spleen cells from IL-1RI^{-/-} mice (Fig. 4.17). This is consistent with previous studies, which demonstrated that the induction of IL-17, by TLR ligands, was mediated via IL-1 production from DC or macrophages (Higgins *et al.*, 2006).

4.2.9 TNF- α , but not IL-1 and IL-23, up-regulates the expression of IL-1RI

One explanation for the IL-1RI dependent effect of TNF- α , IL-1 or IL-23-induced IL-17 is that it may enhance IL-1RI expression, especially given that TNF- α has already been associated with regulation of IL-1RI (Friedman, 2001). Therefore, the effects TNF- α and IL-23 on IL-1RI expression were examined. Spleen cells from C57BL/6 mice were stimulated with IL-1 and IL-23 or with TNF- α for 3 days. Immunofluorescent analysis using anti-IL-1RI antibody revealed that TNF- α enhanced

the surface expression of the IL-1RI on both CD4⁺ and CD8⁺ T cells (Fig. 4.18). In contrast IL-1 and IL-23 had no effect on IL-1RI expression (Fig. 4.18).

4.2.10 IL-6 and TGF- β induced IL-17 production by spleen cells is IL-1RI-dependent

During the course of these experiments, papers by Mangan *et al* and Bettelli *et al* were published, demonstrating that the Treg cell associated cytokine, TGF- β , in combination with IL-6, promoted IL-17 production by CD4⁺ T cells, and largely dismissed the role of IL-23 in the development of IL-17 producing T cells (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). Therefore, the role of IL-1 in TGF- β and IL-6 induced IL-17 production by T cells was examined. Following the experimental protocol used by the Mangan and Bettelli groups, which included co-stimulation with anti-CD3 and anti-CD28, wild type and IL-1RI^{-/-} spleen cells were stimulated with TGF- β and IL-6, in the presence and absence of anti-CD3 and anti-CD28. Analysis of IL-17 concentrations in supernatants after three days revealed that IL-6 alone, but not TGF- β , could induce IL-17 production from wild type spleen cells and that IL-6-induced IL-17 was significantly increased in the presence of TGF- β (Fig. 4.19). However IL-17 induced by IL-6 alone or in combination with TGF- β was significantly reduced in spleen cells from IL-1RI^{-/-} mice (Fig. 4.19). Thus providing further evidence of the importance of IL-1 in promoting IL-17 production.

These experiments showed significant induction of IL-17 production from wild type spleen cells following incubation with TGF- β and IL-6, when cells were co-stimulated with anti-CD3 and anti-CD28. However, the data in Fig. 4.11 demonstrated that IL-1 and IL-23 do not require anti-CD3 or anti-CD28 co-

stimulation for the promotion of IL-17 production. Therefore, spleen cells from C57BL/6 and IL-1RI^{-/-} mice were stimulated with IL-6 and TGF- β or IL-1 and IL-23 without anti-CD3 and anti-CD28. IL-17 was induced following stimulation of spleen cells from wild type mice with IL-1 and IL-23 and this IL-17 production was undetectable in spleen cell supernatants from IL-1RI^{-/-} mice (Fig. 4.20). In contrast, TGF- β and IL-6 alone and in combination failed to induce IL-17 in the absence of anti-CD3 and anti-CD28 co-stimulation. This finding was confirmed by intracellular staining for IL-17, TGF- β and IL-6 failing to increase IL-17 producing cells above that of medium treated cells alone (Fig. 4.21). In contrast, stimulation of spleen cells from C57BL/6 mice with IL-1 and IL-23, in the absence of co-stimulation with anti-CD3 and anti-CD28, increased the frequency of IL-17 producing T cells to 30% compared with 12.6% for medium treated cells (Fig. 4.21). In this experiment, stimulation of spleen cells with IL-1 and IL-23 in the presence of anti-CD3 had only a small effect on IL-17 production, whereas TGF- β and IL-6 did stimulate significant IL-17 in the presence of anti-CD3 stimulation (Fig. 4.21).

4.2.11 In the absence of anti-CD3 stimulation, IL-1 and IL-23 increase the production of IL-17 from CD3⁺ but not CD4⁺ or CD8⁺ T cells

The results in Fig. 4.8 demonstrated that both CD4⁺ and CD8⁺ T cells were capable of responding to IL-1 and IL-23 stimulation to produce IL-17 in the presence of anti-CD3. Furthermore, the data in Fig. 4.11 showed that IL-1 and IL-23 could synergise to induce IL-17 production from wild type spleen cells in the presence and absence of anti-CD3 stimulation, although the concentrations were significantly lower in the absence of anti-CD3 stimulation. In order to identify the T cells that secrete IL-

17 in the absence of anti-CD3 stimulation, spleen cells from C57BL/6 mice were cultured with medium alone or with IL-1 and IL-23 in the presence and absence of anti-CD3, these cells were then stained for intracellular IL-17 and surface CD3, CD4 and CD8 α expression. IL-1 and IL-23 enhanced the frequency of IL-17-secreting CD3⁺ cells, to 27% compared with 6% for cells treated with medium alone (Fig. 4.22 A, B). In contrast, IL-1 and IL-23 failed to enhance the frequency of IL-17-secretion by CD4⁺ or CD8 α ⁺ T cells (Fig. 4.22 D, F).

4.2.12 $\gamma\delta$ T cells produce IL-17 in response to IL-1 and IL-23 stimulation

The results in Fig. 4.22 demonstrated that CD3⁺ T cells, but not CD4⁺ or CD8 α ⁺ T cells, secreted IL-17 in response to IL-1 and IL-23 in the absence of anti-CD3 co-stimulation. This suggests that a population of innate CD3⁺ T cells, such as $\gamma\delta$ T cells or NK T cells, may be responsible for IL-1 and IL-23 induced IL-17 production. Therefore, intracellular cytokine staining was used to determine the possible role of $\gamma\delta$ T cells in IL-17 production. The results in Fig. 4.23 show that 31% of $\gamma\delta$ T cells secreted IL-17 following stimulation of spleen cells with IL-1 and IL-23, whereas only 10% of $\gamma\delta$ T cells produce IL-17 when incubated with medium alone. NK T cells were also examined, but they were found not to produce IL-17 in response to stimulation with IL-1 and IL-23.

These results were confirmed by culturing FACS purified $\gamma\delta$ ⁺ T cells ($\gamma\delta$), and $\gamma\delta$ -depleted spleen cells, with IL-1 and IL-23 alone and in combination, in the presence and absence of anti-CD3 stimulation. In the absence of anti-CD3 $\gamma\delta$ ⁺ T cells, but not $\gamma\delta$ -depleted cells, produced IL-17 following stimulation with IL-1 and IL-23

at concentrations significantly above medium treated cells. $\gamma\delta^+$ T cells did not secrete IL-17 in response to IL-1 or IL-23 alone in the absence of anti-CD3 co-stimulation (Fig. 4.24). However, in the presence of anti-CD3 stimulation, $\gamma\delta$ T cells produced concentrations of IL-17 in response to stimulation with IL-1 and IL-23 alone, which were significantly higher than produced in response to anti-CD3 alone (Fig. 4.24). This IL-17 production was significantly increased when the $\gamma\delta^+$ T cells were stimulated with the combination of IL-1 and IL-23. $\gamma\delta$ -depleted spleen cells were capable of producing IL-17 following stimulation with the combination IL-1 and IL-23 in the presence of anti-CD3, and this concentration was significantly higher than that induced by $\gamma\delta$ depleted cells treated with anti-CD3 alone. However, IL-1 and IL-23-induced IL-17 production from $\gamma\delta$ -depleted spleen cells was significantly lower than concentrations of IL-17 induced by IL-1 and IL-23 from $\gamma\delta^+$ T cells (Fig. 4.24). These results were confirmed at the mRNA level after stimulation of $\gamma\delta^+$ T cells with IL-1 and IL-23, increased expression of IL-17 mRNA (Fig. 4.25 A). In contrast, the $\gamma\delta$ -depleted spleen cells did not respond to IL-1 and IL-23 stimulation and expressed 95% less IL-17A mRNA compared with $\gamma\delta^+$ T cells (Fig. 4.25 B). These data indicate that $\gamma\delta^+$ T cells respond to stimulation by IL-1 and IL-23 to produce IL-17, and may therefore be referred to as $\gamma\delta$ T_{IL-17} cells. The role of $\gamma\delta^+$ T cells in IL-1 and IL-23-induced innate IL-17 production was confirmed using cell from $\gamma\delta^{-/-}$ mice. In the absence of anti-CD3 co-stimulation spleen cells from $\gamma\delta^{-/-}$ mice produced significantly less IL-17 when stimulated with IL-1 and IL-23 compared with that produced by spleen cells from C57BL/6 mice (Fig. 4.26). Spleen cells from $\gamma\delta^{-/-}$ mice were capable of producing small concentrations of IL-17 in response to stimulation with either IL-1 or IL-23 in the presence of anti-CD3 although these concentrations were not

significantly above those produced after stimulation with anti-CD3 alone (Fig. 4.26). However, co-incubation with IL-1 and IL-23 significantly increased IL-17 production by these spleen cells. In the presence of anti-CD3, IL-1, IL-23 or IL-1 and IL-23 induced significantly higher concentrations of IL-17 by spleen cells from C57BL/6 mice than those produced by spleen cells from $\gamma\delta^{-/-}$ mice (Fig. 4.26). This result was confirmed by comparing purified CD3⁺ T cells with CD3⁺ T cells depleted of $\gamma\delta$ T cells. In the absence of anti-CD3 stimulation, CD3⁺ T cells produced significant concentrations of IL-17 in response to IL-1 and IL-23 co-stimulation but not in response to either IL-1 or IL-23 alone (Fig. 4.27). In contrast, $\gamma\delta$ depleted CD3⁺ T cells did not produce significant concentrations of IL-17 in response to IL-1, IL-23 or IL-1 and IL-23 stimulation. In the presence of anti-CD3 stimulation, CD3⁺ T cells produced small concentrations of IL-17 in response to both IL-1 and IL-23 alone, although these concentrations were found not to be significantly higher than those induced by anti-CD3 alone. $\gamma\delta$ depleted CD3⁺ T cells did not induce IL-17, in response to IL-23 alone, but did produce small quantities of IL-17 in response to IL-1 stimulation in the presence of anti-CD3. This IL-1 induced IL-17 was not significantly higher than that induced following stimulation of $\gamma\delta$ depleted CD3⁺ T cells with anti-CD3 alone. Stimulation of both CD3⁺ T cells and $\gamma\delta$ depleted CD3⁺ T cells with the combination of IL-1 and IL-23 induced significant concentrations of IL-17 in the presence of anti-CD3 stimulation. However, IL-17 production by CD3⁺ T cells was significantly higher than that produced by $\gamma\delta$ depleted CD3⁺ T cells (Fig. 4.27).

In addition to promoting IL-17 production, IL-1 stimulated the proliferation of $\gamma\delta^{+}$ T cells, especially in the presence of anti-CD3 stimulation. This IL-1-induced proliferation was enhanced on co-stimulation with IL-23 (Fig. 4.28). However, neither

IL-1 nor IL-23 were capable of inducing significant proliferation of the $\gamma\delta$ -depleted spleen cells (Fig. 4.28).

4.2.13 $\gamma\delta$ T cells condition CD4/CD8 T cells to produce IL-17 via BMDC

$\gamma\delta$ T cells are located primarily at mucosal sites, such as the lung and lower intestine, and act as a link between innate and adaptive immune responses. These $\gamma\delta$ T cells express TLR and are capable of responding to pathogens, by secreting IFN- γ and activating other T cell populations (Kaufmann, 1996; Umemura *et al.*, 2007). Therefore, the role of IL-1 and IL-23 activated $\gamma\delta^+$ T cells in stimulating other T cell responses was examined. Since DC play a major role in directing T cell responses, the role of DC in $\gamma\delta$ T_{IL-17} cell activation of CD4⁺ and CD8⁺ T cells was examined. $\gamma\delta^+$ T cells were FACS sorted from C57BL/6 spleen cells and incubated with medium or IL-1 and IL-23 for three days. Stimulated $\gamma\delta$ T cells were then transferred to a fresh well containing FACS sorted CD4⁺ and CD8 α ⁺ T cells, with or without bone marrow-derived DC. The stimulated $\gamma\delta$ T cells were separated from the BMDC, CD4⁺ and CD8 α ⁺ cells by a nylon mesh and incubated for three days. Supernatants were then collected and tested for IL-17 and IFN- γ production by ELISA. CD4⁺ and CD8 α ⁺ T cells alone did not produce IL-17 nor did they produce the cytokine when stimulated with $\gamma\delta$ T cells or BMDC (Fig. 4.29). However, IL-17 was produced by CD4⁺ and CD8 α ⁺ T cells following co-incubation with $\gamma\delta^+$ T cells, which had been primed with IL-1 and IL-23 and this concentration of IL-17 was found to be significantly above that produced by CD4⁺ and CD8 α ⁺ T cells alone. IL-17 produced by CD4⁺ and CD8 α ⁺ T cells primed with $\gamma\delta$ T_{IL-17} cells was significantly increased when DC were

added to the co-culture (Fig. 4.29). $CD4^+$ and $CD8\alpha^+$ T cells induced a small concentration of IFN- γ by themselves, which were not found to be significant. This IFN- γ production was, however, significantly increased when cells were co-incubated with DC. $\gamma\delta$ T cells decreased $CD4^+$ and $CD8\alpha^+$ induced IFN- γ produced but only in the absence of DC (Fig. 4.29).

4.2.14 IL-17 promotes IL-23, IL-1 β and IL-12p40 production from BMDC

The data in Fig. 4.29 demonstrated a link between $\gamma\delta$ T_{IL-17} cells and DC in the promotion of IL-17 production by $CD4^+$ and $CD8\alpha^+$ T cells. It is possible that IL-17 produced by IL-1 and IL-23 activated $\gamma\delta$ T_{IL-17} cells to promote inflammatory cytokine production by DC, which in turn enhanced IL-17 production from conventional $\alpha\beta^+$ T cells. DC were therefore stimulated with IL-17, and concentrations of IL-23, IL-12p70, IL-6 and IL-1 β determined in the supernatants after 24 hours. DC produced IL-23 and IL-1 β but not IL-12p70 or IL-6 in response to IL-17 stimulation. The highest concentrations of IL-1 β and IL-23 were produced in response to low concentrations of IL-17 (Fig. 4.30).

4.2.15 Pro-inflammatory cytokine stimulation of BMDC induces IL-6 and IL-23 production

The results in Figs. 4.3, 4.15, 4.16 and 4.23 suggested a role for IL-1 β , TNF- α and IL-6, in the stimulation of IL-17 production by spleen cells and $\gamma\delta^+$ T cells. Since DC secrete these cytokines and were further required for IL-1 and IL-23 to

stimulate $\gamma\delta^+$ T cells to activate $CD4^+$ and $CD8^+$ T cells, these cytokines were therefore examined for their ability to activate BMDC. IL-1 β induced the production of IL-6 and IL-23 from BMDC. IL-6 was induced in a dose dependent manner with the highest concentration of IL-6 produced in response to the highest concentration of IL-1 β examined. However the production of IL-23 from BMDC in response to IL-1 β was greatest when BMDC were stimulated with the lowest dose of IL-1 β examined (1 ng/ml) (Fig. 4.31). Stimulation of BMDC with either TNF- α or IL-6 induced IL-23 and IL-1 β production (Fig. 4.32, Fig. 4.33), while TNF- α also induced IL-6 production by DC. Induction of IL-1 β and IL-6 by TNF- α was in a dose dependent manner with highest concentrations of cytokine produced in response to stimulation with the highest dose of TNF- α examined (50 ng/ml). However, the highest concentration of IL-23 was induced with the lowest dose of TNF- α and IL-6 examined (0.5 ng/ml and 1 ng/ml respectively) (Figs. 4.32, 4.33), this pattern was also found for IL-6 induced IL-1 β production from DC. IL-1 β , TNF- α and IL-6 did not induce IL-12p70 production from DC.

4.2.16 IL-1RI is required for the development of severe EAE

IL-1 has previously been implicated in the development of various autoimmune diseases, with monoclonal antibodies against IL-1 and IL-1Ra used as treatments for rheumatoid arthritis (RA) and multiple sclerosis (MS) (Badovinac *et al.*, 1998; Yang *et al.*, 2005). The data from this study have demonstrated a role for IL-1 in the development of IL-17 producing cells. It has previously been shown that T_{IL-17} cells mediate inflammation during the development of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), a mouse model for MS

(Chen *et al.*, 2006). Therefore, the role of IL-1 in the development of EAE was determined. C57BL/6 mice were immunised with myelin oligodendrocyte glycoprotein (MOG) in combination with complete Freund's adjuvant (CFA) and pertussis toxin (PT). Mice began displaying symptoms of EAE 10 days later, with disease severity increasing thereafter and peaking at day 20-post immunisation. The incidence of disease in C57BL/6 mice was 100%, with an average disease severity score of 4 (Fig. 4.34). In contrast, the incidence of EAE was only 6.7% in IL-1RI^{-/-} mice, with only 1 mouse out of 15 developing EAE. The day of onset of disease in the IL-1RI^{-/-} mouse was also much delayed with symptoms not developing until 22 days post induction of EAE.

4.2.17 Induction of antigen-specific T_{IL-17} cells during EAE is dependent upon functional IL-1RI

Next, the possibility that the reduced severity of EAE in IL-1RI^{-/-} mice was associated with the failure to induce T_{IL-17} cells was examined. Spleen cells from C57BL/6 and IL-1RI^{-/-} mice immunised with MOG, CFA and PT as described in Fig. 4.34 were re-stimulated *in vitro* with MOG for 3 days and cytokine production determined by ELISA. There were no significant differences between MOG-specific IL-6, TNF- α and IL-10 production or cell proliferation between spleen cells from wild type and IL-1RI^{-/-} mice (Fig. 4.35). Although MOG-specific IL-6 was higher in spleen cells from IL-1RI^{-/-} mice, this was not significantly different to that observed in supernatants of antigen-stimulated spleen cells from C57BL/6 mice. In contrast, significantly lower MOG-specific IFN- γ and IL-17 concentrations were found in supernatants of antigen-stimulated spleen cells from IL-1RI^{-/-} mice when compared

with C57BL/6 mice (Fig. 4.35). MOG-specific IL-17 production was almost completely absent in spleen cells from IL-1RI^{-/-} mice. In contrast MOG-specific IFN- γ production by spleen cells from IL-1RI^{-/-} mice was only reduced by 50%. The significantly lower concentration of MOG-specific IL-17 produced by spleen cells from IL-1RI^{-/-} mice following induction of EAE was consistent with significantly lower serum IL-17 concentrations in IL-1RI^{-/-} mice compared with C57BL/6 mice (Fig. 4.36).

4.2.18 IL-1RI is required up-stream of T cell activation in the induction of EAE

The role of IL-1 in EAE has traditionally been associated with inflammation down stream of T cell activation. However, the experiments described in this study have demonstrated a role for IL-1 in the development of T_{IL-17} cells. Therefore the role of IL-1 up-stream of T cell activation was determined in the development of EAE. EAE was induced in C57BL/6 mice and once these mice had reached grade 4 disease severity, T cells were isolated from spleens. These wild-type MOG-specific T cells were then adoptively transferred into naïve IL-1RI^{-/-} mice and disease progression monitored daily. IL-1RI^{-/-} mice, which received MOG-specific T cells, developed EAE. The symptoms of EAE in these mice were first detected at nine days post-transfer peaking at 22 days-post transfer with 100% incidence of disease (Fig. 4.37). The development of EAE following transfer of wild type MOG-specific T cell into IL-1RI^{-/-} mice, correlated with MOG-specific IL-17 production by spleen cells from IL-1RI^{-/-} mice (Fig. 4.38).

4.2.19 Development of EAE in IL-1RI^{-/-} mice requires transfer of both wild type $\alpha\beta^+$ and $\gamma\delta^+$ T cells

The data in Fig. 4.37 demonstrated a role for T cells and also functional IL-1RI on T cells in the development of EAE however, the data in Fig. 4.23 showed that $\gamma\delta$ T cells were the main cells which secrete IL-17 in response to stimulation with IL-1 and IL-23. Therefore it was examined whether functional IL-1RI was required on all T cells or only $\gamma\delta$ T cells for the development of EAE. $\gamma\delta$ T cells and CD3⁺ T cells were purified from the spleens of wild type C57BL/6 mice, and adoptively transferred into two separate groups of IL-1RI^{-/-} mice. These mice were subsequently immunised, 24 hours after transfer, for the induction of EAE. Control groups included C57BL/6 and IL-1RI^{-/-} mice, which did not receive transferred cells. Wild-type mice began to display symptoms of EAE 7 days post immunisation, with maximal disease severity observed at day 10 post immunisation. Neither IL-1RI^{-/-} mice, nor IL-1RI^{-/-} mice, which received wild-type $\gamma\delta$ T cells, developed EAE up to 21 days post immunisation. However, IL-1RI^{-/-} mice, which received wild-type CD3⁺ T cells prior to immunisation with MOG, CFA and PT did develop EAE, although the day of onset was delayed compared with that displayed by wild type mice. The incidence of disease in these IL-1RI^{-/-} mice was 100% and was significantly higher compared with that displayed by IL-1RI^{-/-} mice transferred with wild type $\gamma\delta$ T cells (Fig. 4.39). This demonstrates that functional IL-1RI is required on T cells for the development of EAE in IL-1RI^{-/-} mice but that IL-1RI expression on $\gamma\delta$ T cells alone cannot induce EAE in IL-1RI^{-/-} mice. However, this does not rule out a role for $\gamma\delta$ T cells in the development of EAE altogether, Fig. 4.29 suggests that $\gamma\delta$ T cells may promote the production of IL-17 by conventional $\alpha\beta$ T cells via DC. This indicates that $\gamma\delta$ T cell could promote

$\alpha\beta$ T cells in the development of EAE. Therefore the role of $\gamma\delta$ T cells in the priming of $\alpha\beta$ T cells in vivo for the development of EAE was determined. For this experiment $CD3^+$ T cells were isolated from C57BL/6 spleen cells, and transferred directly into IL-1RI^{-/-} mice ($CD3^+$). A second group of IL-1RI^{-/-} mice were transferred with $\gamma\delta$ T cell depleted $CD3^+$ T cells ($CD3^+ \gamma\delta^-$) from C57BL/6 mice. Twenty-four hours post cell transfer mice were immunised with MOG, CFA and PT for the development of EAE. MOG, CFA and PT immunised C57BL/6 and IL-1RI^{-/-} mice, which did not receive cells, acted as positive and negative controls respectively. Symptoms of EAE were first detected in C57BL/6 mice at day-10 post immunisation, with 100% disease incidence, peaking at day 16 post disease induction (Fig. 4.40). IL-1RI^{-/-} mice, which received $CD3^+$ T cells, developed EAE, with symptoms of EAE first observed at day 16 post disease induction. EAE in mice that received total $CD3^+$ T cells was significantly greater than that observed in IL-1RI^{-/-} mice (Fig. 4.40) at day 22 post immunisation. However, both IL-1RI^{-/-} mice, which did not receive transfer of cells, and IL-1RI^{-/-} mice, which received $\gamma\delta$ -depleted $CD3^+$ T cells, developed EAE with symptoms first detected at day 23 post immunisation. This data demonstrates that that $\gamma\delta$ T cells prime $\alpha\beta$ T cells to significantly increase onset of EAE in IL-1RI^{-/-} mice.

4.3 DISCUSSION

IL-17 has been identified as a crucial mediator of inflammation in autoimmune diseases, such as multiple sclerosis (MS) and rheumatoid arthritis (RA) (Koenders *et al.*, 2005; Koenders *et al.*, 2005; Park *et al.*, 2005). The role of IL-17 in autoimmune diseases is attributed to neutrophil recruitment, and the induction of pro-inflammatory cytokines, IL-1 and TNF- α (Andoh *et al.*, 2005; Koenders *et al.*, 2005). It is well established that IL-1 and TNF- α play a major role in autoimmune diseases with pro-inflammatory therapies that inhibit IL-1 and TNF- α being used successfully against a number of autoimmune diseases (Eriksson *et al.*, 2003; McCulloch *et al.*, 2006). It has been assumed that IL-1 and TNF- α act downstream of T cell activation in the development of autoimmune disease. However, findings of this study suggest that this may not be the case, since it was found that functional IL-1RI was required for the development of antigen-specific and PMA and anti-CD3 induced IL-17 producing T cells. Development of other T cell subsets, such as Th1, Th2 and IL-10 producing T cells, did not require IL-1RI, demonstrating a T_{IL-17} cell specific defect. This suggested that IL-1 is required upstream for T_{IL-17} activation as well as downstream to mediate inflammation.

The development of T_{IL-17} cells has already been associated with the IL-12 family member, IL-23 (Langrish *et al.*, 2005; Oppmann *et al.*, 2000). Having demonstrated that IL-1RI was also required, this study examined the possible synergy between IL-1 and IL-23 in the development of T_{IL-17} cells. It was found that, in the absence of IL-1, IL-23 was unable to induce IL-17 production from spleen cells. However co-stimulation of spleen cells with both IL-23 and IL-1 lead to the induction of IL-17 at concentrations 100 fold higher than that observed with IL-23 alone. The

failure of spleen cells from IL-1RI^{-/-} mice to secrete IL-17 in response to IL-1 and IL-23 stimulation was not a general defect in these mice, since IL-17 was produced following stimulation with PMA and anti-CD3. However, the concentration of IL-17 produced by cells from IL-1RI^{-/-} mice was significantly less compared with those produced by cells from wild type mice. Purified CD3⁺ T cells were also found to secrete IL-17 in response to IL-1 and IL-23, in an IL-1RI dependent manner. However, unlike spleen cells, CD3⁺ T cells were unable to produce IL-17 in response to stimulation with IL-23 alone. Therefore IL-23-induced IL-17, produced by spleen cells from C57BL/6 mice, may have resulted from synergy with IL-1 produced by innate cells present in spleen cell cultures. Furthermore, IL-1 and IL-23 were found to promote IL-17 production from both CD4⁺ and CD8⁺ T cells in the presence of anti-CD3 stimulation, although concentrations of IL-17 induced were somewhat reduced in CD8⁺ compared with CD4⁺ T cells. These data demonstrate that both helper and cytotoxic T cells secrete IL-17 in response to IL-1 and IL-23 stimulation in the presence of TCR activation. Interestingly IL-1 and IL-23 synergy for IL-17 protein production was not observed at the mRNA level. IL-23 alone was capable of inducing IL-17 mRNA expression, as well as the IL-17 associated transcription factor RORγT in CD3⁺ T cells. It has previously been reported that the Th1 and Th2 associated cytokines, IFN-γ and IL-4 respectively, inhibit differentiation of T_{IL-17} cells and subsequent IL-17 production (Park *et al.*, 2005; Rangachari *et al.*, 2006). Our findings support this observation, since inhibition of IFN-γ with a neutralizing antibody enhanced IL-1 and IL-23 induced IL-17 producing T cells by over 25%. It should be noted that, in contrast to inhibition of T_{IL-17} cell development by IFN-γ, IL-17 has been implicated in the development of Th1 cells, and IFN-γ production, during *mycobacterium bovis* infection (Umemura *et al.*, 2007).

IL-1 and IL-23 synergy for IL-17 production was not confined to a single member of the IL-1 family. IL-1 α and IL-1 β , as well as the more distantly related IL-1 family member, IL-18, synergised with IL-23 for the induction of IL-17 in spleen cells from C57BL/6 mice, in the absence of co-stimulation with anti-CD3. However, IL-17 induced in response to IL-18 and IL-23 was partially dependent on IL-1RI, since the response was reduced by about 50%, in spleen cells from IL-1RI^{-/-} mice compared with spleen cells from C57BL/6 mice. The demonstrated role for IL-18 in IL-17 production is consistent with previous reports, which have highlighted a role for IL-18 in the development of autoimmune diseases (Ishikura *et al.*, 2003; Jander and Stoll, 1998). The observation that IL-18 and IL-23-induced IL-17 production by spleen cells is partially dependent on IL-1RI may be explained by the fact that IL-1 up-regulates the expression of IL-18R (Krasna *et al.*, 2005), which would result in enhancing the synergy between IL-18 and IL-23 for IL-17 production. This synergy between IL-1 family members and IL-23 for the induction of IL-17 was not demonstrated by all IL-1 family members, as the more distantly related member of the IL-1 family, IL-33, was unable to synergise with IL-23 to increase IL-17 production in spleen cells from either wild type or IL-1RI^{-/-} mice in the absence of anti-CD3 stimulation. However, IL-33 was capable of inducing IL-17 production by spleen cells from C57BL/6 mice in the presence of anti-CD3 stimulation, however, this was found to be IL-1RI-dependent.

Other pro-inflammatory cytokines, such as TNF- α and IL-6, as well as the TLR4 and TLR2 agonists, LPS and Pam3Cys, were also capable of enhancing IL-23-induced IL-17 production by spleen cells from C57BL/6 mice, and this also depends on IL-1 signalling. In contrast to the other TLR agonists examined, the TLR9 agonist

CpG did not synergise with IL-23 to induce IL-17 production by spleen cells from either wild type or IL-1RI^{-/-} mice.

The synergy between IL-23 and the pro-inflammatory cytokines, TNF- α and IL-6, in the induction of IL-1-dependent IL-17 production may explain the role for IL-6 and TNF- α in the development of autoimmune diseases (Baker *et al.*, 1994; Okuda *et al.*, 1999; Selmaj *et al.*, 1991; Selmaj *et al.*, 1991). It appears that IL-6 and TNF- α can enhance IL-17 production, in the presence of IL-1, as well as modulating pro-inflammatory responses downstream of T_{IL-17} cells. In the case of TNF- α , the induction of IL-1-dependent IL-17 is amplified through the up-regulation of IL-1RI expression on T cells. Up-regulation of IL-1RI expression was confined to TNF- α , as neither IL-1 nor IL-23 increased expression of the receptor on T cells. The role of TNF- α in the development of EAE and MS is still not clear (Steinman, 2005) with conflicting data from studies with TNF- α inhibitors and knock out mice, some of which showed that blocking TNF- α lead to exacerbated symptoms of EAE (Liu *et al.*, 1998). These discordant findings could be due to the method by which neutralising antibodies against TNF- α are delivered or due to the janus-like nature of certain pro-inflammatory cytokines associated with autoimmune diseases, where they are initially associated with disease development but are required for recovery from disease at later time points.

The findings of this study have demonstrated that IL-1 is a crucial factor in the development of T_{IL-17} cells and at least partially, explain why targeting IL-1 has been an effective treatment in certain human autoimmune diseases, such as systemic juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (Burger *et al.*, 2006; Cinek *et al.*, 2004). Evidence in support of this is provided by the observation that

development of JIA has been associated with over-expression of IL-1 as a result of IL-1 gene mutations (Cimaz *et al.*, 2007).

Papers published during the course of these experiments have disputed the role for IL-23 in the development of IL-17 producing T cells. Veldhoen *et al* contended that it was the Treg associated cytokine, TGF- β , which was responsible for the development of IL-17 producing T cells (Veldhoen *et al.*, 2006). The hypothesis put forward by Veldhoen was supported by two studies, published concurrently by Mangan *et al* and Bettelli *et al* (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). The study, by Bettelli *et al*, concluded that the pro-inflammatory cytokine IL-6 as well as TGF- β was required for T_{IL-17} polarisation and activation. This report dismissed the role of IL-23 in T_{IL-17} development but failed to account for the fact that IL-23^{-/-} (IL-12p19^{-/-}) mice do not develop autoimmune diseases, due to their inability to generate T_{IL-17} cells (Langrish *et al.*, 2005; Murphy *et al.*, 2003). The present study therefore examined the role of both TGF- β and IL-6 in the development of IL-17 producing T cells, with regards to IL-1 signalling. The findings demonstrated that TGF- β and IL-6 promoted the production of IL-17 by spleen cells from C57BL/6 mice in the presence of anti-CD3 and anti-CD28 co-stimulation, agreeing with the published findings (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). However, this IL-17 production was found to be, at least partially, IL-1RI dependent, as TGF- β and IL-6 stimulated spleen cells, from IL-1RI^{-/-} mice, produced significantly less IL-17 compared with spleen cells from wild type mice. In the absence of anti-CD3 and anti-CD28, TGF- β and IL-6 failed to induce IL-17 production in spleen cells from wild type or knock out mice, in contrast to IL-1 and IL-23 stimulation, which promoted the production of IL-17 in spleen cells from wild type mice. These results were confirmed by intracellular FACS analysis of C57BL/6 spleen cells, which demonstrated that TGF- β and IL-6 could not

enhance the frequency of IL-17 producing T cells in the absence of anti-CD3 stimulation. In the presence of anti-CD3, TGF- β and IL-6 did increase IL-17 producing cells to 23%, from 13% with anti-CD3 alone. This is in contrast to IL-1 and IL-23 stimulation, which increased the frequency of IL-17 producing T cell population to greater than 30% in the absence of co-stimulation, approximately 150 times higher than medium treated cells alone. IL-1 and IL-23 induced IL-17 production by CD3⁺ T cells, in the absence of anti-CD3 co-stimulation, would therefore appear to be promoted in an atypical manner, given that classical T cell activation requires ligation of the CD3 complex on the T cell surface. Furthermore, this IL-17 producing CD3⁺ T cell population was found to be neither CD4⁺ nor CD8 α ⁺. CD4⁺ and CD8 α ⁺ cells were capable of responding to stimulation with IL-1 and IL-23 for the induction of IL-17, but this was found only in the presence of anti-CD3 co-stimulation. The results demonstrated that a CD3⁺CD4⁻CD8 α ⁻ population of T cells secreted IL-17 in response to IL-1 and IL-23 stimulation. Therefore, both NK T cells and $\gamma\delta$ T cells were examined for the ability to produce IL-17 after stimulation with IL-1 and IL-23. It was found that stimulation of $\gamma\delta$ T cells, but not NK T cells, with IL-1 and IL-23 induced an IL-17 producing population. Indeed over 30% of $\gamma\delta$ T cells produced IL-17 in response to IL-1 and IL-23 stimulation.

In the absence of anti-CD3 stimulation, IL-1 and IL-23 promoted IL-17 production by purified $\gamma\delta$ T cells and spleen cells from C57BL/6 mice, but not in spleen cells depleted of $\gamma\delta$ T cells or in spleen cells from $\gamma\delta^{-/-}$ mice. This study also demonstrates that IL-1 acts as a proliferation factor for the expansion for $\gamma\delta$ T cells but not $\gamma\delta$ depleted spleen cells. This proliferation was greatly increased upon co-incubation with either IL-23 or anti-CD3 and would enable the rapid expansion of $\gamma\delta$ T_{IL-17} cells and a corresponding increase in IL-17 production in the presence of IL-1

and IL-23 stimulation. Recently the IL-2 homologue, IL-21, has been linked with IL-17 production from CD4⁺ T cells (Korn *et al.*, 2007). This factor is a known $\gamma\delta$ T cell differentiation and proliferative factor, which unlike IL-2 does not stimulate IFN- γ production. This may explain why IL-21, and not IL-2, has been associated with IL-17 production by possibly promoting the expansion of IL-1 and IL-23 promoted $\gamma\delta$ T_{IL-17} cells (Eberl *et al.*, 2002).

It is possible that $\gamma\delta$ T_{IL-17} cells do not act in isolation and may co-localise with innate immune cells, such as DC and macrophages, subsequently promoting IL-17 production by $\alpha\beta$ T cells. Co-culture experiments in this study revealed that IL-1 and IL-23-activated $\gamma\delta$ T cells promoted IL-17 production by CD4⁺ and CD8⁺ T cells through interaction with DCs. Furthermore, this study found that DC produced IL-1 and IL-23 in response to stimulation with IL-17. The pro-inflammatory cytokines, IL-1, TNF- α and IL-6 synergise with IL-23 to enhance the production of IL-17 from spleen cells, however these cytokines were also capable of promoting the production of IL-23 from DC, while TNF- α and IL-6 also induced IL-1 β production by DC. Thus providing an amplification loop through which IL-1 and IL-23 stimulate $\gamma\delta$ and $\alpha\beta$ T cells and consequently enhancement IL-17 production by both of these T cell subsets.

It has already been reported that IL-17 and IL-1 play a role in the development of EAE (Jacobs *et al.*, 1991; Komiyama *et al.*, 2006) neutralising IL-1 or IL-17 abrogated symptoms of EAE. This was confirmed by our studies, which demonstrated a reduced incidence of EAE in IL-1RI^{-/-} compared with wild type C57BL/6 mice, with a corresponding reduction in serum and MOG-specific IL-17 in IL-1RI^{-/-} mice immunised for the development of EAE. This study went on to demonstrate that IL-1 is required for the development of antigen-specific IL-17 producing T cells, since IL-1RI^{-/-} mice were capable of developing EAE after transfer of MOG-specific T cells

from C57BL/6 mice. This finding indicates that IL-1 is dispensable downstream of T cell activation, perhaps due to redundancy in the actions of pro-inflammatory cytokines and chemokines in the induction of inflammatory pathology in EAE.

This study has shown that $\gamma\delta$ T cells are the major T cell subset responding to stimulation with IL-1 and IL-23 to produce IL-17. However, their role in EAE has been widely disputed, with conflicting publications citing a resolution in disease with the removal of $\gamma\delta$ T cells (Odyniec *et al.*, 2004), an increase in disease severity in the absence of $\gamma\delta$ T cells (Kobayashi *et al.*, 1997) or $\gamma\delta$ T cells having no effect on the development of disease (Clark and Lingenheld, 1998). Given the link found between IL-1 and IL-23 activated $\gamma\delta$ T_{IL-17} cells and subsequent development of $\alpha\beta$ T_{IL-17} cells, this study examined the requirement for IL-1 promoted $\gamma\delta$ T_{IL-17} cells in the initiation of EAE. It was found that IL-1RI^{-/-} mice developed EAE following transfer of total CD3⁺ T cells but not $\gamma\delta$ ⁺ T cells from C57BL/6 mice, suggesting that IL-1 does not work solely on $\gamma\delta$ T cells. However, IL-1RI^{-/-} mice could not develop EAE after transfer of $\gamma\delta$ T cell-depleted C57BL/6 CD3⁺ T cells, indicating that IL-1 is required on both $\alpha\beta$ and $\gamma\delta$ T cells in the development of EAE.

In conclusion, this study provides evidence of a crucial role for the pro-inflammatory cytokine IL-1 in the development of IL-17 producing T cells. Through synergy with IL-23, IL-1 can promote the development of both conventional, $\alpha\beta$ T cells, and unconventional, $\gamma\delta$ T cells, into T_{IL-17} cells. Indeed IL-1 and IL-23 promoted $\gamma\delta$ T_{IL-17} cells are capable of priming $\alpha\beta$ T_{IL-17} cells, potentially through inducing production of pro-inflammatory cytokines such as IL-1, TNF- α and IL-6 from DC, each of which this study has found to promote IL-17 production, in

combination with IL-23. This study therefore provides an alternative explanation for the effectiveness of targeting IL-1 in alleviating autoimmune diseases.

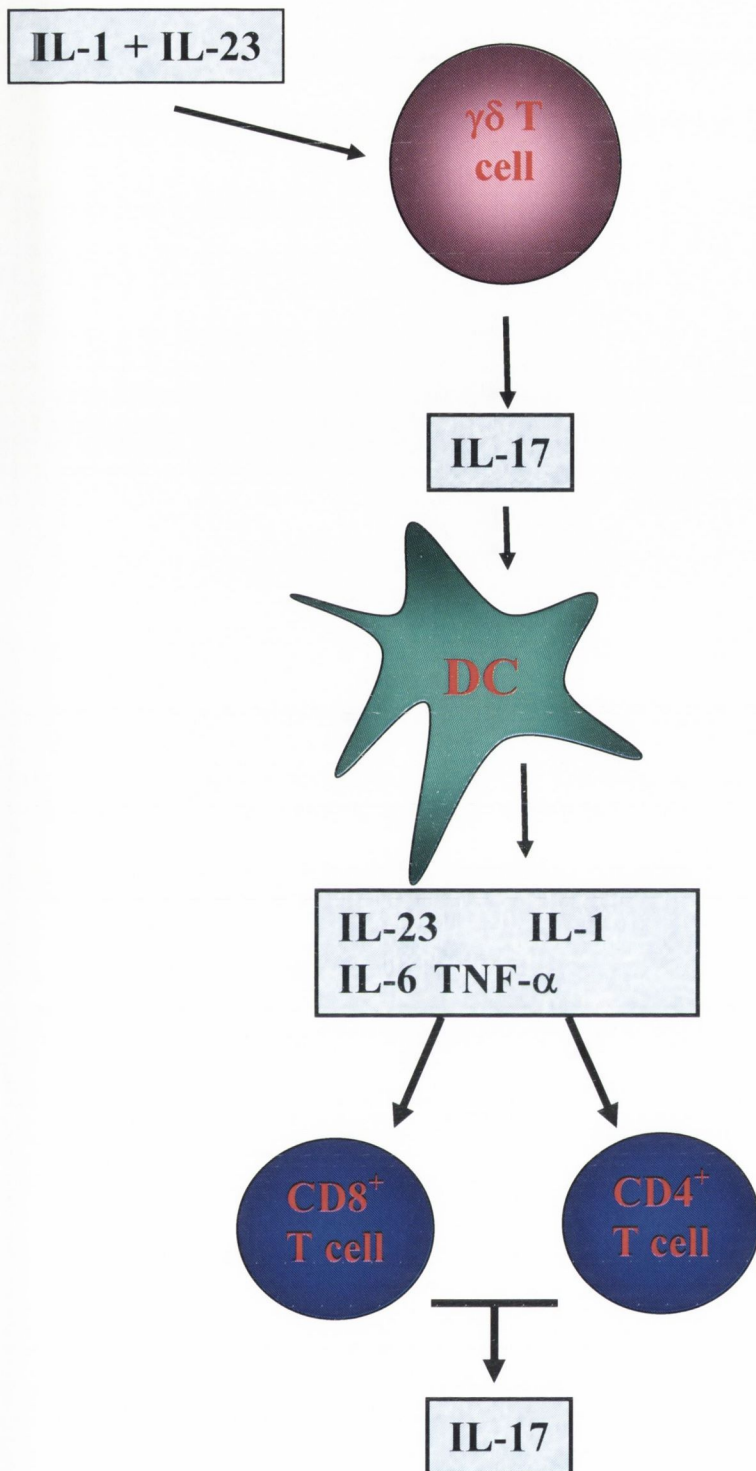


Diagram 4.1: Proposed role for IL-1 and IL-23 stimulated $\gamma\delta$ T cells in the generation of IL-17 producing $CD4^+$ and $CD8^+$ T cells via DC.

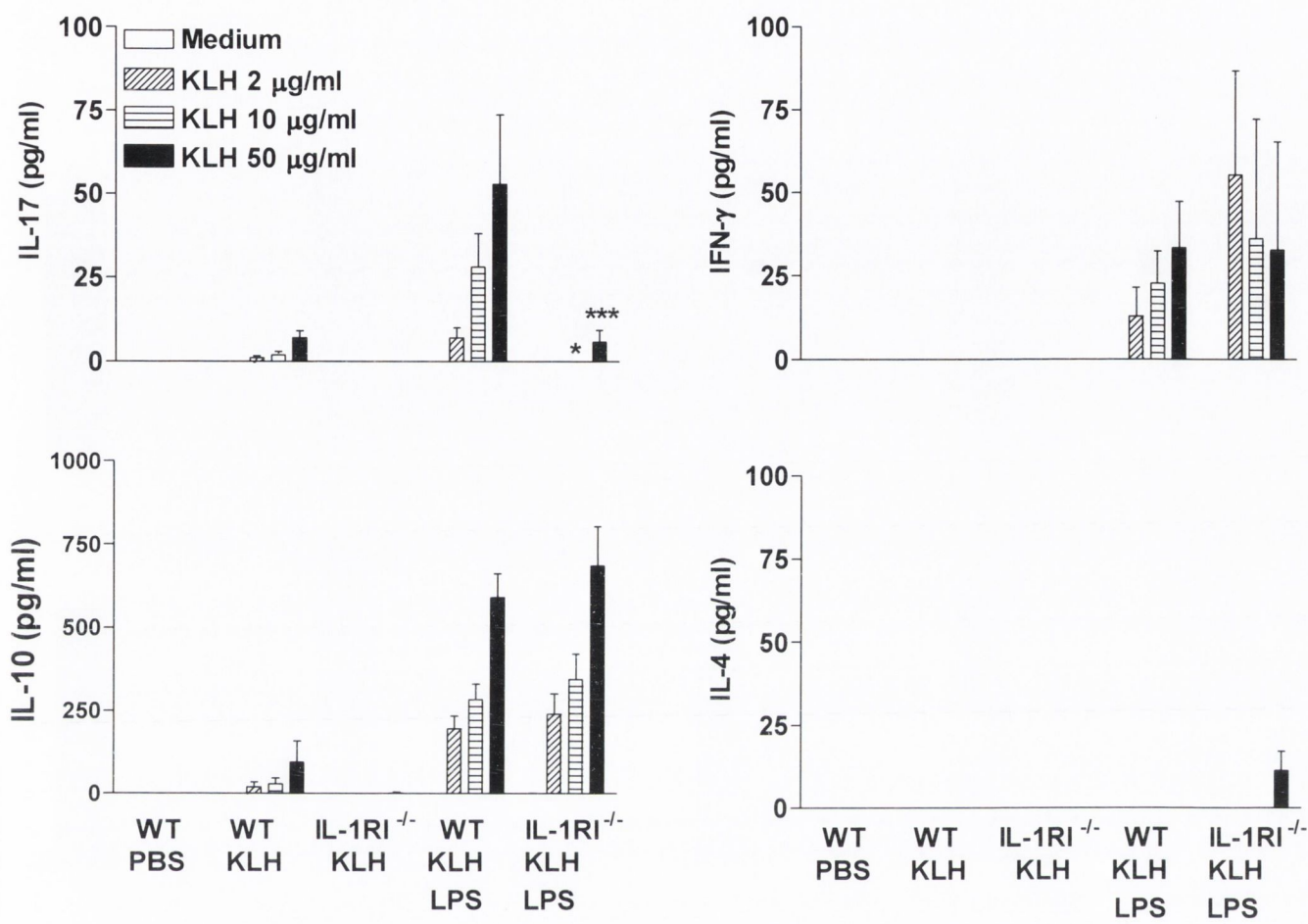


Figure 4.1: IL-1RI is required for induction of antigen-specific T_{IL-17} cells. C57BL/6 (WT) or IL-1RI^{-/-} mice were immunized s.c. with 10 μg KLH either alone or with 10 μg LPS or with PBS only. Inguinal lymph nodes were harvested after 7 d and cells were re-stimulated with medium or 2, 10 or 50 μg/ml KLH. Supernatants were collected after 3 d, and IL-4, IL-10, IL-17 and IFN-γ concentrations determined by ELISA. C57BL/6 versus IL-1RI^{-/-}: *, P < 0.05; ***, P < 0.001.

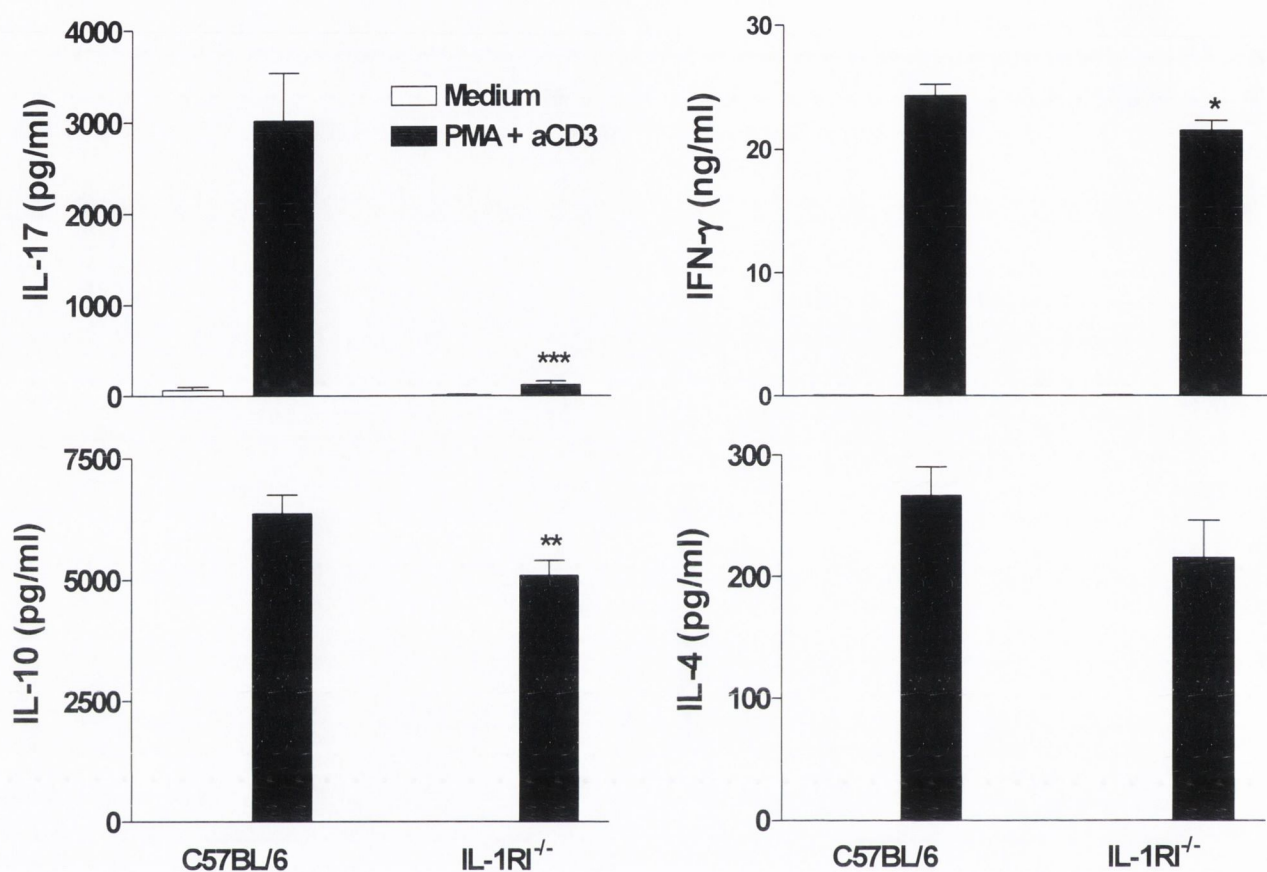


Figure 4.2: IL-1RI is required for the antigen-independent induction of IL-17. Inguinal lymph node cells from C57BL/6 or IL-1RI^{-/-} mice immunised with PBS were collected after 7 d and re-stimulated with medium or 20 ng/ml PMA and 1 µg/ml anti-CD3. Supernatants were collected after 3 d and IL-4, IL-10, IL-17 and IFN-γ concentrations determined by ELISA. C57BL/6 versus IL-1RI^{-/-}: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

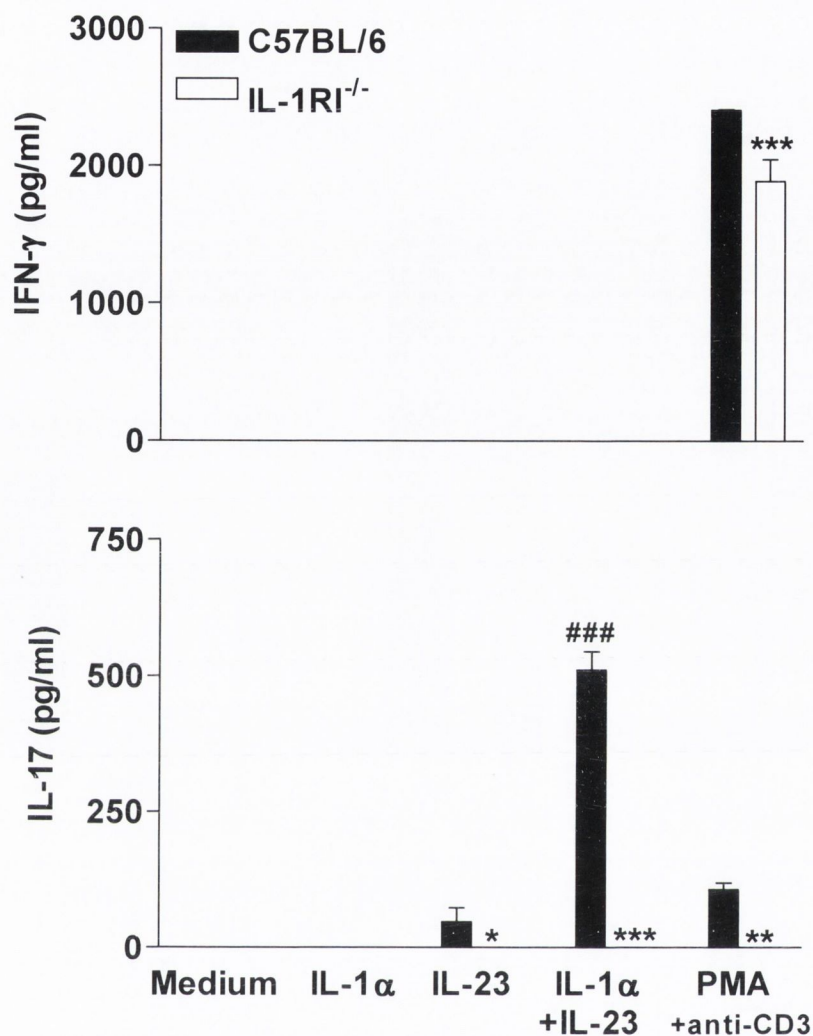


Figure 4.3: IL-1 synergises with IL-23 to promote IL-17 production from spleen cells. Spleen cells (10^6 cells/ml) from C57BL/6 or IL-1RI^{-/-} mice were cultured with IL-23 (100 pg/ml), IL-1α (1 ng/ml), IL-1α+IL-23, PMA (1 ng/ml) and anti-CD3 (1 μg/ml) or with medium alone. Supernatants were collected after 3 d and analysed for IL-17 and IFN-γ production by ELISA. C57BL/6 versus IL-1RI^{-/-}: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. IL-1α + IL-23 versus medium alone: ###, $P < 0.001$.

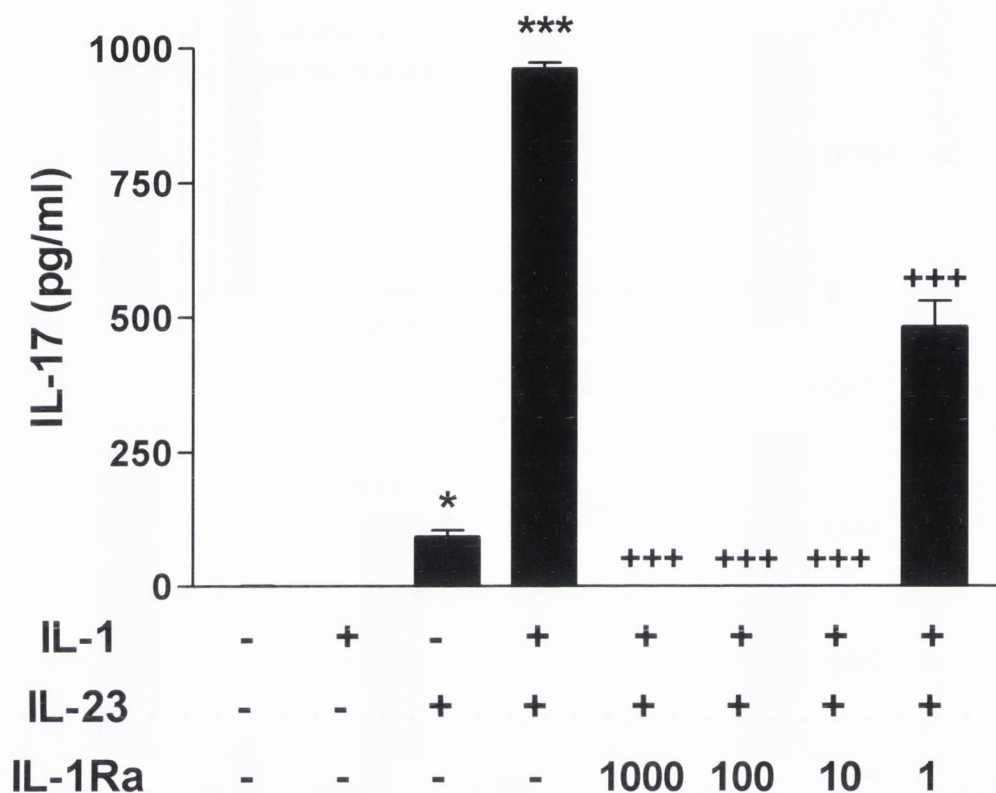


Figure 4.4: IL-1 synergy with IL-23 for IL-17 production from spleen cells is inhibited by IL-1Ra. Spleen cells (10^6 cells/ml) from C57BL/6 mice were cultured with IL-1 β (10 ng/ml), IL-23 (10 ng/ml) and IL-1 + IL-23 in the presence and absence of IL-1Ra (1000-1 μ g/ml) or with medium alone. Supernatants were collected after 3 d and analysed for IL-17 production by ELISA. Treatment versus medium alone: *, $P < 0.05$; ***, $P < 0.001$. Treatment + IL-1 + IL-23 versus IL-1 + IL-23 alone: +++, $P < 0.001$.

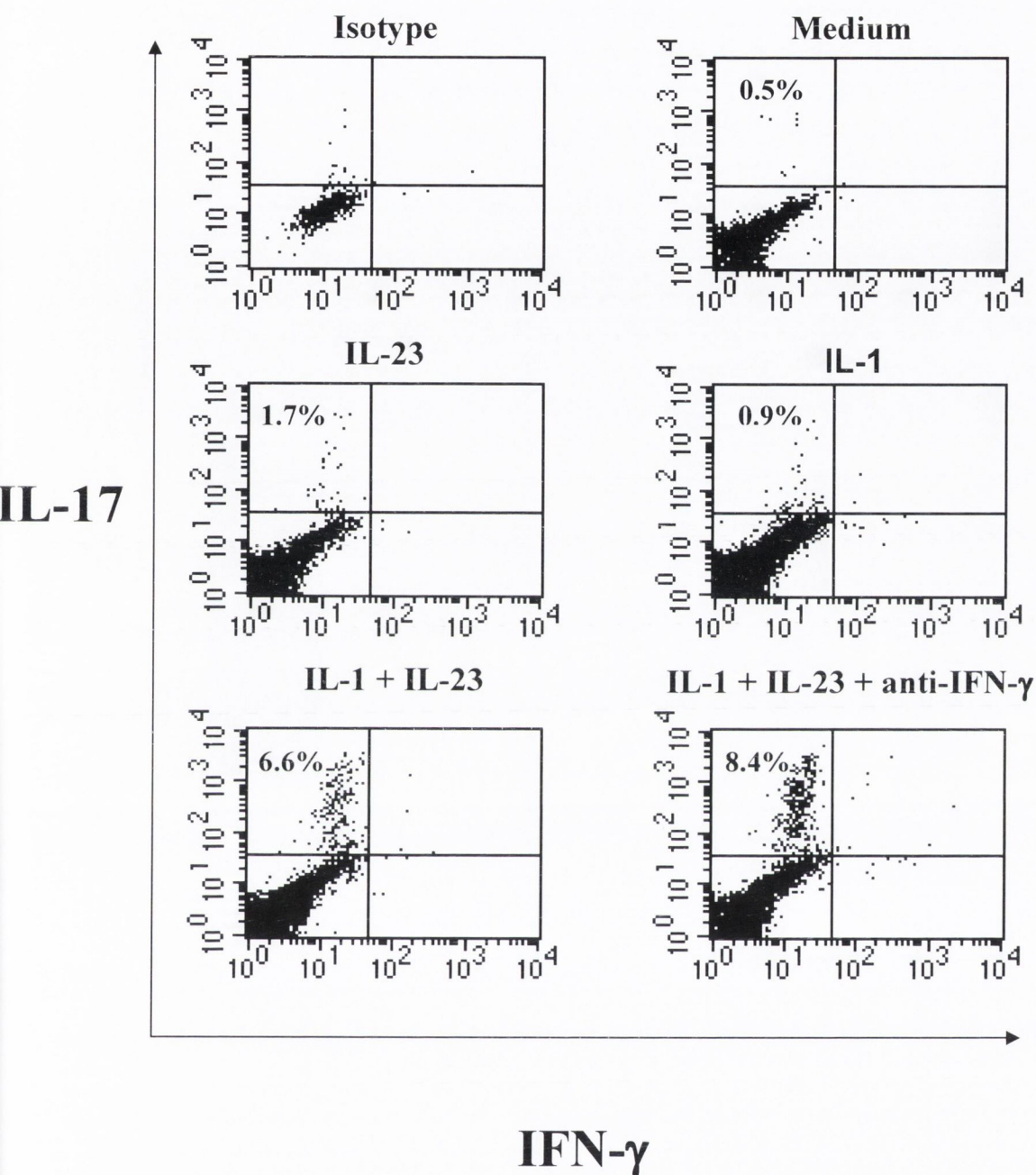


Figure 4.5: IL-1 synergises with IL-23 to promote T_{IL-17} cells. Spleen cells from C57BL/6 mice (2.25×10^6 cells/ml) were cultured for 48 h with IL-23 (10 ng/ml), IL-1 α (10 ng/ml), IL-1 α + IL-23 in the presence or absence of anti-IFN- γ (10 μ g/ml) or with medium alone in the presence of anti-CD3 (1 μ g/ml) and for the last 4 h in the presence of 10 μ g/ml brefeldin A. Cells were then surface stained for CD3 and stained for intracellular IL-17 and IFN- γ . Percentages refer to cells gated on CD3.

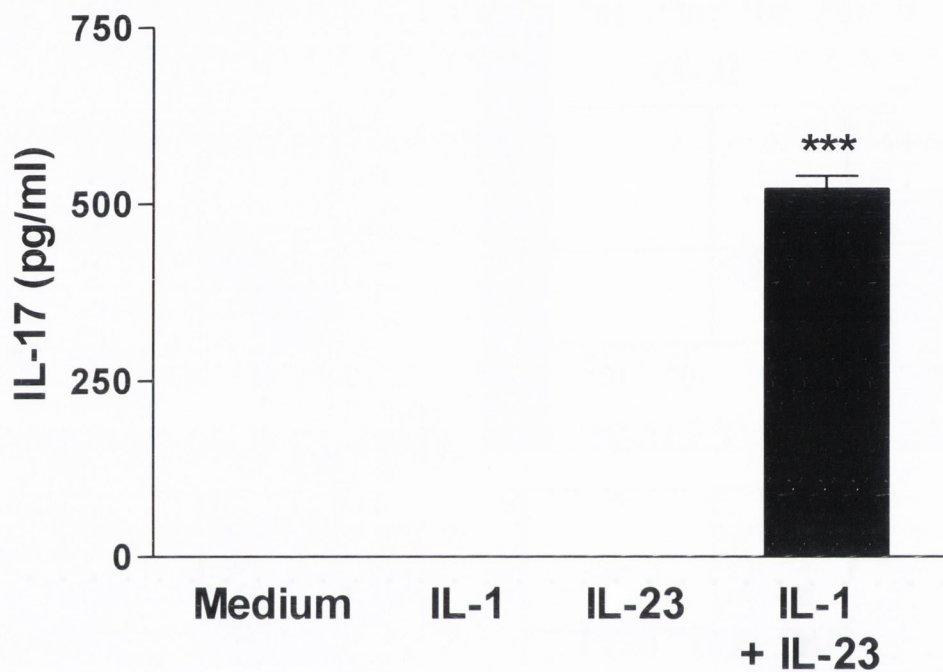


Figure 4.6: IL-1 synergises with IL-23 to promote IL-17 production from CD3⁺ T cells. CD3⁺ T cells from C57BL/6 mice (5×10^5 cells/ml) were incubated with IL-1 β (10 ng/ml), IL-23 (10 ng/ml), IL-1 β and IL-23 or with medium alone in the presence of anti-CD3 (1 μ g/ml). Supernatants were removed after 3 d and tested for IL-17 production by ELISA. Treatment versus medium alone: ***, $P < 0.001$.

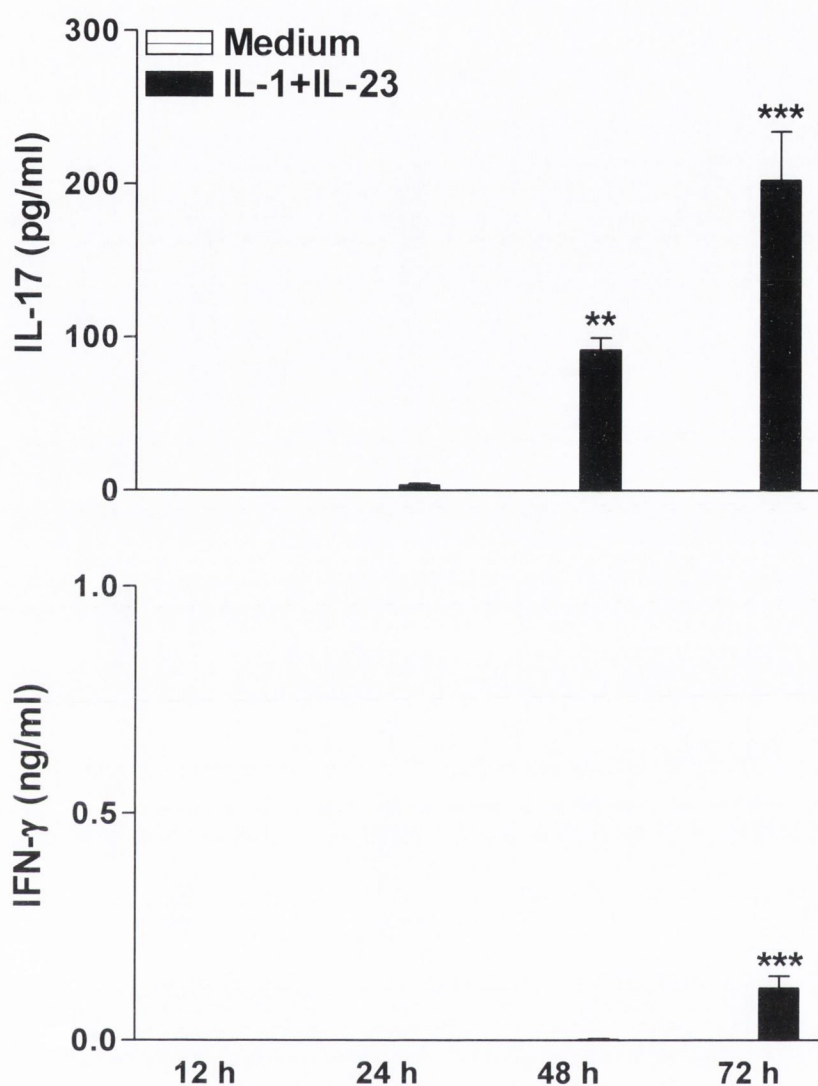


Figure 4.7: Splenic CD3⁺ cells require 48 h stimulation with IL-1 and IL-23 to induce IL-17 production. CD3⁺ cells from C57BL/6 mice (5×10^5 cells/ml) were incubated with IL-1 β (10 ng/ml) and IL-23 (10 ng/ml), or with medium all in combination with anti-CD3 (1 μ g/ml). Supernatants were removed 12, 24, 48 and 72 h post stimulation and tested for IL-17 and IFN- γ production by ELISA. IL-1 + IL-23 versus medium alone: **, $P < 0.01$ ***, $P < 0.001$.

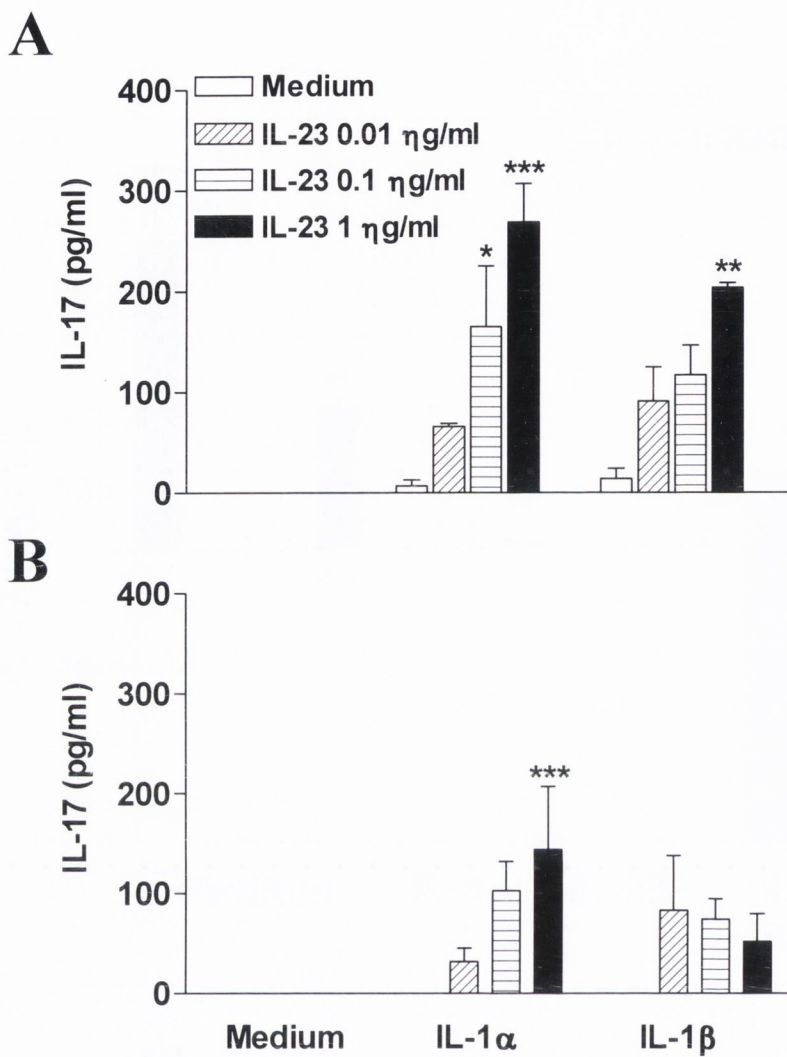


Figure 4.8: Both IL-1 α and IL-1 β synergise with IL-23 to induce IL-17 production from CD4⁺ and CD8⁺ T cells. CD4⁺ (A) and CD8⁺ (B) T cells (5×10^5 cells/ml) from naïve C57BL/6 mice were incubated with 10 U/ml IL-2 and 1 μ g/ml anti-CD3 antibody alone or together with 0.1-1 ng/ml IL-23 and/or 1 ng/ml IL-1 α or IL-1 β . IL-17 concentrations were measured by ELISA in supernatants collected after 3 d. IL-23 + IL-1 versus IL-23 only: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

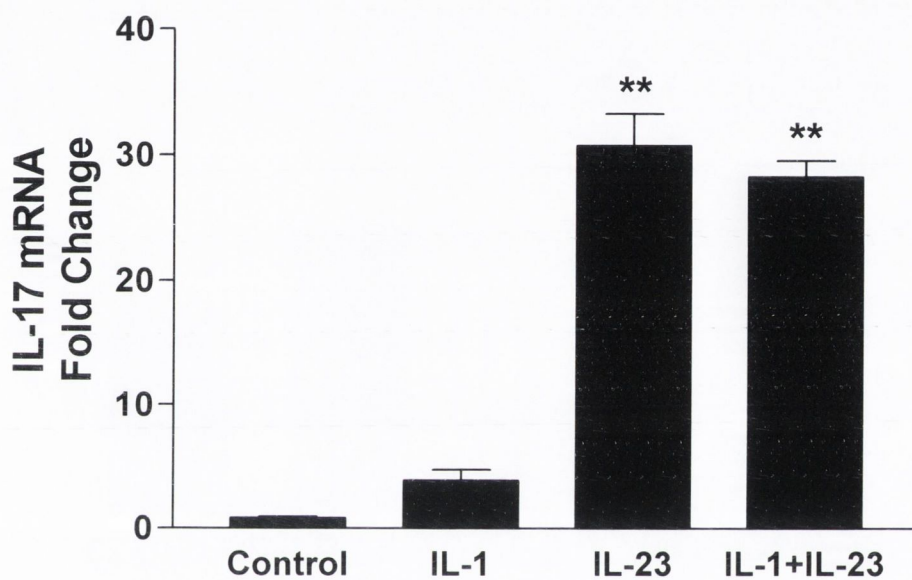


Figure 4.9: IL-23 induces the transcription of IL-17 mRNA. Splenic CD3⁺ T cells (7×10^6 cells/ml) from C57BL/6 mice were incubated with 1 μ g/ml anti-CD3 for 2 h before stimulation with IL-1 β (10 ng/ml), IL-23 (10 ng/ml), IL-1 β and IL-23, or medium alone. IL-17 mRNA expression was evaluated by real-time PCR 48 h after stimulation, and the data represent the fold change for IL-17 mRNA in relation to 18S rRNA. Treatment versus medium alone: **, $P < 0.01$.

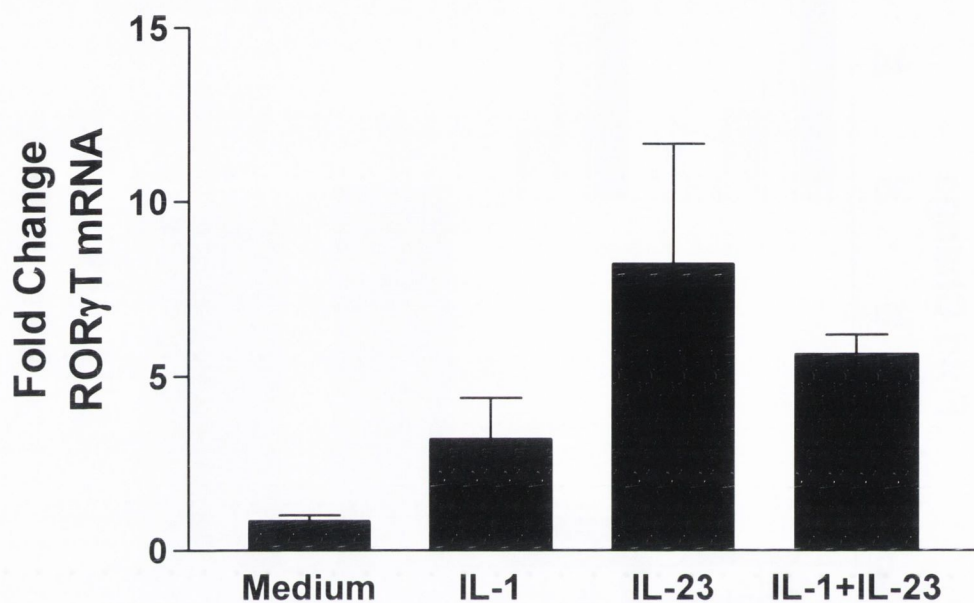


Figure 4.10: IL-23 induces the T_{IL-17} associated transcription factor ROR γ T in $CD3^+$ T cells. Splenic $CD3^+$ T cells (7×10^6 cells) from C57BL/6 mice were stimulated with anti-CD3 (1 μ g/ml) in combination with IL-1 β (10 ng/ml), IL-23 (10 ng/ml) or with medium alone for 2 d. ROR γ T mRNA expression was then evaluated by real-time PCR, the data represent the fold change for ROR γ T mRNA in relation to 18S rRNA.

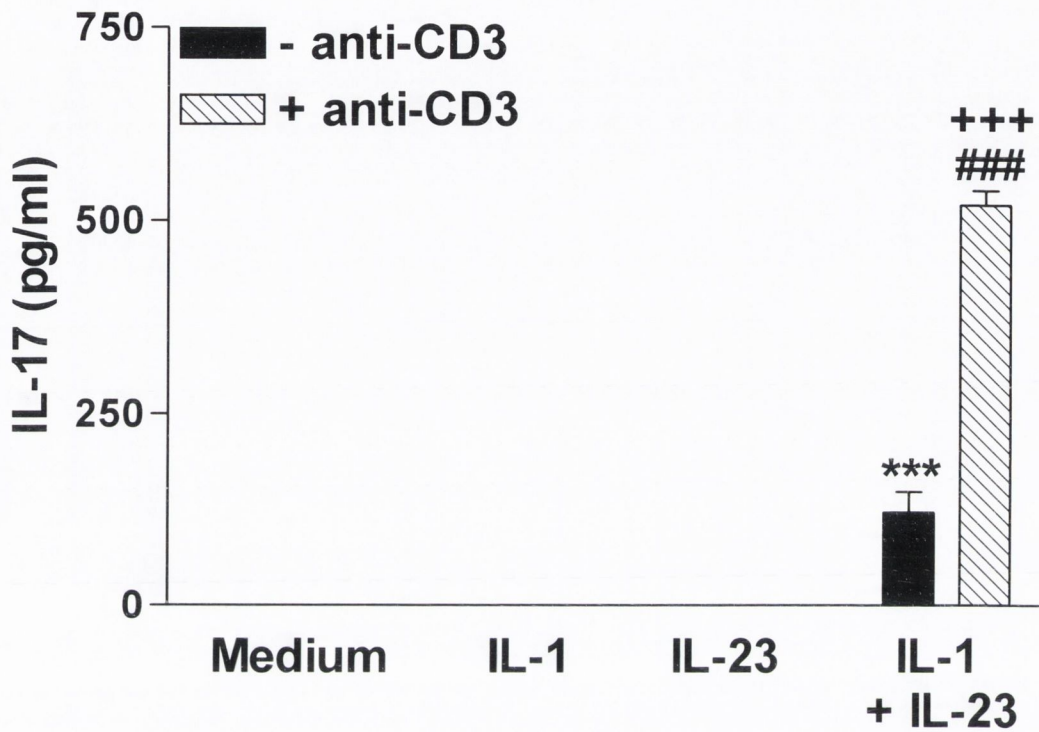


Figure 4.11: IL-1 and IL-23 synergise for IL-17 production in the presence and absence of anti-CD3 stimulation. CD3⁺ T cells from C57BL/6 mice (5×10^5 cells/ml) were incubated with IL-1 (10 ng/ml) and IL-23 (10 ng/ml), or with medium alone in the presence and absence of anti-CD3 (1 μ g/ml). Supernatants were removed after 3 d and tested for IL-17 production by ELISA. Treatment versus medium alone: ***, $P < 0.01$; Treatment + anti-CD3 versus anti-CD3 alone: ###, $P < 0.01$. With anti-CD3 versus without anti-CD3: ++++, $P < 0.001$.

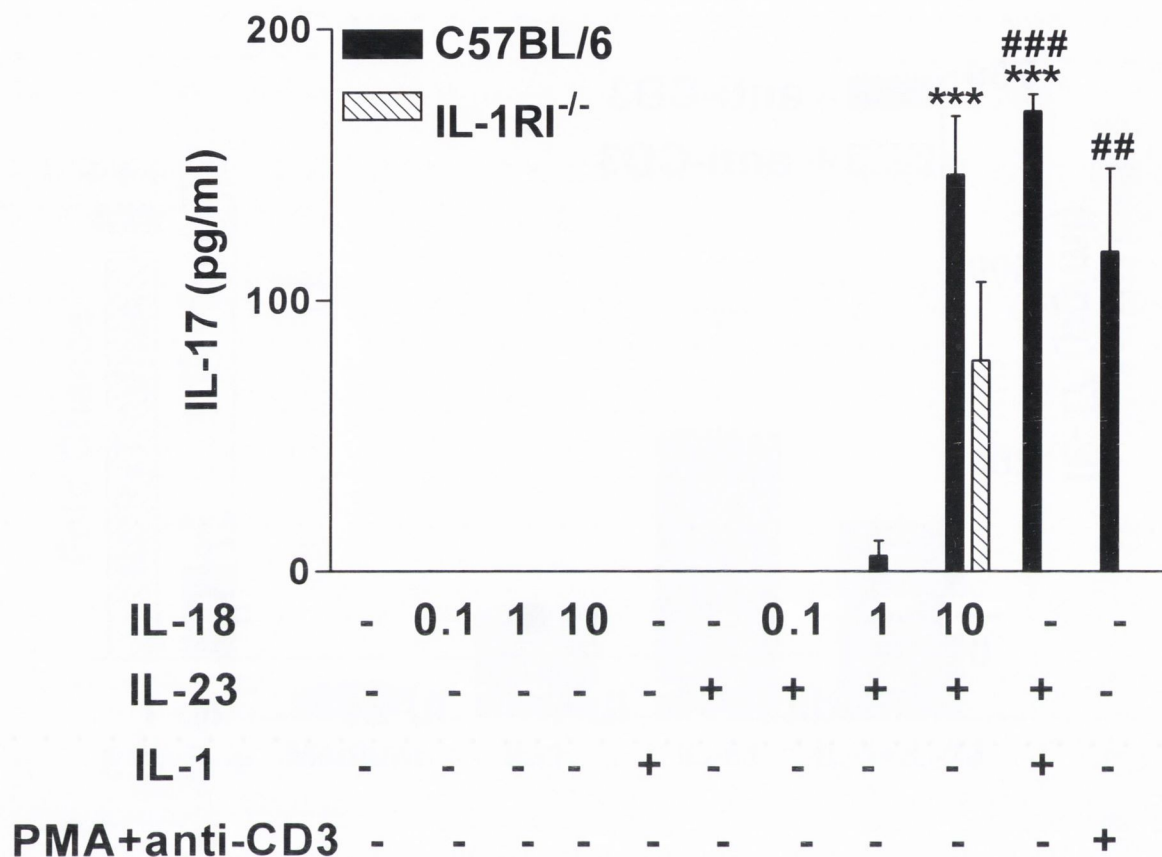


Figure 4.12: IL-18 can synergise with IL-23 for IL-17 production and this effect is partially independent of IL-1 signalling. Spleen cells from C57BL/6 and IL-1RI^{-/-} mice (5×10^5 cells/ml) were incubated with either IL-18 (0.1-10 ng/ml) with and without IL-23 (1 ng/ml), IL-23 alone, IL-1 (0.1 ng/ml), IL-1+IL-23, PMA (1 ng/ml) and anti-CD3 (1 μ g/ml) or with medium alone. Supernatants collected after 3 d tested for IL-17 production by ELISA. Treatment + IL-23 versus IL-23 alone: ***, $P < 0.001$. C57BL/6 versus IL-1RI^{-/-}: ##, $P < 0.01$; ###, $P < 0.001$. Data representative of two separate experiments.

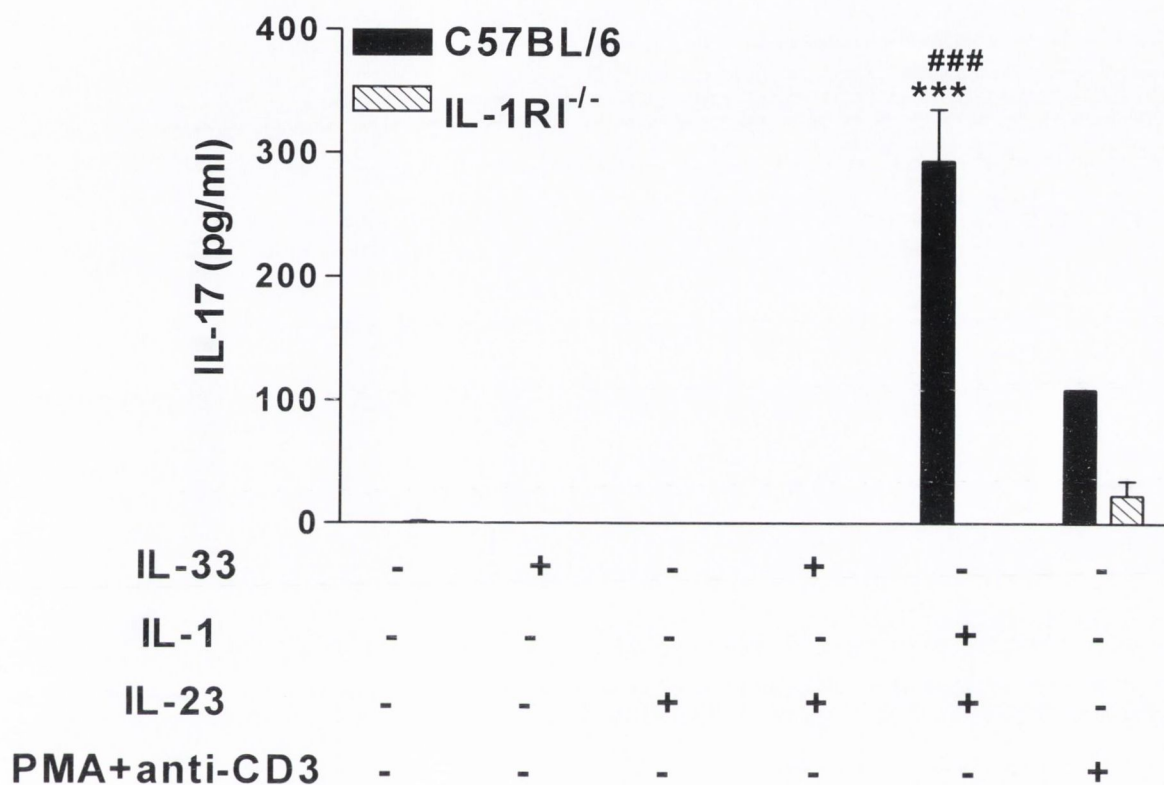


Figure 4.13: IL-33 does not synergise with IL-23 for IL-17 production from wild type or IL-1RI^{-/-} spleen cells in the absence of anti-CD3 stimulation. Spleen cells from C57BL/6 or IL-1RI^{-/-} mice were cultured at 5×10^5 cells/ml with IL-33 (1 ng/ml), IL-23 (1 ng/ml), IL-33 + IL-23, IL-1 β (1 ng/ml) + IL-23, PMA (1 ng/ml) and anti-CD3 (1 μ g/ml) or with medium alone. Supernatants were removed after 3 d and tested for IL-17 production by ELISA. Treatment + IL-23 versus IL-23 alone: ***, $P < 0.001$. C57BL/6 versus IL-1RI^{-/-}: ###, $P < 0.001$.

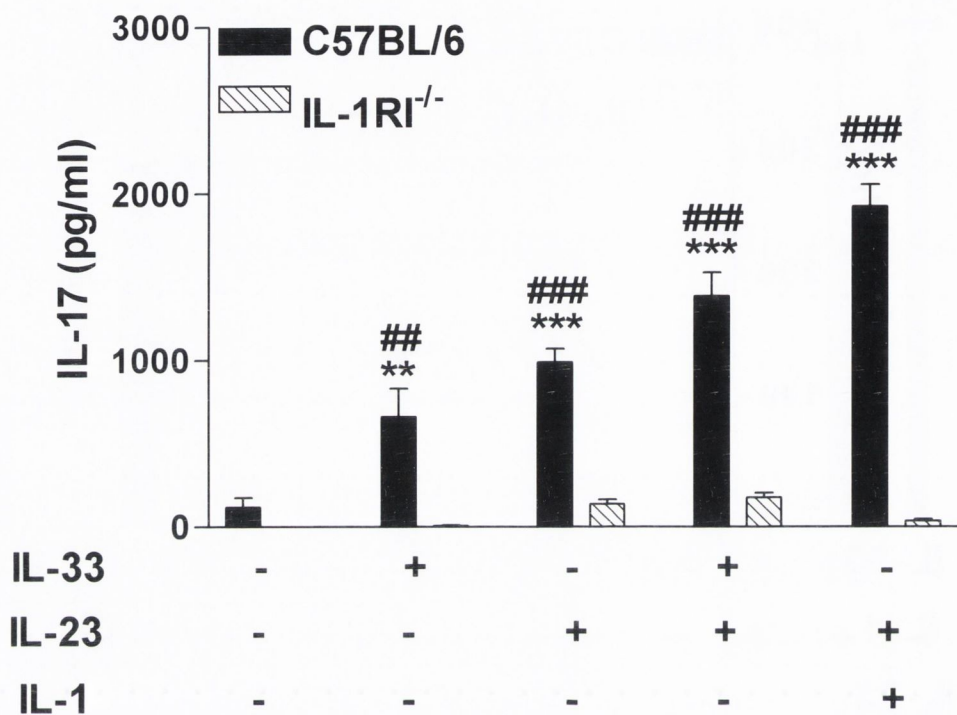


Figure 4.14: IL-33-induced IL-17 production from spleen cells in the presence of anti-CD3 is IL-1RI dependent. Spleen cells from C57BL/6 and IL-1RI^{-/-} mice (5×10^5 cells/ml) were cultured with IL-33 (1 ng/ml), IL-23 (1 ng/ml), IL-33 + IL-23, IL-1 (1 ng/ml) + IL-23 or with medium alone, all in the presence of anti-CD3 (1 μ g/ml) stimulation. Supernatants were removed after 3 days and tested for IL-17 production by ELISA. Treatment versus medium alone: ##, $P < 0.01$; ###, $P < 0.001$. C57BL/6 versus IL-1RI^{-/-}: **, $P < 0.01$; ***, $P < 0.001$.

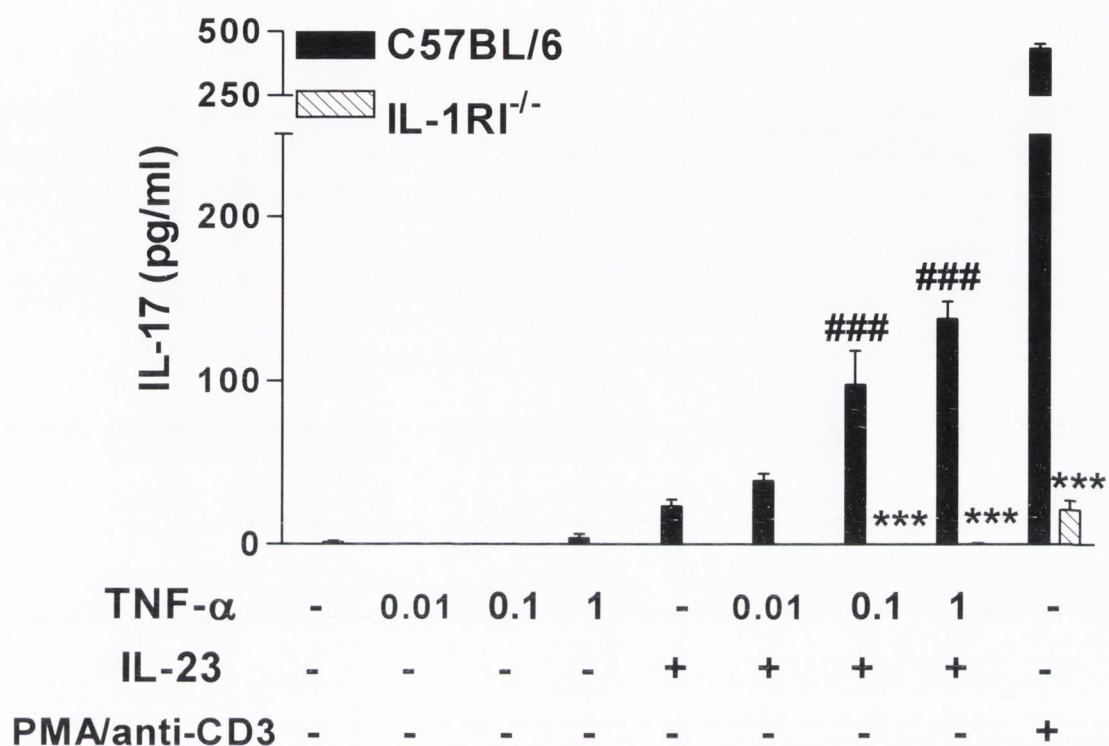


Figure 4.15: TNF- α promotes IL-23-induced IL-17 production in an IL-1-dependent manner. Spleen cells (10^6 cells/ml) from C57BL/6 or IL-1RI^{-/-} mice were cultured with 1 ng/ml IL-23, 0.01-1 ng/ml TNF- α , IL-23 and TNF- α , medium, or with PMA (1 ng/ml) and anti-CD3 (1 μ g/ml). Supernatants were collected after 3 d and analysed for IL-17 production by ELISA. Treatment + IL-23 versus IL-23 alone: ###, $P < 0.001$. C57BL/6 versus IL-1RI^{-/-}: ***, $P < 0.001$.

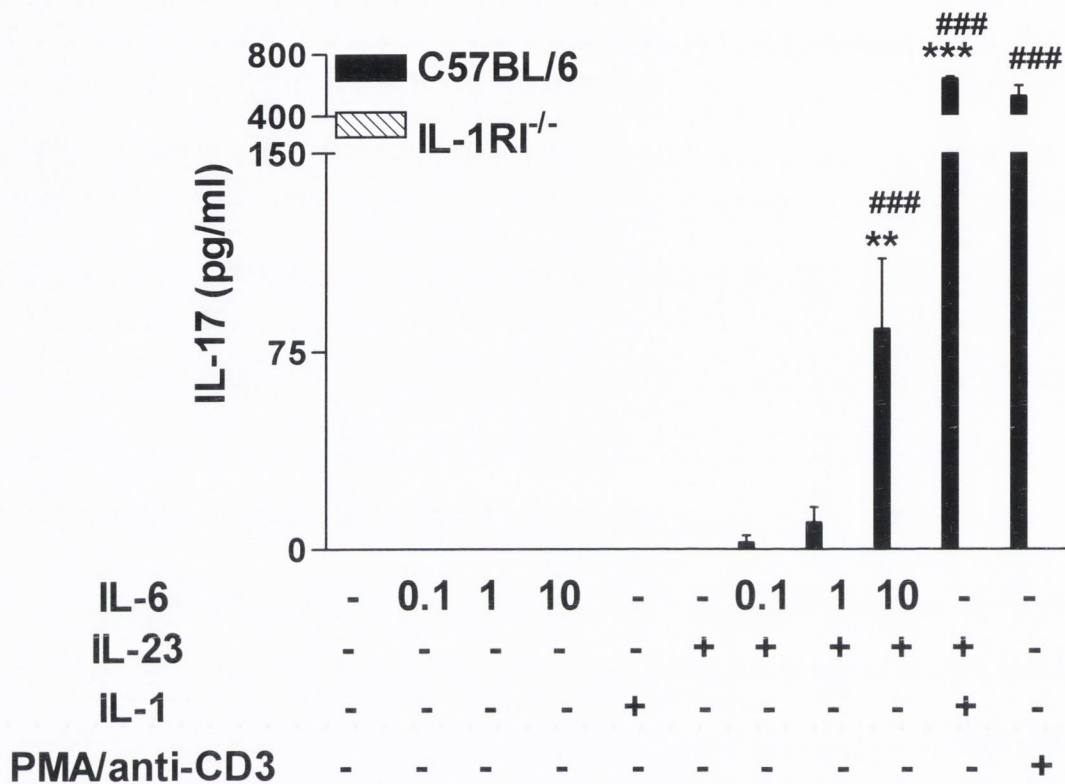


Figure 4.16: IL-6 synergises with IL-23 to promote IL-17 production in an IL-1RI dependent manner. Spleen cells from C57BL/6 and IL-1RI^{-/-} mice (10⁶ cells/ml) were stimulated with either IL-6 (0.1-10 ng/ml) in the presence and absence of IL-23 (1 ng/ml), IL-1 (0.1 ng/ml), IL-1+IL-23, PMA (1 ng/ml) and anti-CD3 (1 µg/ml) or with medium alone. Supernatants were removed after 3 d and tested for IL-17 production by ELISA. Treatment + IL-23 versus IL-23 alone: **, P < 0.01; ***, P < 0.001. C57BL/6 versus IL-1RI^{-/-}: ###, P < 0.001.

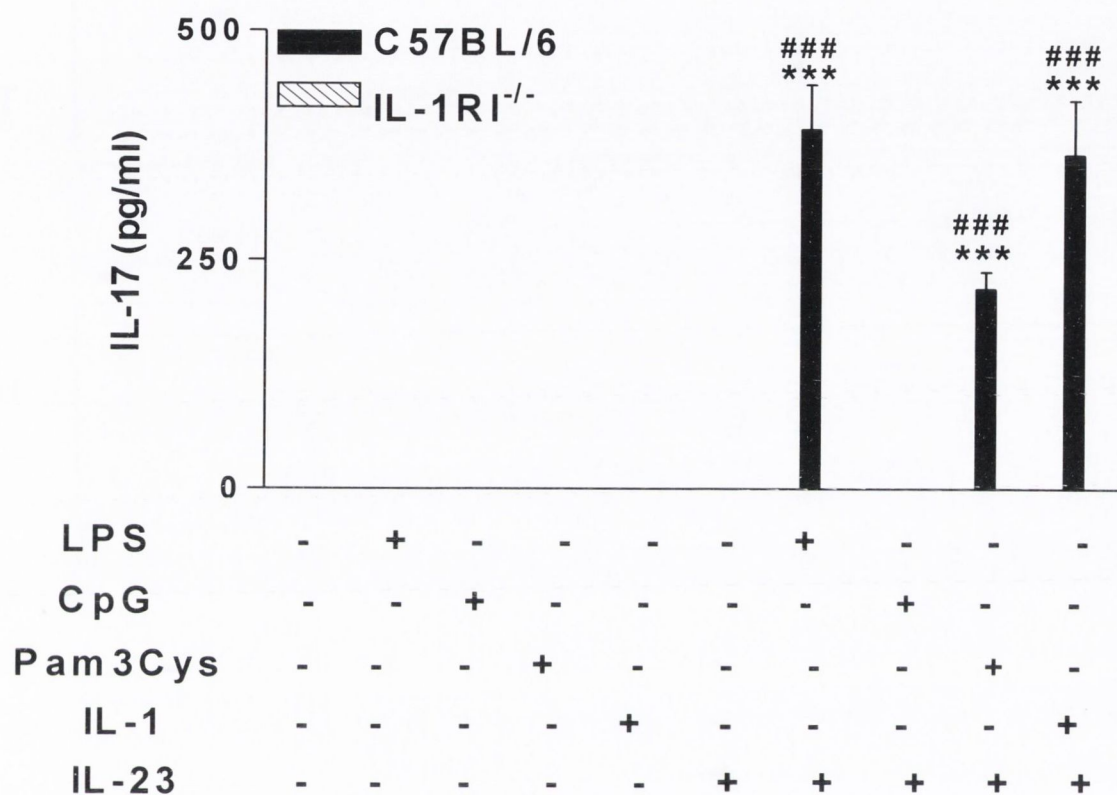


Figure 4.17: The TLR agonists, LPS and Pam3Cys, synergise with IL-23 to promote IL-17 production from spleen cells in an IL-1-dependent manner. Spleen cells from C57BL/6 and IL-1RI^{-/-} mice (5×10^5 cells/ml) were incubated with LPS (100 ng/ml), CpG (10 μ g/ml), Pam3Cys (100 ng/ml) or IL-1 (0.1 ng/ml) alone or in combination with IL-23 (1 ng/ml) or with medium alone. Supernatants were removed after 3 d and tested for IL-17 production by ELISA. Treatment + IL-23 versus IL-23 alone: ***, $P < 0.001$. C57BL/6 versus IL-1RI^{-/-}: ###, $P < 0.001$.

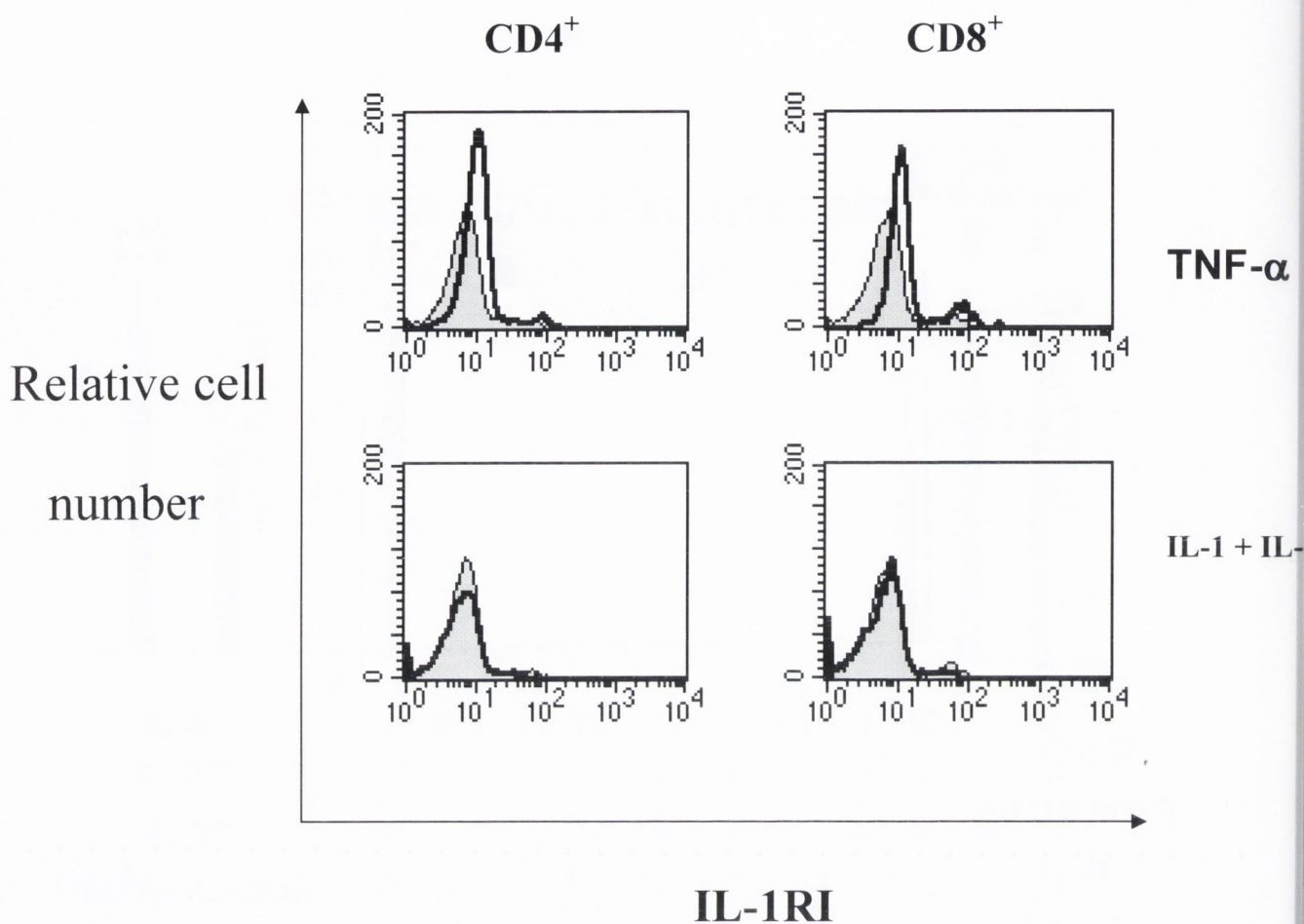


Figure 4.18: $TNF-\alpha$, but not IL-1 and IL-23, up-regulates the expression of IL-1RI on T cells. Spleen cells (2.5×10^6 cells/ml) from C57BL/6 mice were cultured with 10 ng/ml $TNF-\alpha$, IL-23 (10 ng/ml) and IL-1 β (10 ng/ml), or with medium alone for 72 h. Cells were then washed and stained with an antibody specific for IL-1RI. Results from flow cytometric immunofluorescence analysis are shown for treated (black line) compared with untreated (grey histogram) spleen cells.

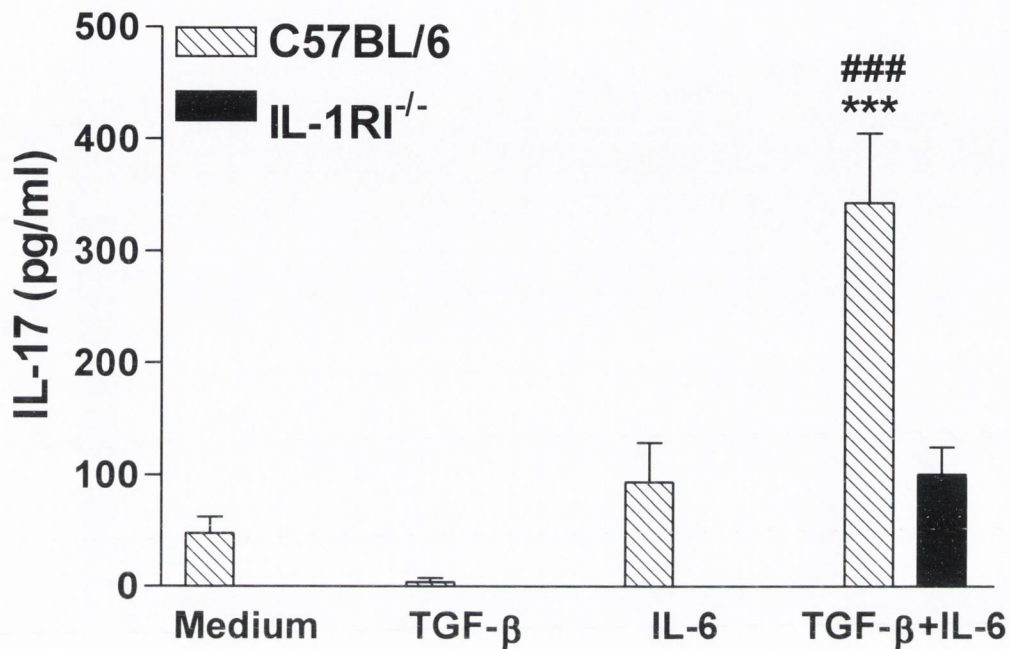


Figure 4.19: The ability of IL-6 and TGF- β to induce IL-17 production is partially IL-1RI dependent. Spleen cells from C57BL/6 and IL-1RI^{-/-} mice (5×10^5 cells/ml) were stimulated with anti-CD3 (1 μ g/ml) and anti-CD28 (10 μ g/ml) in combination with TGF- β (10 ng/ml), IL-6 (20 ng/ml), TGF- β and IL-6 or with medium alone. Supernatants removed 3 d later and tested for IL-17 production by ELISA. Treatment versus medium alone: ***, $P < 0.001$. C57BL/6 versus IL-1RI^{-/-}: ###, $P < 0.001$.

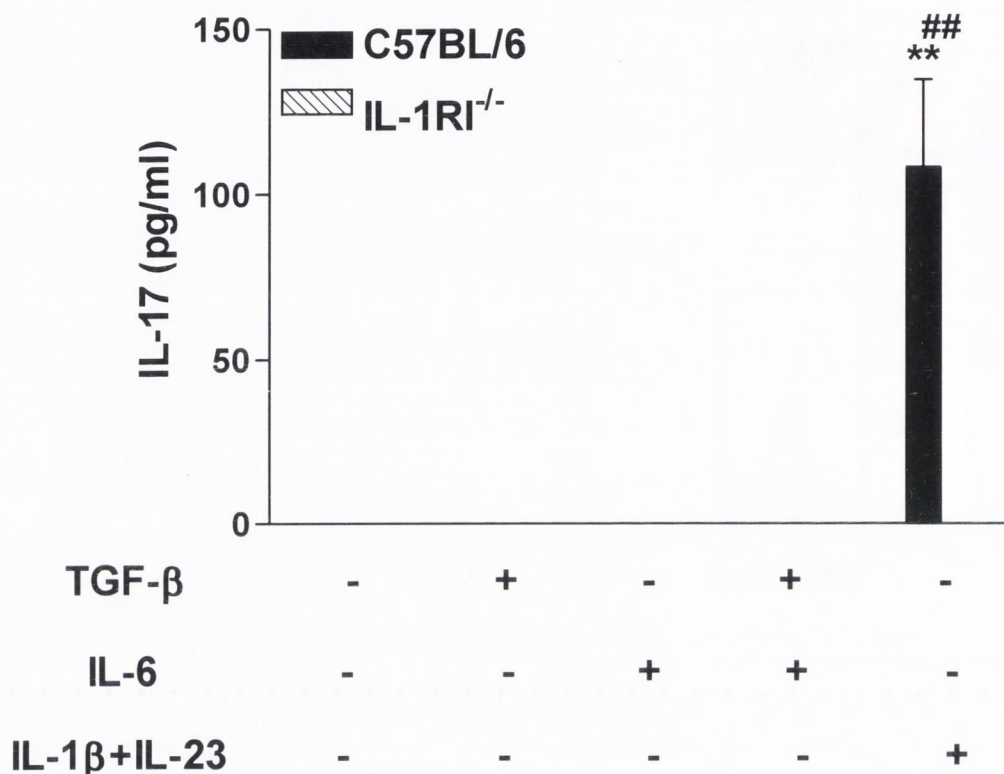


Figure 4.20: In contrast to IL-1 β and IL-23, TGF- β and IL-6 do not induce IL-17 production from spleen cells in the absence of anti-CD3 and anti-CD28 stimulation. Spleen cells from C57BL/6 and IL-1RI^{-/-} mice (5×10^5 cells/ml) were stimulated with TGF- β (10 ng/ml), IL-6 (20 ng/ml), TGF- β and IL-6, IL-1 β (100 ng/ml) and IL-23 (10 ng/ml) or with medium alone. Supernatants were removed 3 d later and tested for IL-17 production by ELISA. Treatment versus medium alone: **, $P < 0.01$. C57BL/6 versus IL-1RI^{-/-}: ##, $P < 0.01$.

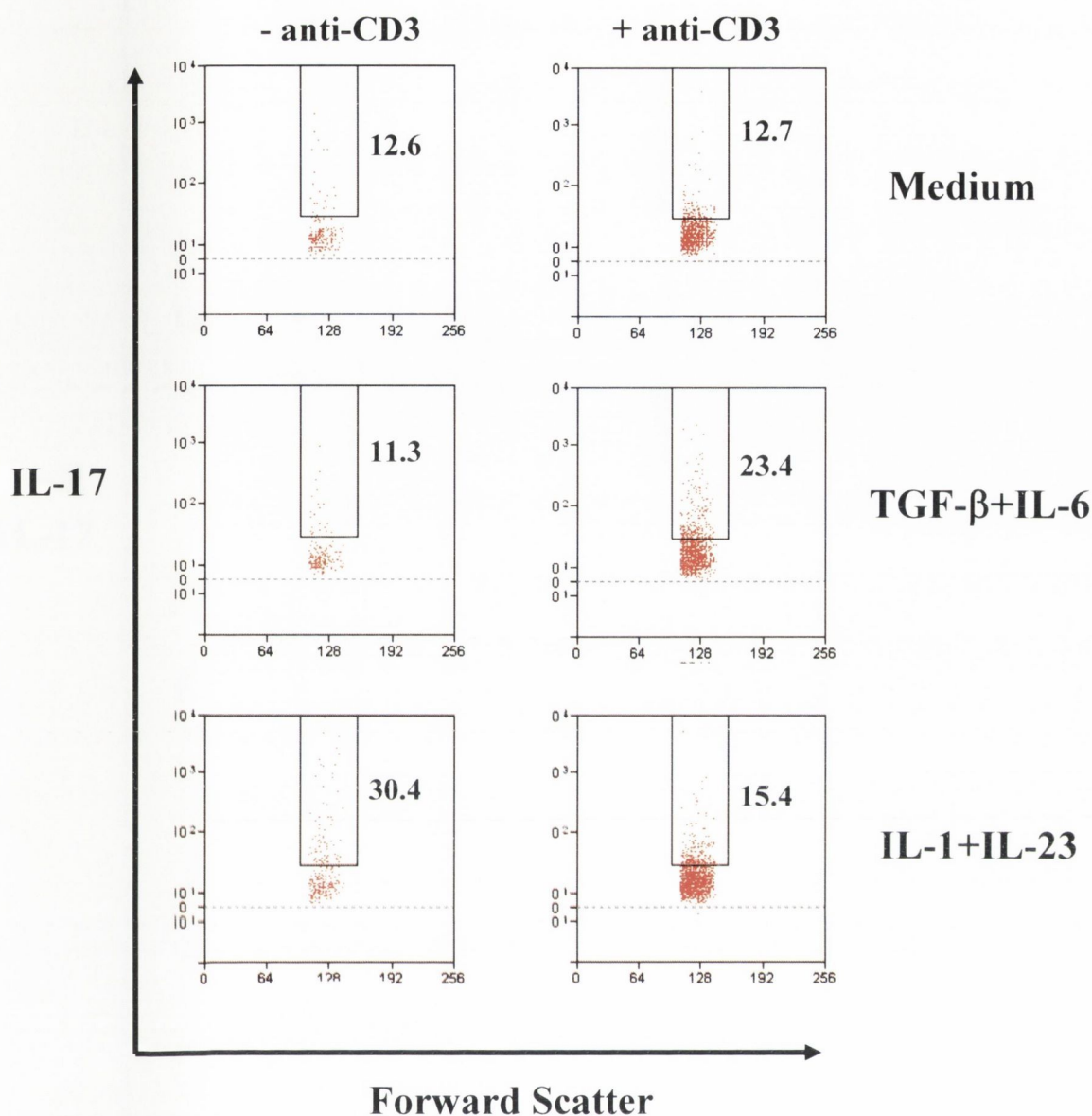


Figure 4.21: In contrast to IL-1 and IL-23 stimulation, TGF- β and IL-6 only increase IL-17 producing cells in the presence of anti-CD3. Spleen cells from C57BL/6 mice (4×10^6 cells) were cultured with IL-1 and IL-23 (both 10 ng/ml), TGF- β (10 ng/ml) and IL-6 (100 ng/ml) or with medium alone, for 3 days in the presence or absence of anti-CD3 (1 μ g/ml). For the final 6 h of culture cells were re-stimulated with PMA (10 ng/ml) and ionomycin (1 μ g/ml), and for the final 4 h with brefeldin A (10 μ g/ml). Cells were then harvested, washed, blocked and stained for intracellular IL-17. Percentages refer to live IL-17 producing cells.

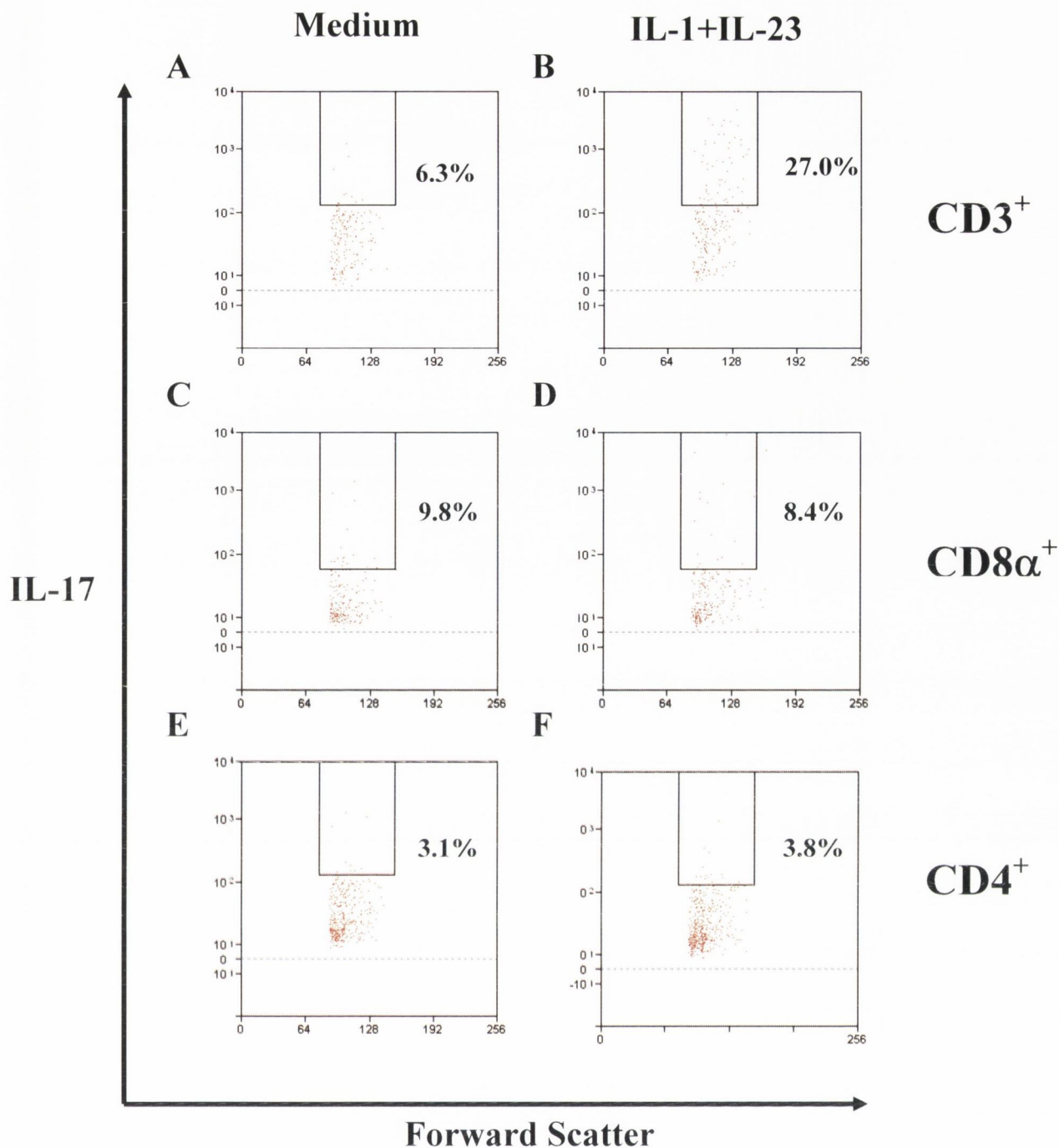


Figure 4.22: IL-1 and IL-23 increase the production of IL-17 from a CD3⁺, but non-CD4⁺ or CD8α⁺ cell in the absence of anti-CD3 stimulation. Spleen cells from C57BL/6 mice (4×10^6 cells) were cultured with IL-1 (10 ng/ml) and IL-23 (10 ng/ml) (**B, D, F**) or with medium alone (**A, C, E**), for 3 days. For the final 6 h of culture cells were re-stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml), and for the final 4 h with brefeldin A (10 μg/ml). Cells were then harvested, washed, blocked and surface stained for CD3, CD8α or CD4 and stained for IL-17 intracellularly. Percentages refer to live CD3⁺ (**A, B**), CD8α⁺ (**C, D**) or CD4⁺ (**E, F**), IL-17 producing cells.

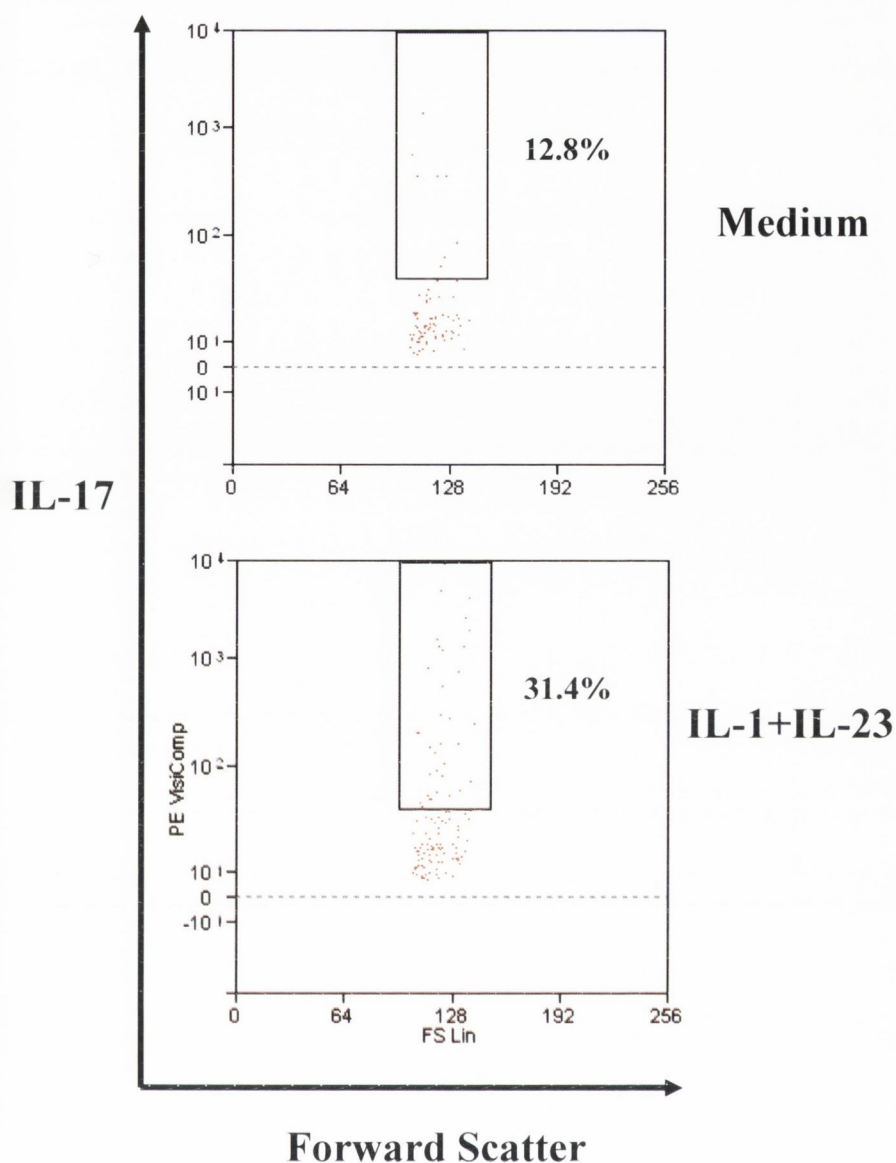


Figure 4.23: $\gamma\delta$ T cells secrete IL-17 in response to stimulation with IL-1 and IL-23. Spleen cells from C57BL/6 mice (4×10^6 cells) were stimulated with IL-1 (10 ng/ml) and IL-23 (10 ng/ml), or with medium alone for 3 d. Cells were cultured for the final 6 h with PMA (10 ng/ml) and ionomycin (1 μ g/ml), and for the final 4 h with brefeldin A (10 μ g/ml). Cells were then harvested, washed, blocked and stained for $\gamma\delta$ T cell surface expression and intracellular IL-17. Percentages refer to live IL-17 producing cells, gated on $\gamma\delta$ T cells.

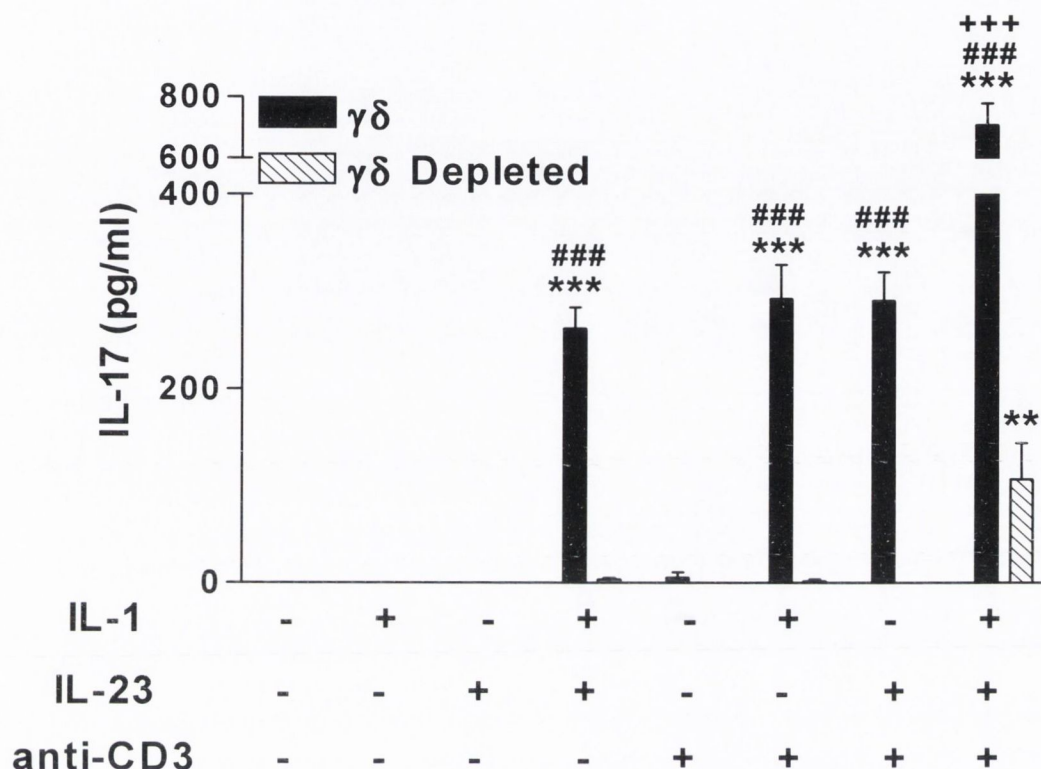


Figure 4.24: IL-1 and IL-23 can induce IL-17 production by $\gamma\delta$ T cells in an anti-CD3- independent manner. Spleen cells from C57BL/6 mice were FACS sorted into $\gamma\delta$ positive ($\gamma\delta$) and $\gamma\delta$ negative ($\gamma\delta$ Depleted) cells. $\gamma\delta$ and $\gamma\delta$ depleted T cells (3×10^5 cells/ml) were stimulated with IL-1 β (10 ng/ml), IL-23 (10 ng/ml), IL-1 β + IL-23 or with medium alone, in the presence or absence of anti-CD3 (1 μ g/ml). Supernatants were removed after 3 d and tested for IL-17 production by ELISA. Treatment versus medium alone: **, $P < 0.01$, ***, $P < 0.001$. $\gamma\delta$ versus $\gamma\delta$ depleted: ###, $P < 0.001$. IL-1 + IL-23 + anti-CD3 versus IL-1 + anti-CD3 alone and IL-23 + anti-CD3 alone: +++, $P < 0.001$.

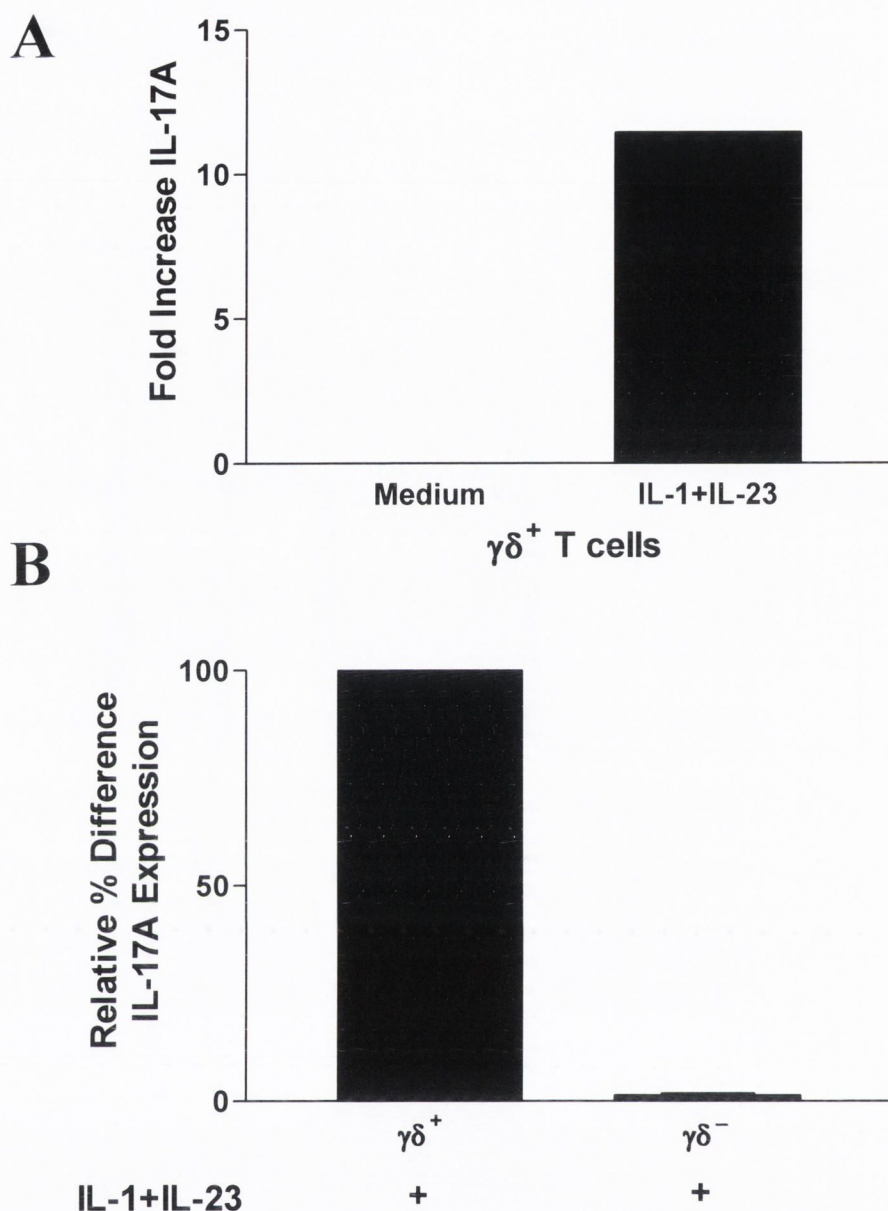


Figure 4.25: IL-1 and IL-23 stimulate IL-17A mRNA expression in $\gamma\delta$ T cells. C57BL/6 spleen cells were FACS sorted into $\gamma\delta^+$ and $\gamma\delta^-$ cells. **(A)** $\gamma\delta^+$ T cells (4×10^5 cells) were stimulated with IL-1 (10 ng/ml) and IL-23 (10 ng/ml), or with medium alone. IL-17A mRNA expression was then evaluated by real-time PCR 48 h after stimulation. The data represent the fold change for IL-17 mRNA in relation to 18S rRNA. **(B)** $\gamma\delta^+$ and $\gamma\delta^-$ T cell fractions were stimulated with IL-1 and IL-23 as above. The data represents the relative expression of IL-1 and IL-23 induced IL-17A produced by $\gamma\delta^+$ T cells versus $\gamma\delta^-$ T cells.

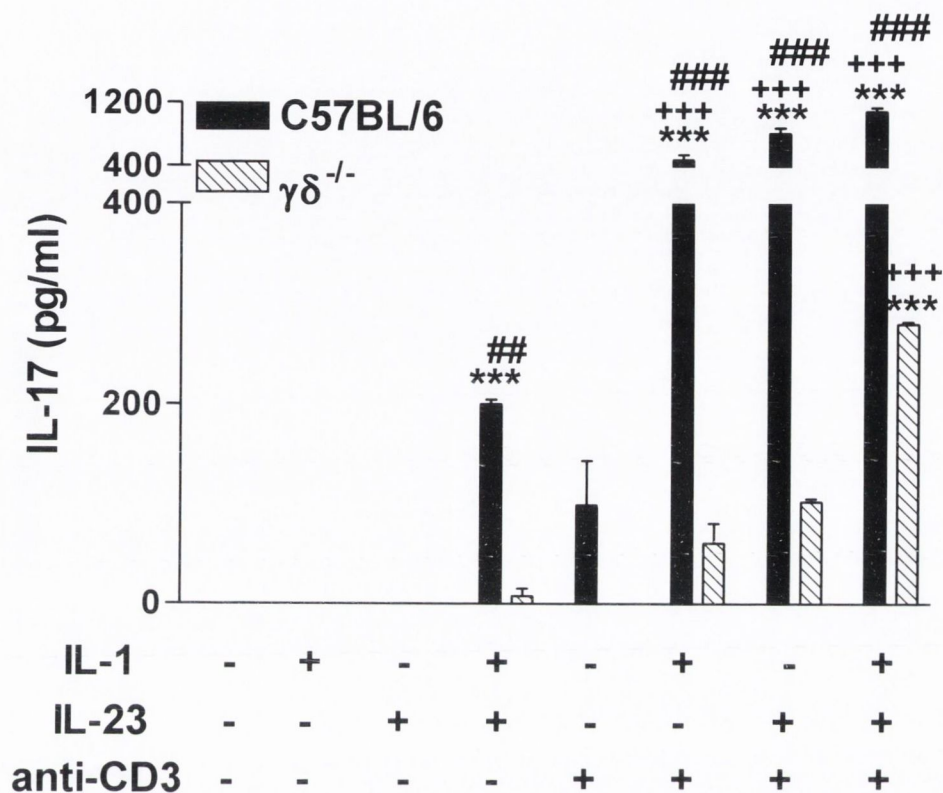


Figure 4.26: In the absence of $\gamma\delta$ T cells, splenocytes produce significantly less IL-17 in response to stimulation with IL-1 and IL-23. Spleen cells from C57BL/6 and $\gamma\delta^{-/-}$ mice (5×10^5 cells/ml) were incubated with IL-1 β (10 ng/ml), IL-23 (10 ng/ml) or IL-1 and IL-23 in the presence or absence of anti-CD3 (1 μ g/ml). Supernatants were removed after 3 d and tested for IL-17 production by ELISA. Treatment versus medium alone: ***, $P < 0.001$. With anti-CD3 versus without anti-CD3: +++, $P < 0.001$. C57BL/6 versus $\gamma\delta^{-/-}$: ##, $P < 0.01$; ###, $P < 0.001$.

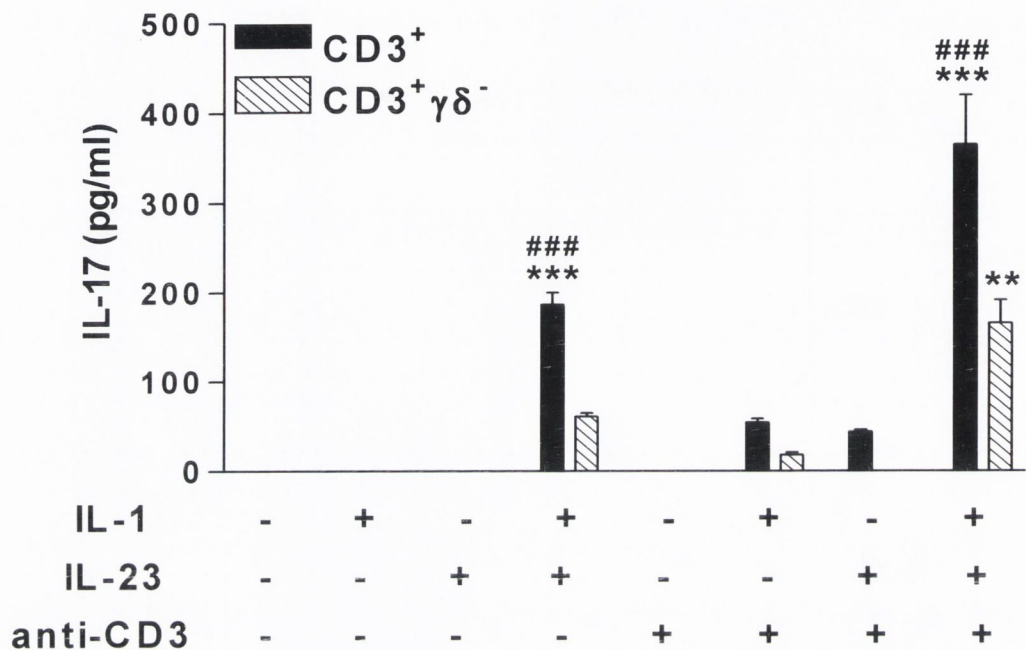


Figure 4.27: In the absence of $\gamma\delta$ T cells, $CD3^+$ T cells produce significantly less IL-17 in response to stimulation with IL-1 and IL-23. $CD3^+$ T cells were isolated from C57BL/6 spleen cells and directly incubated (5×10^5 cells/ml) or depleted of $\gamma\delta$ T cells by FACS sorting (5×10^5 cells/ml), prior to incubation, with IL-1 (10 ng/ml), IL-23 (10 ng/ml), IL-1+IL-23 or with medium alone. Supernatants were removed after 3 d and tested for IL-17 production by ELISA. Treatment versus medium alone: **, $P < 0.01$; ***, $P < 0.001$. $CD3^+$ versus $CD3^+\gamma\delta^-$: ###, $P < 0.001$.

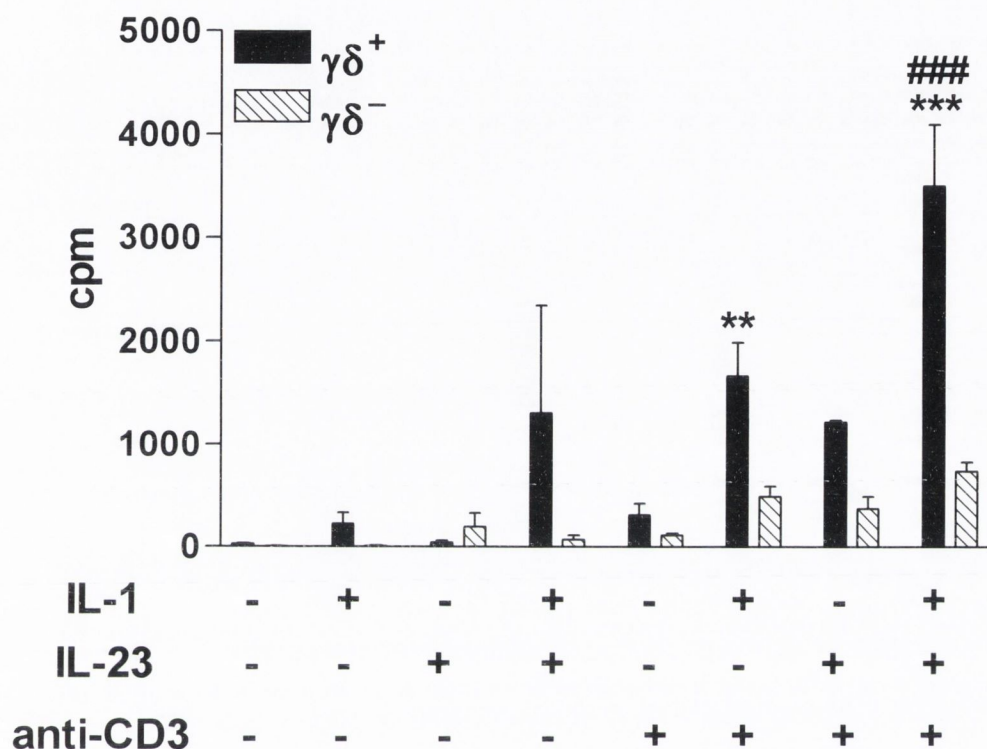


Figure 4.28: IL-1 induced $\gamma\delta$ T cell proliferation is enhanced by IL-23. Spleen cells from C57BL/6 mice were FACS sorted into $\gamma\delta$ positive ($\gamma\delta^+$) and $\gamma\delta$ negative ($\gamma\delta^-$) cells (3×10^5 cells/ml) and stimulated with medium alone, IL-1 β (10 ng/ml), IL-23 (10 ng/ml) or IL-1 β + IL-23 in the presence or absence of anti-CD3 (1 μ g/ml). After 3 d stimulation, cells were incubated for a further 6 h in the presence of thymidine [3 H]. Data represents [3 H] incorporation from isolated cells. Treatment + anti-CD3 versus anti-CD3 alone: **, $P < 0.01$, ***, $P < 0.001$. IL-1 + IL-23 + anti-CD3 versus IL-1 + anti-CD3 alone and IL-23 + anti-CD3 alone: ###, $P < 0.001$.

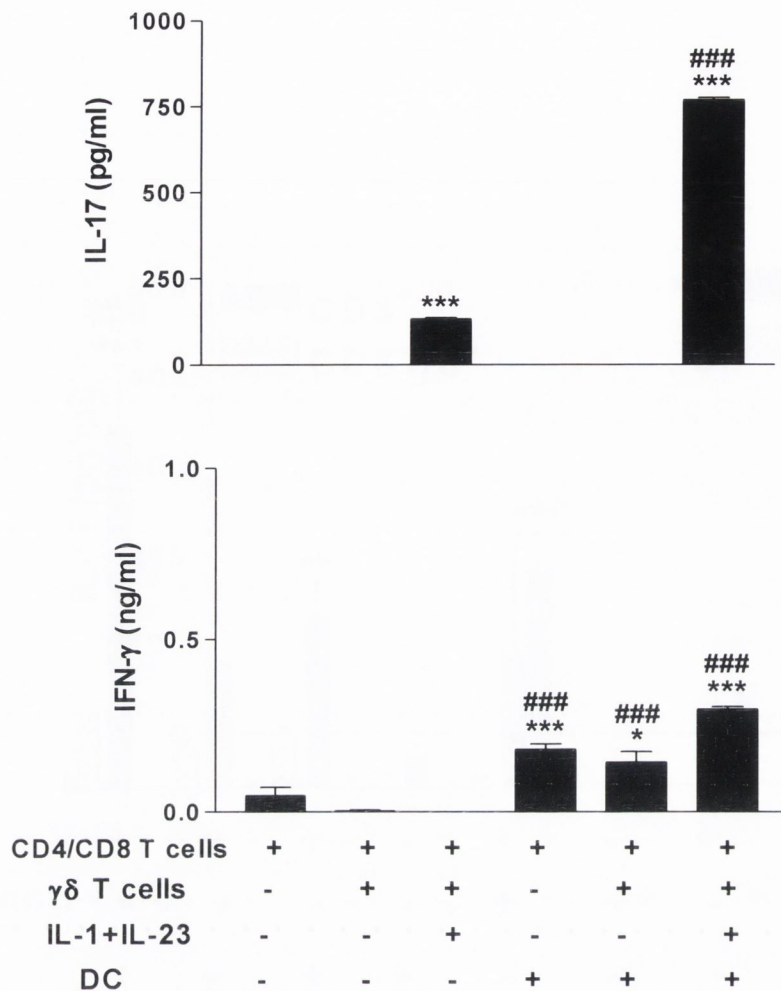


Figure 4.29: IL-1 and IL-23 stimulated $\gamma\delta$ T cells promote IL-17 production from CD4 and CD8 T cells through interaction with BMDC. $\gamma\delta^+$ T cells (4×10^5 cells/ml) were FACS sorted from C57BL/6 spleen cells and incubated with IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) or with medium alone, within a trans-well insert. After 3 d the trans-well insert was removed to a fresh well containing a combination of purified CD4 $^+$ and CD8 $^+$ T cells (6×10^5 cells/ml) with anti-CD3 (1 μ g/ml) with or without BMDC (6×10^5 cells/ml). Cells were incubated for a further 3 d, after which supernatants from outside of the trans-well were collected and tested for IL-17 and IFN- γ production by ELISA. CD4 $^+$ /CD8 $^+$ cells + treatment versus CD4 $^+$ /CD8 $^+$ cells alone: *, $P < 0.05$; ***, $P < 0.001$. CD4 $^+$ /CD8 $^+$ + $\gamma\delta$ T cells + BMDC versus CD4 $^+$ /CD8 $^+$ and $\gamma\delta$ T cells alone: ###, $P < 0.001$.

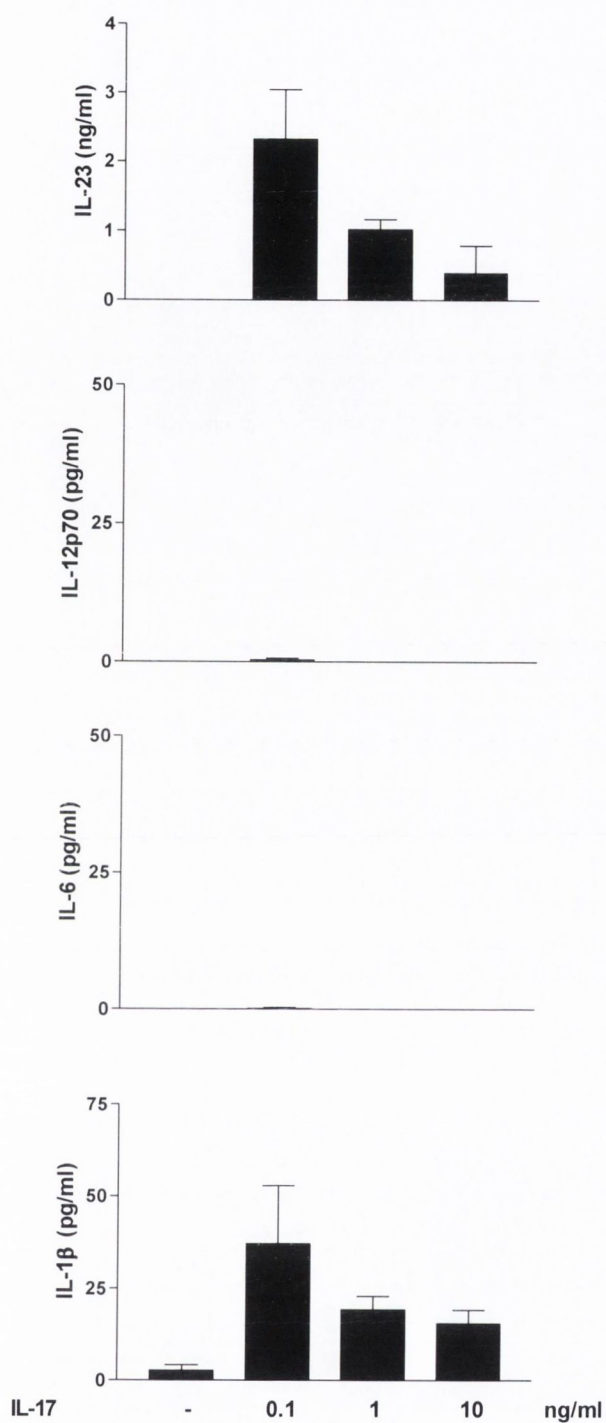


Figure 4.30: IL-17 promotes the production of IL-1 β and IL-23 from BMDC. BMDC (5×10^5 cells/ml) from C57BL/6 mice were incubated with IL-17 (0.1-10 ng/ml) or with medium alone for 24 h. Supernatants were removed and tested for IL-12p70, IL-23, IL-6 and IL-1 β production by ELISA.

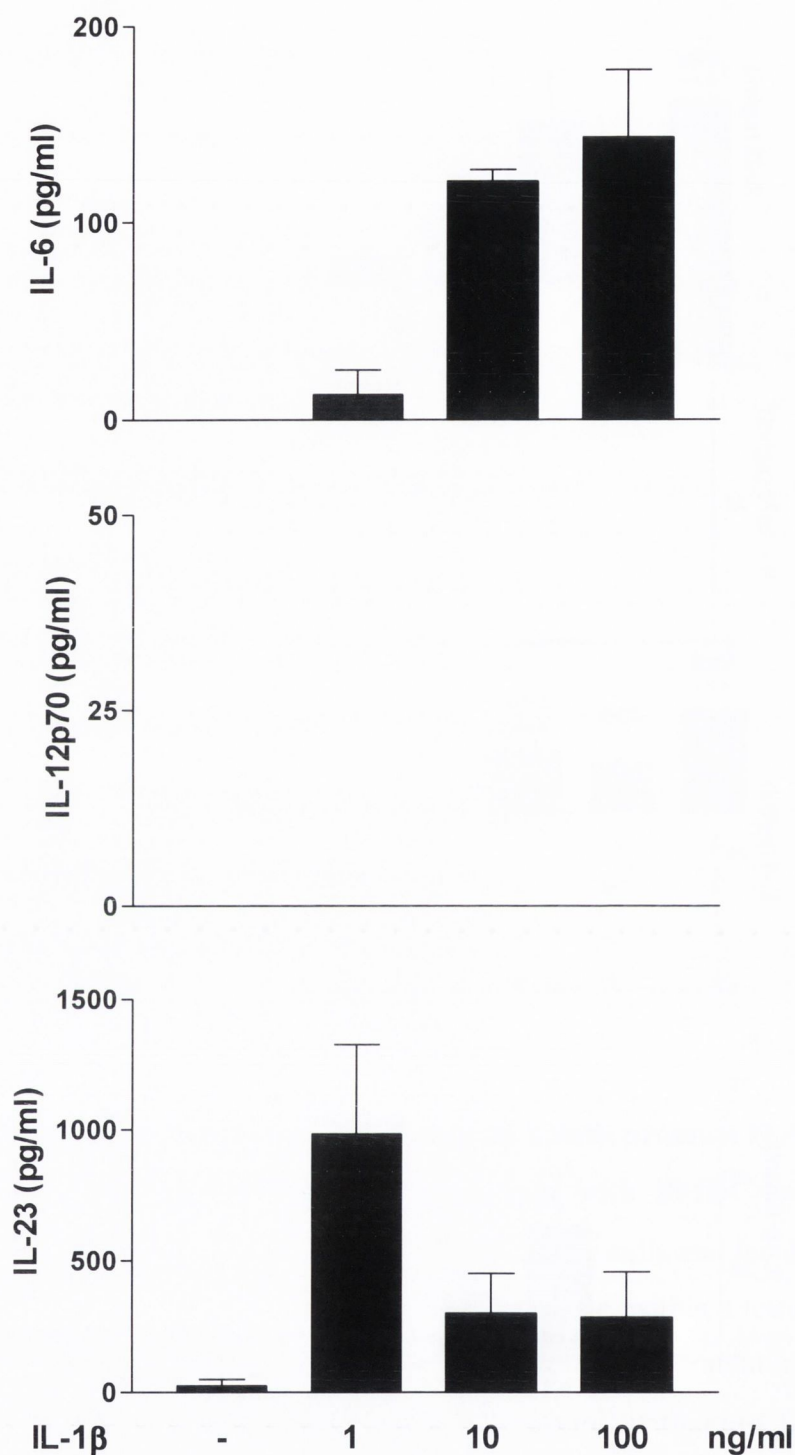


Figure 4.31: IL-1 β induces the production of IL-6 and IL-23 from BMDC. BMDC (5×10^5 cells/ml) from C57BL/6 mice, were incubated with IL-1 β (1-100 ng/ml) or with medium alone for 24 h. Supernatants were removed and tested for IL-23, IL-12p70 and IL-6 production by ELISA.

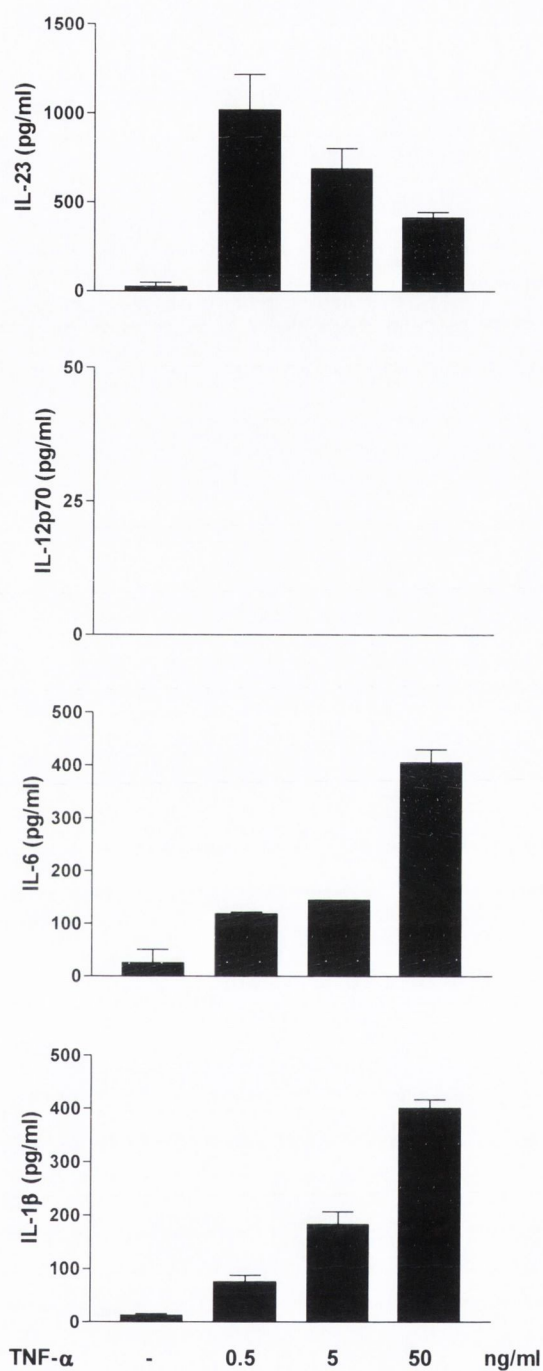


Figure 4.32: TNF- α induces the production of IL-6, IL-1 β and IL-23 from BMDC. BMDC (5×10^5 cells/ml) from C57BL/6 mice were incubated with TNF- α (0.5-50 ng/ml) or with medium alone for 24 h. Supernatants were removed and tested for IL-1 β , IL-23, IL-12p70 and IL-6 production by ELISA.

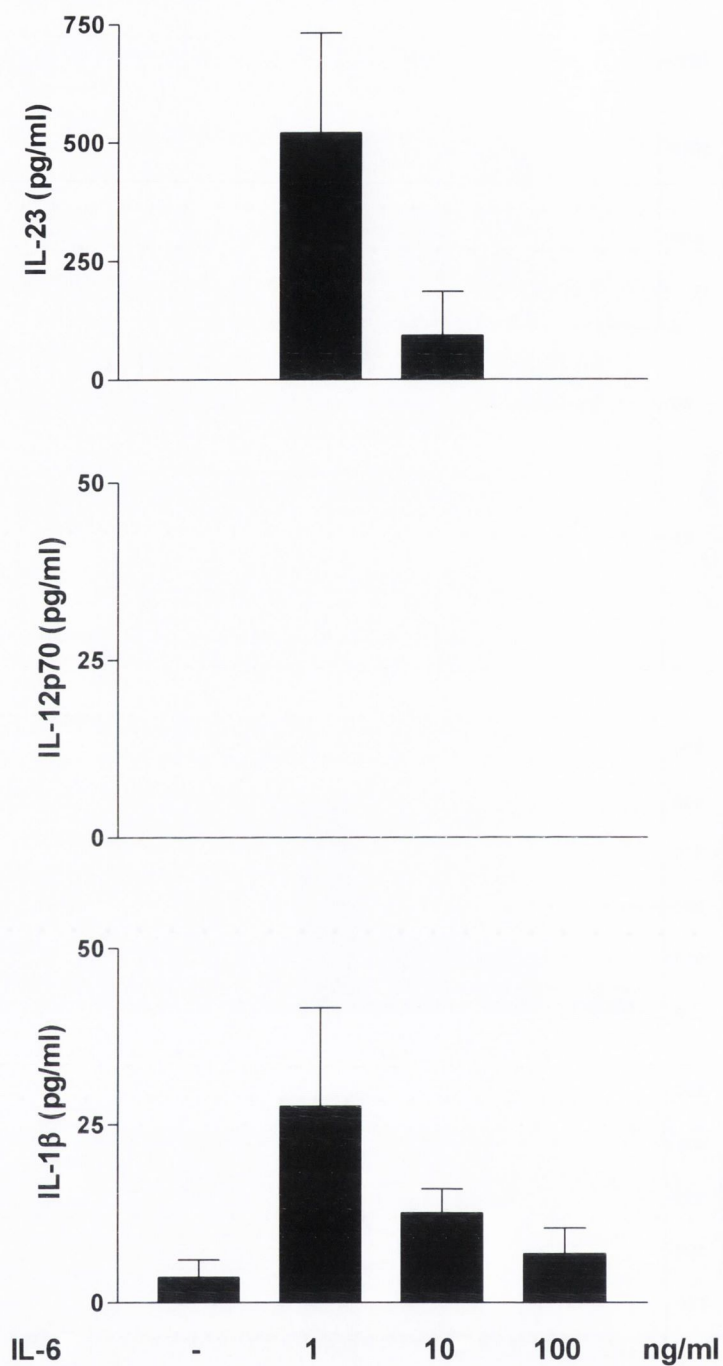


Figure 4.33: IL-6 induces the production of IL-1 β and IL-23 from BMDC. BMDC (5×10^5 cells/ml) from C57BL/6 mice were incubated with IL-6 (1-100 ng/ml) or with medium alone for 24 h. Supernatants were removed and tested for IL-1 β , IL-12p70 and IL-23 production by ELISA.

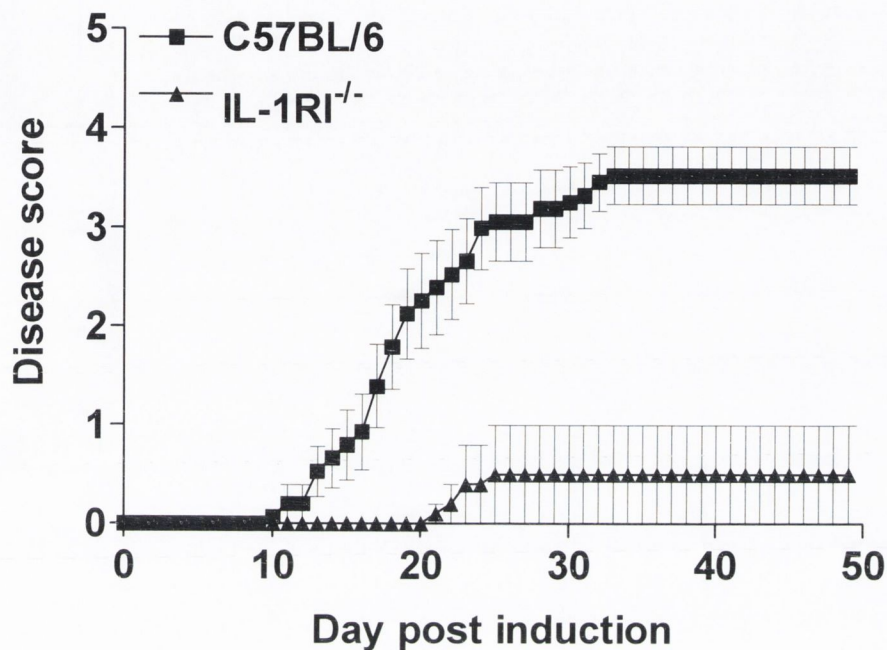


Figure 4.34: IL-1RI is required for the development of severe EAE. C57BL/6 or IL-1RI^{-/-} mice (n = 15/group) were injected with 150 µg MOG in 100 µl CFA containing 400 µg *m. tuberculosis* and 500 ng PT followed 2 d later with a second dose of PT. Mice were observed for clinical symptoms of EAE daily for 48 d and graded for disease progression +/- SD.

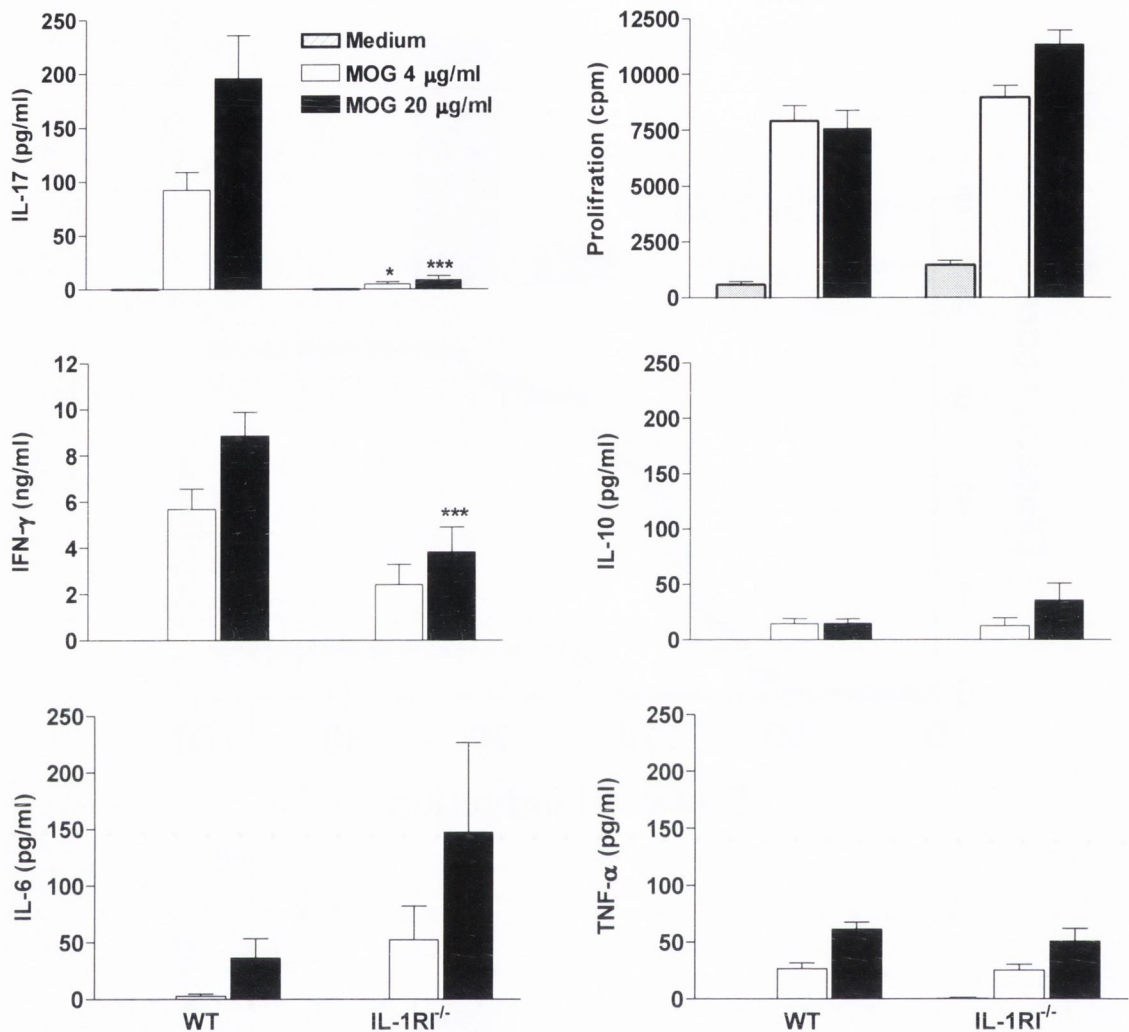


Figure 4.35: The induction of antigen-specific T_{IL-17} cells during EAE is dependent on functional IL-1RI. C57BL/6 or IL-1RI^{-/-} mice (n = 15/group) were injected with 150 µg MOG in 100 µl CFA, containing 400 µg *m. tuberculosis*, and 500 ng PT followed 2 d later with a second dose of PT. Mice were killed on d 21, and spleen cells re-stimulated with either 4 or 20 µg/ml MOG peptide or with medium alone. After 3 d, supernatants were collected and cytokine concentrations determined by ELISA. C57BL/6 versus IL-1RI^{-/-}: *, P < 0.05; *** P < 0.001.

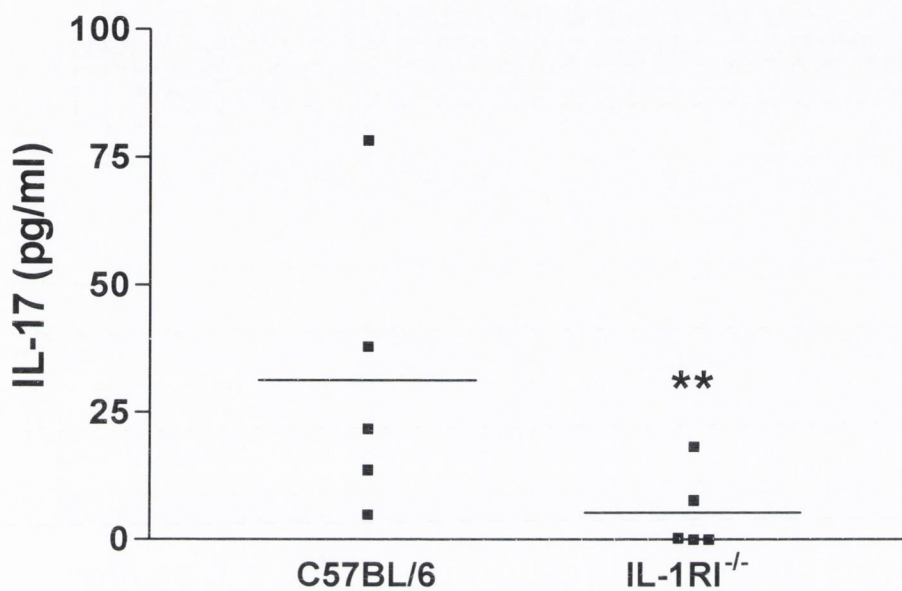


Figure 4.36: Serum IL-17 concentrations are significantly lower in IL-1RI^{-/-} than wild type mice after induction of EAE. EAE was induced in C57BL/6 or IL-1RI^{-/-} mice (n = 5/group), as described in Fig 4.29. Mice were killed on d 20 and the serum assayed for the presence of IL-17 by ELISA. C57BL/6 versus IL-1RI^{-/-}: **, P < 0.01.

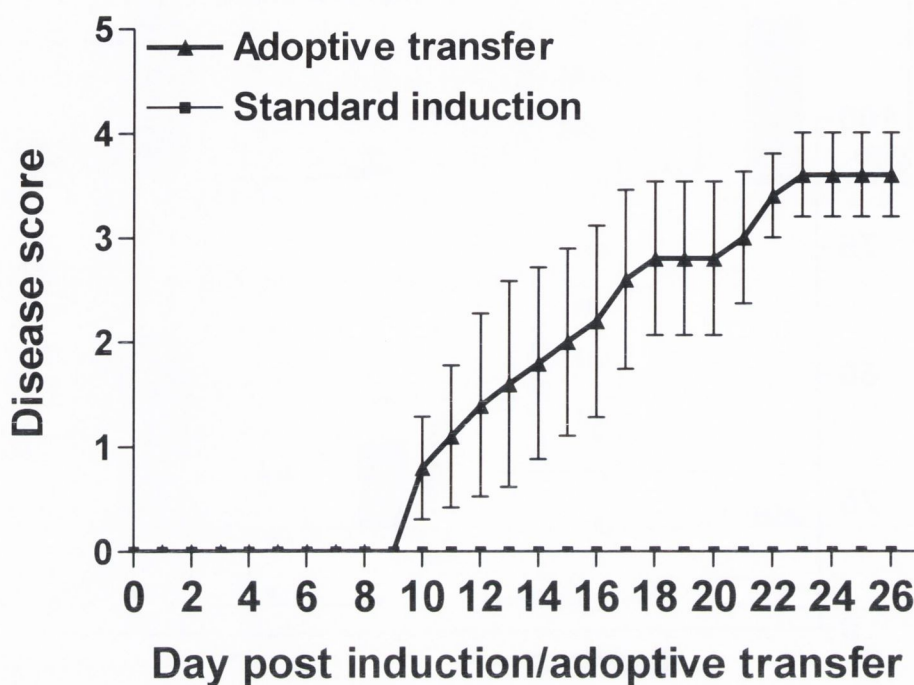


Figure 4.37: IL-1RI is required up-stream of T cell activation in the induction of EAE. IL-1RI^{-/-} mice (n = 7) were injected with 150 µg MOG in 100 µl CFA, containing 400 µg *m. tuberculosis*, or with MOG-specific T cells (9×10^6 cells/mouse) generated from the spleens of C57BL/6 mice with EAE, and 500 ng PT followed 2 d later with a second dose of PT. Mice were graded daily for 26 d for disease development +/- SD.

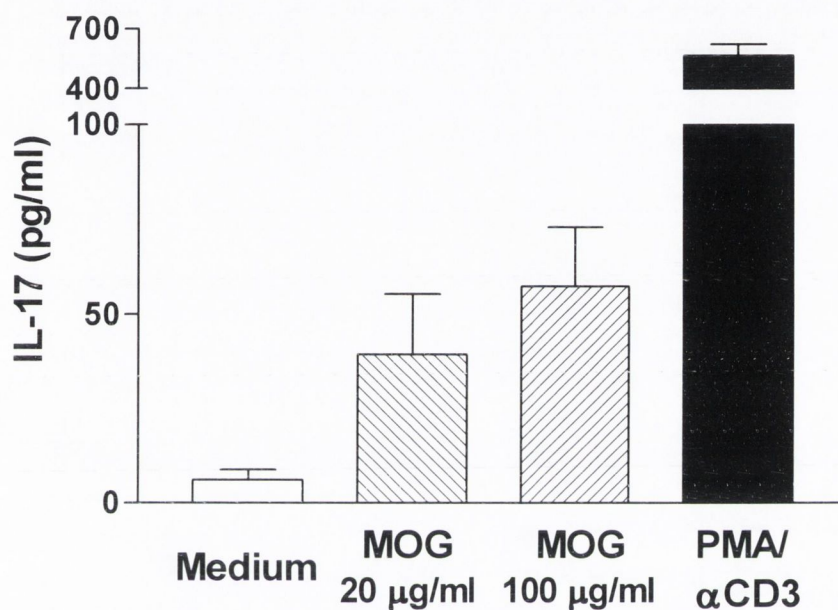


Figure 4.38: Development of EAE in IL-1RI^{-/-} mice, induced by transfer of MOG-specific T cells, correlates with antigen-specific IL-17 production. IL-1RI^{-/-} mice (n = 7) were injected with MOG-specific T cells (9×10^6 cells/mouse, generated from the spleens of C57BL/6 mice with EAE), and 500 ng PT followed 2 d later with a second dose of PT. Spleen cells were harvested when mice reached disease grade 4 and cells re-stimulated with medium, 20 or 100 µg/ml MOG or with PMA (1 ng/ml) and anti-CD3 (1 µg/ml), for 3 d. Supernatants were collected and tested for IL-17 production by ELISA.

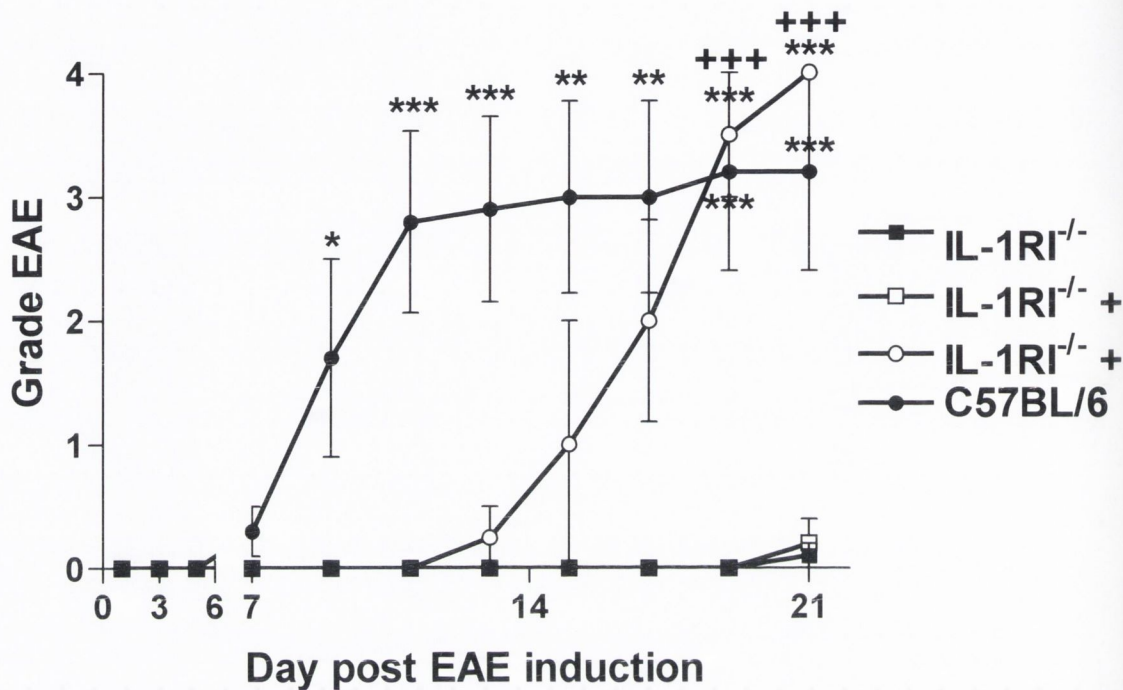


Figure 4.39: IL-1RI on $\gamma\delta$ T cells alone cannot transfer EAE in IL-1RI^{-/-} mice. $\gamma\delta^+$ and CD3⁺ cells were FACS sorted from C57BL/6 spleen cells. $\gamma\delta$ T cells or CD3⁺ cells were then adoptively transferred into IL-1RI^{-/-} mice. 24 h post adoptive transfer, transferred mice, as well as IL-1RI^{-/-} mice, which did not receive cells (negative control), and C57BL/6 mice (positive control), were immunised for EAE induction with 150 μ g MOG in 100 μ l CFA, containing 400 μ g *m. tuberculosis*, and 500 ng PT followed 2 d later with a second dose of PT. Mice were observed for clinical symptoms of EAE daily for 21 d and graded for disease progression $\pm$ SD, n = 5. C57BL/6 versus IL-1RI^{-/-}: *, P < 0.05; **, P < 0.01; ***, P < 0.001. IL-1RI^{-/-} + CD3⁺ versus IL-1RI^{-/-} + $\gamma\delta^+$: +++, P < 0.001.

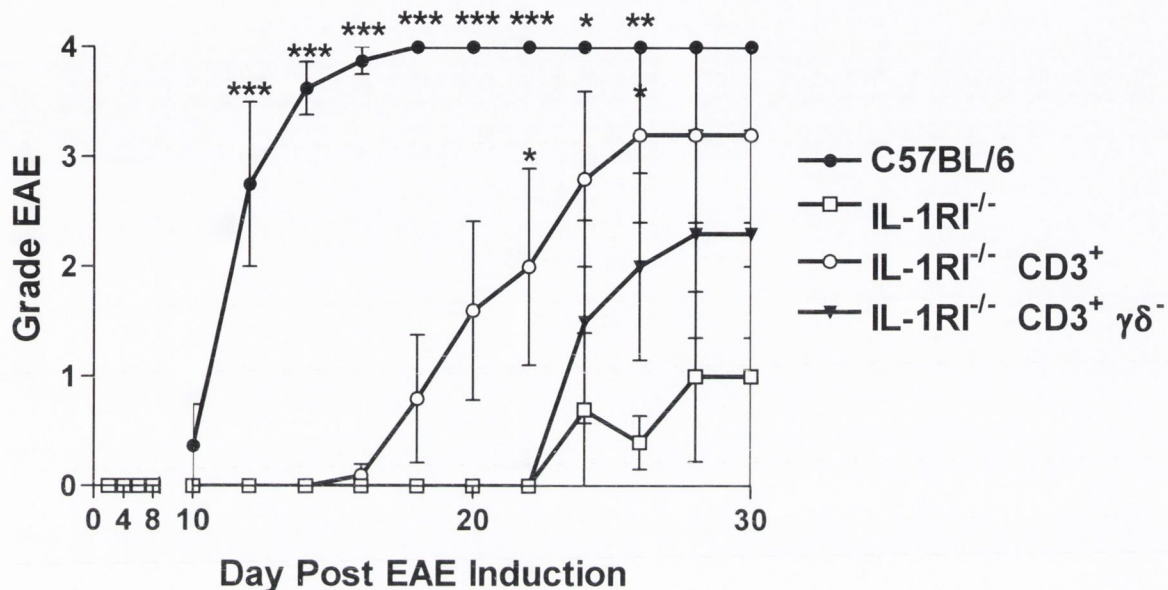


Figure 4.40: Functional IL-1RI is required on both $\gamma\delta$ T cells and $\alpha\beta$ T cells for the development of EAE in IL-1RI^{-/-} mice. CD3⁺ T cells were isolated from C57BL/6 spleen cells and either adoptively transferred into IL-1RI^{-/-} mice (IL-1RI^{-/-} CD3⁺ $\gamma\delta$ ⁺) or depleted of $\gamma\delta$ T cells by FACS sorting and then adoptively transferred into IL-1RI^{-/-} (IL-1RI^{-/-} CD3⁺ $\gamma\delta$ ⁻). 24 h post adoptive transfer, transferred mice, as well as IL-1RI^{-/-} mice (negative control), and C57BL/6 mice (positive control), which did not receive cells, were immunised for EAE induction with 150 μ g MOG in 100 μ l CFA, containing 400 μ g *m. tuberculosis*, and 500 ng PT followed 2 d later with a second dose of PT. Mice were observed for clinical symptoms of EAE daily for 30 d and graded for disease progression +/- SD. C57BL/6 or IL-1RI^{-/-} CD3⁺ versus IL-1RI^{-/-}: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

		IL-23	IL-1	IL-18	IL-33	TNF- α	IL-6	TGF- β	IL-1 +IL-23	IL-18 +IL-23	IL-33 +IL-23	TNF- α +IL-23	IL-6 +IL-23
	C57BL/6	0–10 [§]	0	0	0	0	0	0	200–500	150	↔	150	100
	IL-1RI ^{-/-}	0	0	0	0	0	0	0	↔	ND	↔	ND	ND
+ α CD3	C57BL/6	>300	250	250	600	ND	0	0	>1000	600	1200	ND	ND
	IL-1RI ^{-/-}	0	0	ND	0	ND	0	0	↔	ND	↔	ND	ND

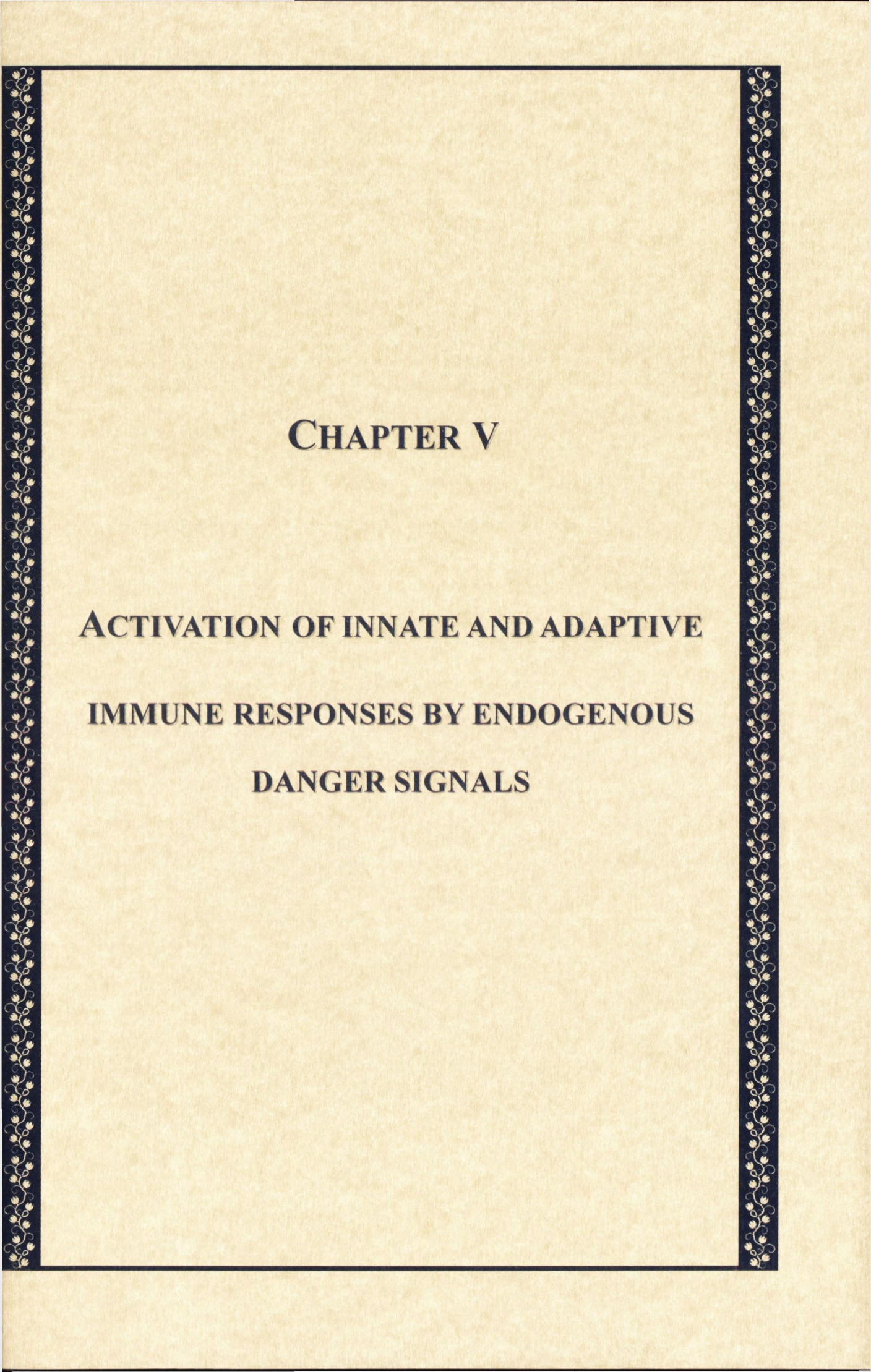
§; pg/ml

↔; no significant difference in IL-17 production compared with recombinant cytokines alone.

↓↓↓; significant reduction compared with IL-23 alone; p < 0.001.

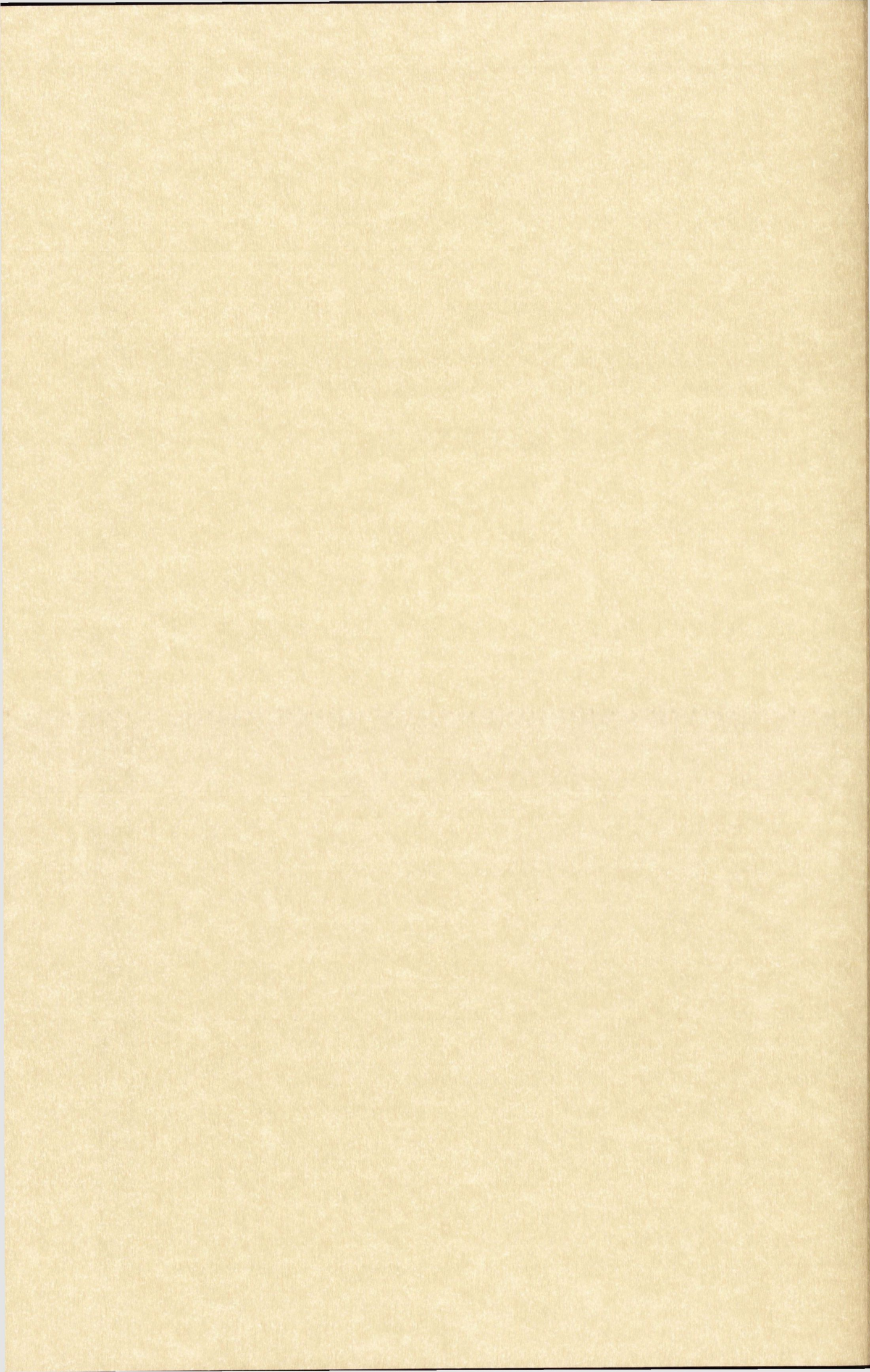
ND; non-defined.

Table 4.1: Concentrations of IL-17 detected by ELISA in spleen cell supernatants from C57BL/6 and IL-1RI^{-/-} mice, following 3 d stimulation with IL-23, IL-1, IL-18, IL-33, TNF- α , IL-6 or TGF- β in the presence and absence of anti-CD3 stimulation.



CHAPTER V

ACTIVATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES BY ENDOGENOUS DANGER SIGNALS



5.1 INTRODUCTION

Polly Matzinger first proposed the “danger theory” in the mid nineties, putting forward the hypothesis that factors present in the body could prime the immune system and cause inflammation following damage (Matzinger, 1997; Matzinger, 1998). She suggested that in addition to pathogen associated molecular patterns (PAMPs) of bacteria and viruses that the innate immune system also responded to self through recognition of danger associated molecular patterns (DAMPs) from dead or dying cells (Seong and Matzinger, 2004). However, this did not explain immune responses that mediate autoimmune disease, tissue graft rejection or anti-tumour immunity, none of which involve non-self or foreign antigens. Matzinger proposed that endogenous factors were capable of immune activation under certain conditions and that normal stasis sequestered these factors away from the cells of the immune system. In contrast, during uncontrolled cell death, as seen during necrosis, these normally intracellular factors would be released into the intercellular space and encounter innate immune cells, priming immune and inflammatory responses. This immune activation is confined to factors released following cell necrosis and not apoptosis, where the factors are safely removed by scavenging cells (Basu *et al.*, 2000; Fadok *et al.*, 2001; Sauter *et al.*, 2000; Stenfeldt and Wenneras, 2004). However, in environments where apoptosis is prevalent there is the potential for danger signals to escape if there is a shortage of scavenging cells present to remove the apoptotic cells. In this case the apoptotic cells may proceed to secondary necrosis, thus releasing their contents. Indeed innate cells such as macrophages, DC and eosinophils have been shown to be activated upon encountering these necrotically

released factors in vitro (Basu *et al.*, 2000; Fadok *et al.*, 2001; Salio *et al.*, 2000; Stenfeldt and Wenneras, 2004).

This may represent a highly conserved pathway responsible for the detection of damage within the individual, arising prior to that responsible for the detection of PAMPs. An adaptation of this theory has recently proposed that these “self” molecules only become stimulatory when proteins are mis-folded, such as in times of stress or injury, and reveal their hydrophobic residues (Seong and Matzinger, 2004). A study of hyaluronan has provided support for this theory. Under normal stasis hyaluronan, which lines the blood vessels, remains in its non-immunostimulatory high-molecular weight form. However, during tissue damage this high-molecular weight form is broken down due and a low-molecular weight form, where normally hidden hydrophobic residues expressed become immunostimulatory (Nakamura *et al.*, 2004; Seong and Matzinger, 2004).

Among the factors identified as “danger signals” include the nucleic high mobility group box 1 (HMGB1), cytoplasmic heat shock proteins and hyaluronic acid, although many others are emerging (Basu *et al.*, 2000; Gallucci *et al.*, 1999; Gallucci and Matzinger, 2001; Shi *et al.*, 2003). However, the significance of these factors in immune stimulation is subject to debate. Contamination of samples with bacterial products, especially by the TLR4 agonist LPS, is an issue, since LPS is a potent activator of innate cells. Indeed the claims in many papers suggesting a role for TLR-activation by endogenous factors have been contested due to possible contamination of samples with bacterial components, such as LPS and the TLR2 agonist peptidoglycan (Salio *et al.*, 2000). Therefore, there is still much debate surrounding the danger theory with many divided as to whether there are indeed any endogenous danger signals capable of immune activation. The aim of this project was to examine

the immune activating capacity of endogenous factors, such as pancreatic elastase, HMGB1 and histamine releasing factor (HRF), in the absence of LPS-contamination. In order to out-rule the possible contribution of any contaminating LPS all experiments with the potential DAMPs were preformed in TLR4-defective C3H/HeJ mice, as well as in the wild type C3H/HeN mice, which retain the ability to respond to LPS. CpG was used as control PAMP, to ensure that responses with cells from both C3H/HeN and C3H/HeJ were comparable. This study also aimed to examine whether the endogenous danger signals were capable of promoting DC maturation in a similar fashion to that displayed by the TLR agonists. Furthermore, experiments were carried out to examine possible synergy between endogenous danger signals and LT for cytokine production as it was shown in Chapter 3 that LT synergised with the TLR agonist LPS to induce cytokine production from DC. Since LT acts as an adjuvant *in vivo* in the absence of TLR agonists synergy with DAMPs may explain the adjuvant properties of the toxin.

5.2 RESULTS

5.2.1 Endogenously-derived danger signals activate BMDC

To assess whether DC from C3H/HeN and C3H/HeJ mice responded comparably, DC from both mouse strains were incubated with the TLR9 agonist, CpG. Stimulation of BMDC from C3H/HeN mice with CpG enhanced surface expression of B7-H1, ICAM-1, CD40 and MHCII. Furthermore, CpG-induced up-regulation was comparable for DC from C3H/HeN and C3H/HeJ mice (Fig. 5.1). The endogenous danger signals, elastase, HMGB1, HRF or the cleaved version of this protein cHRF, were next examined for their ability to mature DC through assessment of surface marker expression. Elastase, HMGB1, HRF and cHRF each enhanced the expression of B7-H1, ICAM-1, CD40 and MHCII on DC from both C3H/HeN and C3H/HeJ mice (Fig. 5.2, Fig. 5.3).

5.2.2 Endogenously-derived danger signals induce pro-inflammatory cytokine production from BMDC

The data in Fig. 5.2 and Fig. 5.3 demonstrated that endogenous danger signals up-regulated expression of MHCII and co-stimulatory markers on DC independently of TLR4. Therefore elastase, HMGB1, HRF and cHRF were examined for their ability to induce cytokine production from DC. The TLR9 agonist, CpG, was included as a positive control. Stimulation of BMDC with CpG induced the production of significant concentrations of the pro-inflammatory cytokines IL-1 β , TNF- α , IL-6, IL-12, the chemokine MIP-1 α , as well as the anti-inflammatory cytokine IL-10 in DC

from both C3H/HeN and C3H/HeJ mice (Fig. 5.4, Fig. 5.5). Similarly, s elastase induced significant concentrations of IL-1 β , TNF- α , IL-6 and MIP-1 α by DC from both C3H/HeN and C3H/HeJ mice (Fig. 5.4), although concentrations produced by DC from C3H/HeJ mice were reduced compared with those produced by DC from C3H/HeN mice. Stimulation of DC from C3H/HeJ mice with the highest dose of elastase examined, induced concentrations of IL-1 β , IL-6 and TNF- α , which were comparable with those generated after stimulation with CpG (Fig. 5.4). However, in contrast to stimulation with CpG, elastase induced low concentrations of IL-10, IL-12p40 and IL-12p70 in DC from C3H/HeN mice, and undetectable concentrations of these cytokines in DC from C3H/HeJ mice (Fig. 5.5).

Stimulation of DC with the endogenous factor, HMGB1, induced significant concentrations of TNF- α , IL-6, IL-10 and IL-12p40 by DC from C3H/HeN mice, and generated IL-1 β production above that produced by un-stimulated DC (Fig. 5.6, Fig. 5.7). However, HMGB1-induced IL-10 production in DC from C3H/HeJ mice was not significantly above that induced by medium alone. HMGB1-induced cytokine production was almost identical in DC from C3H/HeN and C3H/HeJ mice, demonstrating that the responses were not mediated by LPS or by signalling through TLR4. Indeed the concentration of IL-6 induced by the highest concentration of HMGB1 examined, 25 μ g/ml, was comparable with CpG-induced IL-6 production by DC. HMGB1 did not induce MIP-1 α or IL-12p70 production in DC from either C3H/HeN or C3H/HeJ mice (Fig. 5.6, Fig. 5.7). Furthermore, stimulation with CpG, elastase and HMGB1 induced significant concentrations of IL-23 in DC from C3H/HeJ mice (Fig. 5.8). However, this IL-23 production was only observed for the two highest doses of elastase (4 and 20 μ g/ml) and HMGB1 (5 and 25 μ g/ml) examined.

Stimulation of DC from both C3H/HeN and C3H/HeJ mice with HRF and cHRF induced production of the pro-inflammatory cytokines IL-1 β , TNF- α and IL-6, as well as the chemokine MIP-1 α at concentrations, which were significantly above that induced by medium alone (Fig. 5.9). The concentration of MIP-1 α produced by DC from C3H/HeJ mice in response to stimulation with HRF and cHRF, was much lower than concentrations produced by DC from C3H/HeN mice (Fig. 5.9). The uncleaved HRF protein was also capable of inducing small concentrations of IL-10 in DC from both C3H/HeN and C3H/HeJ mice, which were significantly higher than those induced with medium alone (Fig. 5.10). However, cHRF was unable to induce IL-10 in DC from C3H/HeJ mice. Neither HRF nor cHRF induced IL-12 production in DC from C3H/HeJ mice (Fig. 5.10).

5.2.3 Immunisation with antigen in the presence of HRF or cHRF as an adjuvant induces a Th2 type response

In order to test the adjuvant effect of HRF *in vivo*, mice were immunised with KLH in the presence or absence of HRF and cHRF. Immunisation with KLH alone induced antigen-specific IFN- γ and IL-10 producing T cells in draining lymph nodes, but this was not significantly above those induced after immunisation with PBS alone (Fig. 5.11). Antigen-specific T cells from mice immunised with KLH and either HRF or cHRF produced significantly higher concentrations of IL-5 and IL-4 when compared with T cells from mice immunised with KLH alone (Fig. 5.11). However, IL-10 production was not affected by the presence of HRF or cHRF, while KLH-specific IFN- γ production was reduced by co-administration of KLH with HRF, but not cHRF (Fig 5.11).

5.2.4 Immunisation with KLH in the presence of HRF or cHRF enhances antigen-specific antibody production

Having demonstrated an adjuvant effect of HRF and cHRF on T cell responses, experiments were performed to examine the effect of immunisation with KLH and either HRF or cHRF on antibody responses. Immunisation with KLH in the presence of either HRF or cHRF enhanced the production of KLH-specific antibody in the serum of immunised mice (Fig. 5.12). The concentrations of IgG2a titres produced after immunisation with KLH and either HRF or cHRF were not significantly higher than that induced after immunisation with KLH alone (Fig. 5.12). In contrast, immunisation with KLH in the presence of either HRF or cHRF significantly enhanced IgG1 antibody titres in the serum, above that seen after immunisation with KLH alone (Fig. 5.12).

5.2.5 Immunisation with antigen in the presence of HMGB1 as an adjuvant induces antigen-specific IL-10 and IL-5 production

Subsequently, the adjuvant effect of HMGB1 was examined *in vivo*. C57BL/6 mice were immunised with KLH in the presence or absence of HMGB1. Immunisation with KLH alone induced a small number of antigen-specific IL-4 and IL-5, but not IFN- γ or IL-10, producing T cells in draining lymph nodes. KLH-specific IL-4 and IL-5 producing T cells were not significantly above those induced after immunisation with PBS alone (Fig. 5.13). Immunisation with KLH and HMGB1 produced higher concentrations of antigen-specific IL-5 and IL-10 when compared with mice immunised with KLH alone, while concentrations of antigen specific IL-4

and IFN- γ were not increased upon immunisation with HMGB1 and KLH compared with KLH alone.

5.2.6 *E. coli* heat labile enterotoxin modulates DAMP-induced DC cytokine production

In Chapter 3 *E. coli* heat labile enterotoxin (LT) was shown to modulate PAMP-induced DC cytokine production, acting as a second signal to enhance LPS-induced IL-10 and IL-1 β , while inhibiting LPS-induced IL-12 and TNF- α (Fig. 3.8). The data presented in Fig. 5.4, Fig. 5.6 and Fig. 5.9 showed that the endogenous danger signals, elastase, HMGB1, HRF and cHRF were capable of inducing pro-inflammatory cytokine production from DC comparable with CpG-induced cytokine production. Therefore, LT was examined for its ability to modulate DAMP-induced cytokine production from DC. LT significantly increased CpG induced IL-1 β production by DC from C3H/HeN and C3H/HeJ mice (Fig. 5.14), indeed concentrations of IL-1 β induced by CpG in combination with LT were comparable for DC from two strains mice. Stimulation of DC, from both C3H/HeN and C3H/HeJ mice, with the DAMPs, elastase, HRF and cHRF induced production of IL-1 β (Fig. 5.14). This IL-1 β production was significantly enhanced when DC were pre-incubated with LT for 6 hours prior to that addition of the endogenous danger signals. HMGB1 did not induce IL-1 β in DC from either C3H/HeN or C3H/HeJ mice, however small concentrations of IL-1 β were produced following co-incubation of DC with HMGB1 and LT, although these concentrations were not significantly higher than stimulation with medium alone. Stimulation of DC from both C3H/HeN and C3H/HeJ mice with

LT also significantly enhanced CpG-induced IL-10 production (Fig. 5.14). Furthermore, DC from C3H/HeN and C3H/HeJ mice produced significant concentrations of IL-10 following stimulation with LT in combination with either elastase, HRF or cHRF even though neither LT nor the endogenous factors alone induced the production of IL-10 from C3H/HeJ DC (Fig. 5.14). Unlike the other endogenous factors studied, HMGB1 was unable to synergise with LT for the production of IL-10 by DC from either C3H/HeN or C3H/HeJ mice (Fig. 5.14).

LT was previously shown to inhibit the production of LPS-induced IL-12 and TNF- α from DC (Fig. 3.8). Pre-treatment of DC from C3H/HeN and C3H/HeJ mice with LT for 6 hours prior to the addition of CpG significantly inhibited CpG-induced IL-12p40, IL-12p70 and TNF- α production (Fig. 5.15). Elastase, HMGB1, HRF and cHRF each induced significant concentrations of TNF- α by DC from C3H/HeN and C3H/HeJ mice (Fig. 5.4, Fig. 5.6, Fig. 5.9), this DAMP-induced TNF- α production was significantly inhibited by LT (Fig. 5.15). None of the DAMPs studied induced significant concentrations of IL-12p70 by DC from C3H/HeJ mice (Fig. 5.5, Fig. 5.6, Fig. 5.10) and HMGB1 alone induced small concentrations of IL-12p40 from DC. LT significantly inhibited HMGB1-induced IL-12p40 production when incubated with DC for 6 hours prior to the addition of the HMGB1 (Fig. 5.15).

5.2.7 Enzymatically inactive LT mutants can modulate endogenous-induced DC cytokines

Data presented in Chapter 3 demonstrated that the enzymatically inactive mutant enterotoxin, LTK63, was capable of modulating PAMP-induced DC cytokines. LTK63 increased expression of TLR-induced IL-1 β and IL-10, while it

inhibited LPS-induced TNF- α , IL-12p40 and IL-12p70. Therefore, experiments were carried out to assess if LTK63 could modulate DAMP-induced cytokine production. LTK63 was found to increase CpG-induced IL-1 β (Fig. 5.16) and significantly inhibited CpG-induced IL-12p40 and IL-12p70 production (Fig. 5.17) in DC from C3H/HeJ mice. LTK63 did not enhance CpG-induced IL-10 production or inhibit CpG-induced TNF- α in DC from C3H/HeJ mice (Fig. 5.16, Fig. 5.17). This result may be due to pre-treatment of DC with LTK63 for 6 hours prior to CpG addition, which was found to be sub-optimal for LTK63 modulation of TLR-induced cytokine production (Fig. 3.10).

LTK63 enhanced elastase-induced IL-1 β production by DC from C3H/HeN mice. HMGB1 alone did not induce IL-1 β production by DC from both C3H/HeN and C3H/HeJ mice. However, following pre-treatment with LTK63, HMGB1 induced IL-1 β production (Fig. 5.16). LTK63 partially inhibited HRF-induced IL-1 β production by DC from both C3H/HeN and C3H/HeJ mice (Fig. 5.16), but did not significantly alter cHRF-induced IL-1 β production. Furthermore, LTK63 significantly enhanced elastase, HRF and cHRF-induced IL-10 in DC from both C3H/HeN and C3H/HeJ mice (Fig. 5.16), although none of the DAMPs or LTK63 alone induced IL-10 production from these cells. LTK63 significantly enhanced HMGB1-induced IL-10 production by DC from C3H/HeN mice (Fig. 5.16). Elastase, HMGB1, HRF and cHRF each induced TNF- α production in DC from C3H/HeN and C3H/HeJ mice (Fig. 5.17). LTK63 significantly inhibited this DAMP-induced TNF- α production in DC from C3H/HeJ mice. Elastase, HRF and cHRF induced small concentrations of IL-12p40 and IL-12p70 in DC from C3H/HeN mice, and these concentrations were significantly inhibited by LTK63. However, LTK63 could not modulate IL-12p70 or IL-12p40 production in DC from C3H/HeJ mice, since none of the endogenous

factors studied induced production of IL-12 from these cells (Fig. 5.17). In contrast CpG induced IL-12p40 and IL-12p70 in DC from both C3H/HeN and C3H/HeJ mice. This CpG-induced IL-12 production was significantly inhibited by LTK63 in DC from C3H/HeJ.

5.3 DISCUSSION

It is now widely accepted that endogenous factors derived from self can activate innate immune responses (Gallucci *et al.*, 1999; Sauter *et al.*, 2000; Seong and Matzinger, 2004; Shi *et al.*, 2003). However, there are persistent claims from critics of the danger hypothesis that most of the data published in the field may be complicated by contamination of the endogenous danger signals with pathogen associated molecules, such as LPS, which are potent activators of innate immune responses (Salio *et al.*, 2000). The aim of this present study was to examine whether the endogenous factors, elastase, HMGB1 and HRF, were capable of innate immune cell activation in the absence of TLR4 signalling. Furthermore, if these endogenous factors were indeed capable of immune activation, were the responses induced similar to or distinct from those generated by PAMPs. DC from the TLR4 defective, LPS hypo-responsive, C3H/HeJ mice, were used to examine, the role of DAMPs in DC activation and maturation, without the influence of LPS. This study also examined whether the bacterial toxin, LT, was capable of modulating DAMP-induced DC cytokine production, similar to that observed for modulation of PAMP-induced DC maturation.

This study found that the endogenous danger signals, elastase, HMGB1 and HRF, were capable of activating DC maturation in the absence of LPS stimulation. Each of the endogenous danger signals examined enhanced the expression of CD40, ICAM-1 and the B7 family member, B7-H1 on the surface of DC. Indeed DAMP-induced surface marker regulation was comparable with that induced by stimulation of DC with CpG. This is consistent with recent reports, which showed that HMGB1

increased expression of another B7 family member, CD80, on DC and ICAM-1 expression on endothelial cells (Messmer *et al.*, 2004; Treutiger *et al.*, 2003).

The up-regulation of co-stimulatory markers, such as CD40, ICAM-1 and B7-H1, is essential in the priming of adaptive immune responses. However, in contrast to B7-1 (CD80) and B7-2 (CD86), expression of B7-H1 has been linked with negative regulation of T cell responses and apoptosis (Dong *et al.*, 2002). This negative regulation appears to depend upon the location and degree of activation of the T cell. Each of the DAMPs examined enhanced expression of MHCII on DC, but not to the same extent as that observed following stimulation with the PAMP, CpG. These results indicate that DAMPs are capable of providing the first two signals required for DC activation, increasing the expression of the antigen presentation complex, MHCII, in combination with up-regulated expression of co-stimulatory markers.

DAMPs were also examined for their ability to induce cytokine production from DC. Elastase, HMGB1 and HRF induced significant production of the pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 from DC. This finding is consistent with previous reports, which demonstrated that elastase and HMGB1 induced TNF- α production by macrophages (Andersson and Erlandsson-Harris, 2004; Andersson *et al.*, 2000; Geisler *et al.*, 2005; Jaffray *et al.*, 2000) as well as the human erythroleukemic cell line, K562 (Kang *et al.*, 2007) and monocyte/macrophage cell line THP-1 (El Mezayen *et al.*, 2007). Indeed in the study by El Mezayen *et al.*, it was found that necrotic cell lysates were capable inducing IL-6, TNF- α and IL-8 from THP-1 cells, and this pro-inflammatory cytokine production was increased in the presence of LPS. Furthermore, this report demonstrated that blocking HMGB1 and heat shock protein-70 (Hsp70) significantly decreased the pro-inflammatory cytokine production. However, concentrations of LPS-induced TNF- α , IL-6 and IL-8 in the

presence of anti-HMGB1 and anti-Hsp70 were higher than LPS treated THP-1 cells alone. This finding indicates that other DAMPs, contained within the necrotic cell lysate induce pro-inflammatory cytokine production from THP-1 cells.

The present study demonstrated that HMGB1, HRF and cHRF induced significant concentrations of the chemokine MIP-1 α (CCL3), a chemoattractant for T cells as well as DC and NK cells, from both C3H/HeN and C3H/HeJ DC. Although the DAMPs were capable of inducing pro-inflammatory cytokines and chemokines, comparable with a TLR agonist, the overall cytokine profile induced by the DAMPs was different to that induced by CpG. None of the DAMPs studied induced significant levels of IL-10, IL-12p40 or IL-12p70, key T cell polarising cytokines, which are significantly increased after PAMPs stimulation (Iwasaki and Medzhitov, 2004; Jakob *et al.*, 1998). This is consistent with a previous report on HMGB1 stimulation of human monocytes, which found pro-inflammatory cytokine production in the absence of either IL-10 or IL-12 induction (Andersson *et al.*, 2000). However, the present study found that HMGB1 and elastase did induce significant concentrations of the T_{IL-17} polarising cytokine, IL-23, from DC. DAMP-induced IL-23 production was, however, significantly lower than that induced by CpG. Since DAMPs enhanced surface marker expression and cytokine production in DC from C3H/HeJ mice, contamination of DAMPs with LPS can be ruled out as an explanation for DAMP activity. Furthermore, the differences in DAMP and PAMP induced IL-10 and IL-12 production from DC, suggests that the samples were not contaminated with a PAMP.

In addition to the influence of DAMPs on DC maturation, the endogenous factors, HRF, cHRF and HMGB1 were found to be effective adjuvants, when co-administered with the antigen, KLH. Both HMGB1 and the HRFs promoted a predominantly Th2 response, with antigen-specific IL-5 produced by re-stimulated T

cells, and in the case of HRF and cHRF this was associated with significantly enhanced KLH-specific IgG1 production. In addition HMGB1 increased KLH-specific IL-10 producing T cells, although innate IL-10 helps to promote the induction of Treg cells HMGB1 failed to induce IL-10 production from DC. However, this antigen-specific IL-10 production was associated with up-regulation of B7-H1 on DC, which has previously been shown to enhance T cell proliferation and IL-10 production (Dong *et al.*, 1999). The present findings contrast with previous studies, which demonstrated that immunisation with an antigen and HMGB1 as adjuvant, promoted a Th1-polarised response (Messmer *et al.*, 2004). However, the Th1 response, observed by Messmer *et al.*, may be due to TLR contamination of HMGB1.

A final aim of this study was to determine whether, DAMP-induced DC maturation, was modulated by a bacterial enterotoxin, such as LT or its enzymatically inactive mutant LTK63. The results presented in Chapter 3 showed that LT and LTK63 alone were unable to induce cytokine production or increase MHCII expression on DC. Nevertheless, both LT and LTK63 have been shown to have potent adjuvant activity when co-administered with antigens (Gagliardi *et al.*, 2000; Richards *et al.*, 2001; Ryan *et al.*, 1999). However, it has been suggested that adjuvant activity requires innate cell activation, including up-regulation of MHC and co-stimulatory molecule expression, in combination with enhanced regulatory cytokine production. This suggests that LT and LTK63 may require a second signal, in order to promote MHC expression and cytokine production, and thereby mediate its adjuvant activity. The data in Chapter 3 also demonstrated that both LT and LTK63 were capable of modulating PAMP-induced cytokine production from DC, however LT acted as an adjuvant *in vivo* in the absence of an added PAMP.

The results of the present study demonstrate that both LT and LTK63 are capable of modulating DAMP-induced pro-inflammatory cytokine production by DC. Pre-incubation of DC with LT enhanced DAMP-induced IL-1 β and IL-10 production and inhibited DAMP-induced IL-12p40 and TNF- α production. Furthermore, the enzymatically inactive mutant of LT, LTK63, was also capable of modulating the DAMP-induced cytokine production enhancing DAMP-induced IL-1 β and IL-10 and inhibiting DAMP-induced TNF- α production. Thus, the adjuvanticity of LT and LTK63 may rely upon a second signal provided by DAMPs *in vivo*.

Similarly, a link between PAMP-induced immune activation and development of autoimmune disease has not been firmly established. However, DC maturation is required for development of certain autoimmune diseases, such as EAE (Hansen *et al.*, 2006). DAMP-induced DC maturation may therefore be involved in the development of autoimmune diseases. This is supported by the observation in the present study that DAMP-induced pro-inflammatory cytokine production is comparable with that induced after PAMP stimulation. In conclusion, this study has demonstrated that DAMPs induce IL-23 production from DC. IL-23 is a T_{IL-17} polarising cytokine, which has been directly linked with the development of autoimmune diseases (Langrish *et al.*, 2005). This provides further evidence that DAMPs may play a role in precipitating autoimmune diseases.

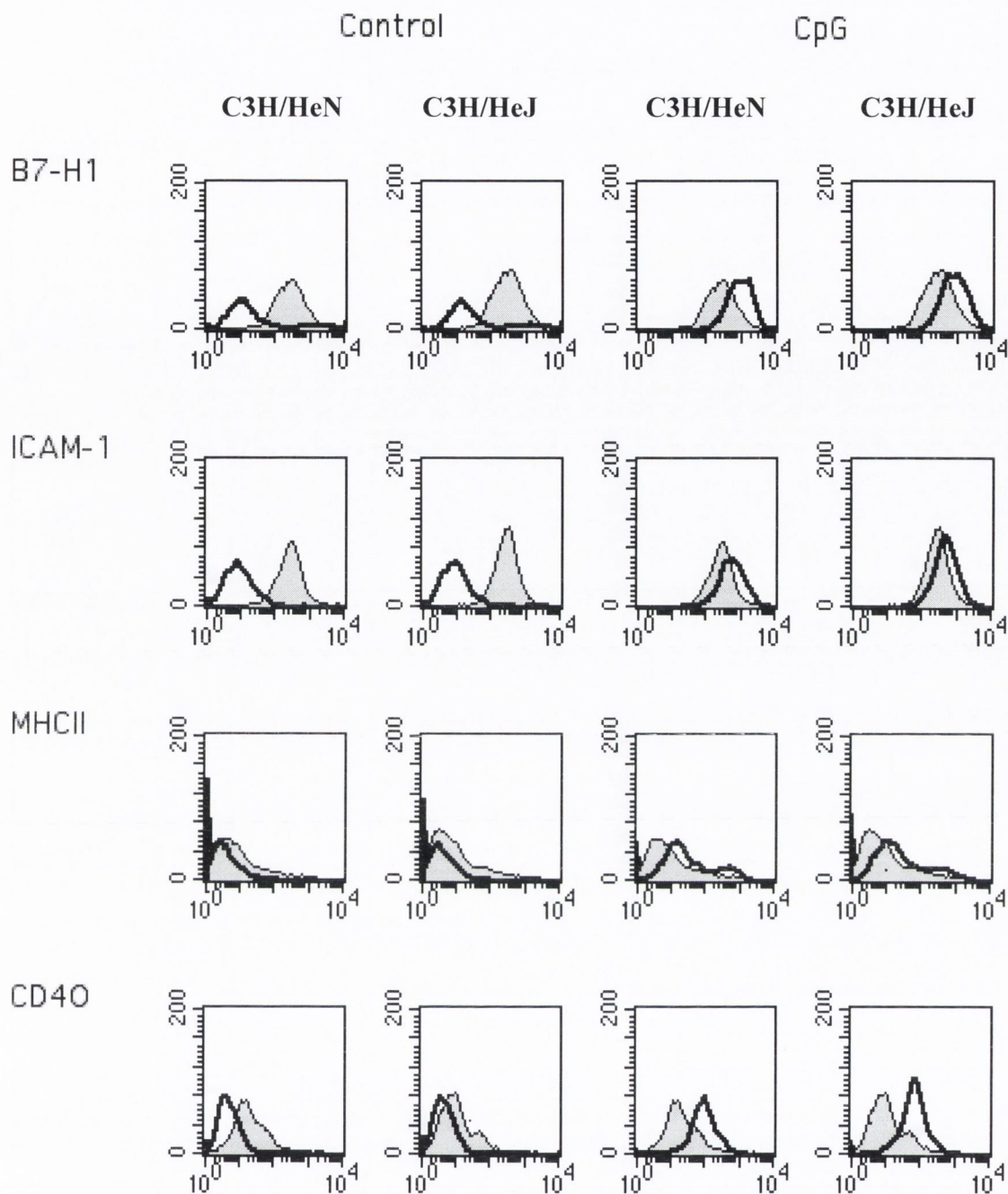


Figure 5.1: The TLR9 agonist, CpG, can mature DC from C3H/HeN and C3H/HeJ mice. BMDC from C3H/HeN and C3H/HeJ mice (1×10^6 cells/ml) were stimulated with CpG (10 μ g/ml) or with medium alone (control). After 24 h cells were washed and stained with antibodies specific for B7-H1, ICAM-1, MHCII, CD40 or with isotype matched controls (black line, control column). Results from flow cytometric immunofluorescence analysis are shown for treated (black line) compared with untreated (grey histogram) DC, gated on CD11c⁺ cells.

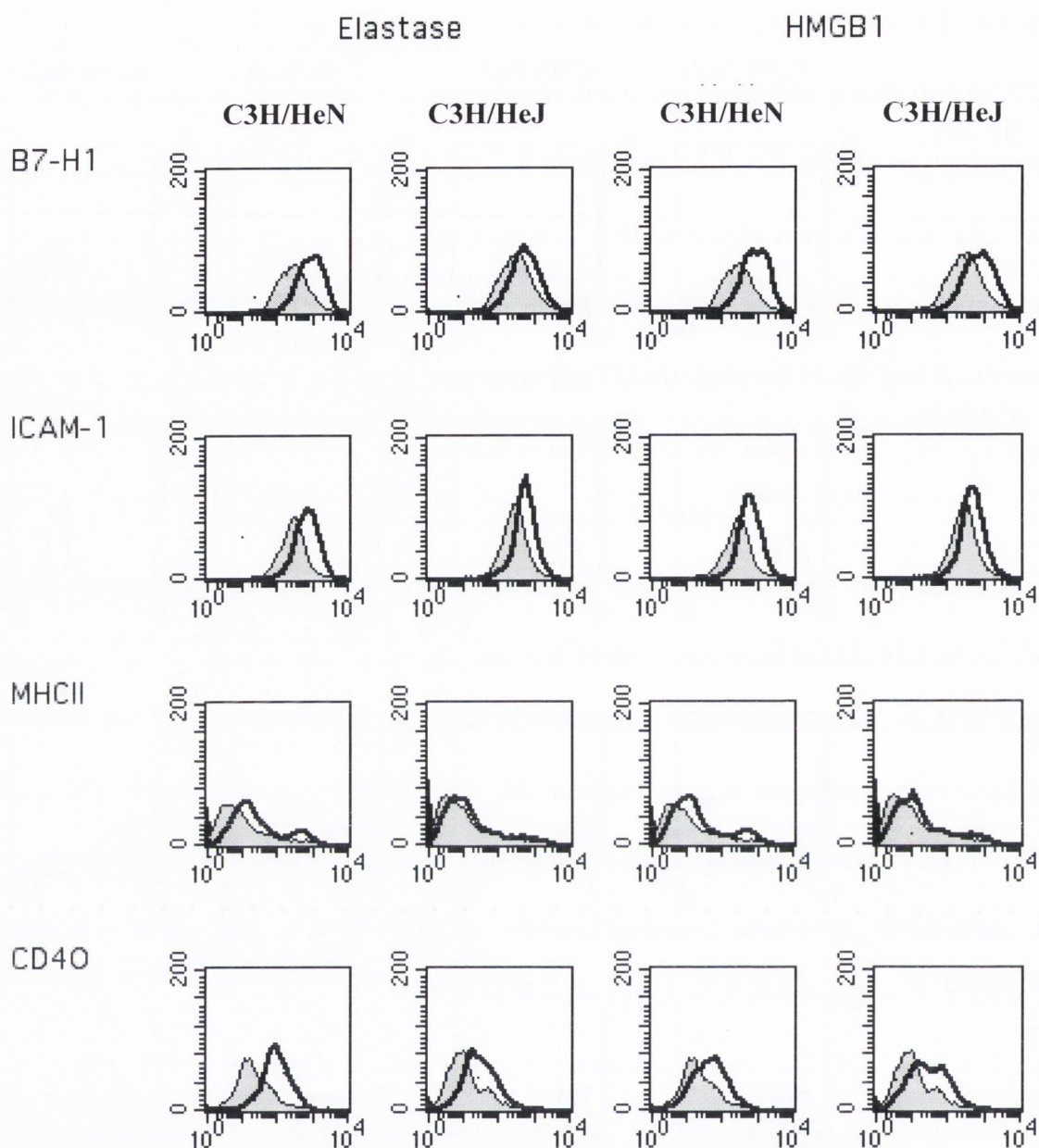


Figure 5.2: Elastase and HMGB1 can mature DC in a TLR4 independent manner. BMDC from C3H/HeN and C3H/HeJ mice (1×10^6 cells/ml) were stimulated with elastase ($5 \mu\text{g/ml}$), HMGB1 ($20 \mu\text{g/ml}$) or with medium alone. After 24 h cells were washed and stained with antibodies specific for B7-H1, ICAM-1, MHCII or CD40. Results from flow cytometric immunofluorescence analysis are shown for treated (black line) compared with untreated (grey histogram) DC, gated on CD11c⁺ cells.

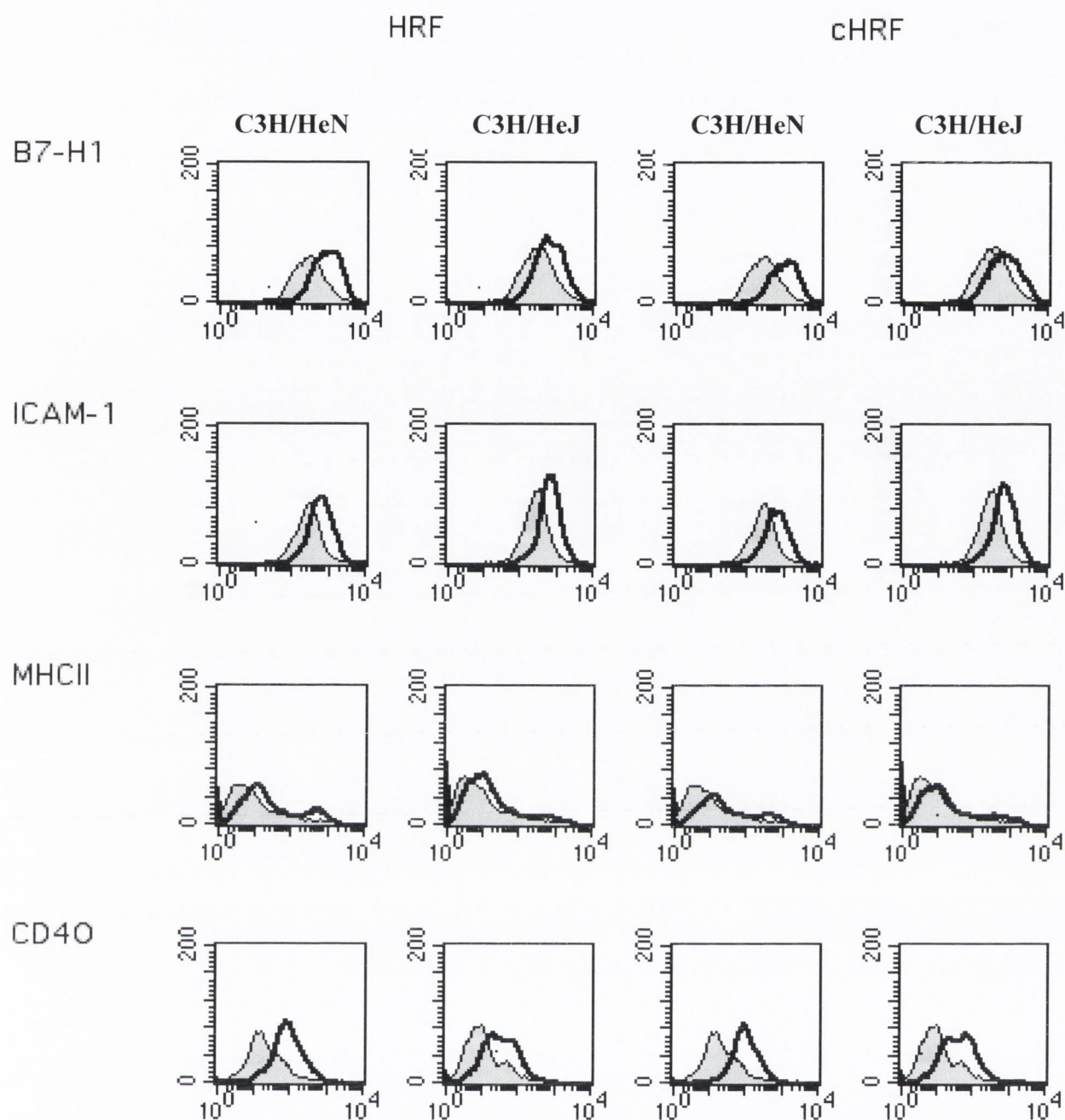


Figure 5.3: Both HRF and the cleaved version of the molecule, cHRF, can mature DC independently of TLR4. BMDC from C3H/HeN and C3H/HeJ mice (1×10^6 cells/ml) were stimulated with HRF (1 μ g/ml), cHRF (1 μ g/ml) or with medium alone. After 24 h cells were washed and stained with antibodies specific for B7-H1, ICAM-1, MHCII or CD40. Results of immunofluorescence analysis are shown for treated (black line) compared with untreated (grey histogram) DC, gated on CD11c⁺ cells.

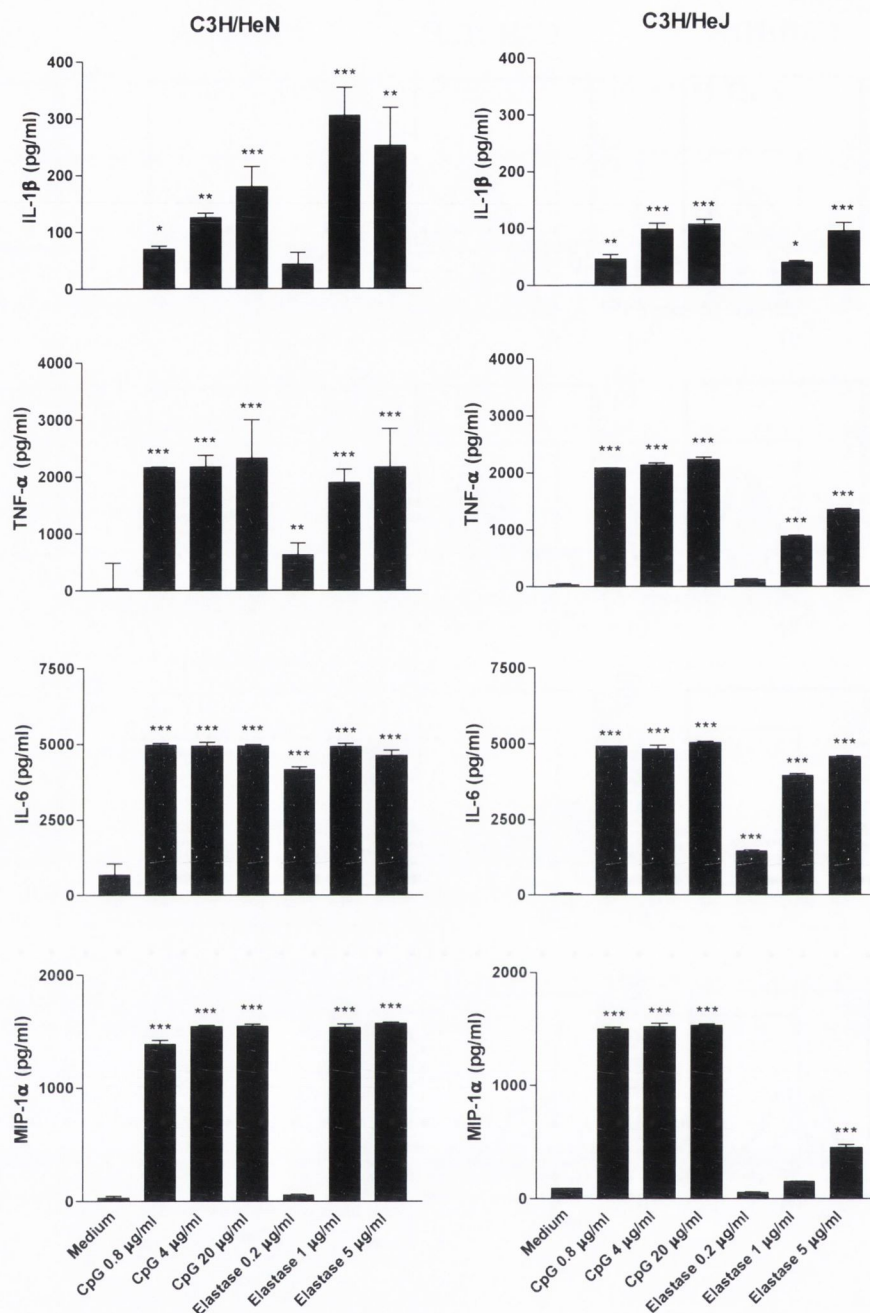


Figure 5.4: Elastase induced the secretion of IL-1 β , IL-6, TNF- α and MIP-1 α from DC independently of TLR4. BMDC from both C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with CpG (0.8, 4 or 20 μ g/ml), elastase (0.2, 1 or 5 μ g/ml) or with medium alone. Supernatants were removed after 24 h and tested for IL-1 β , TNF- α , IL-6 and MIP-1 α production by ELISA. Treatment versus medium alone: *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

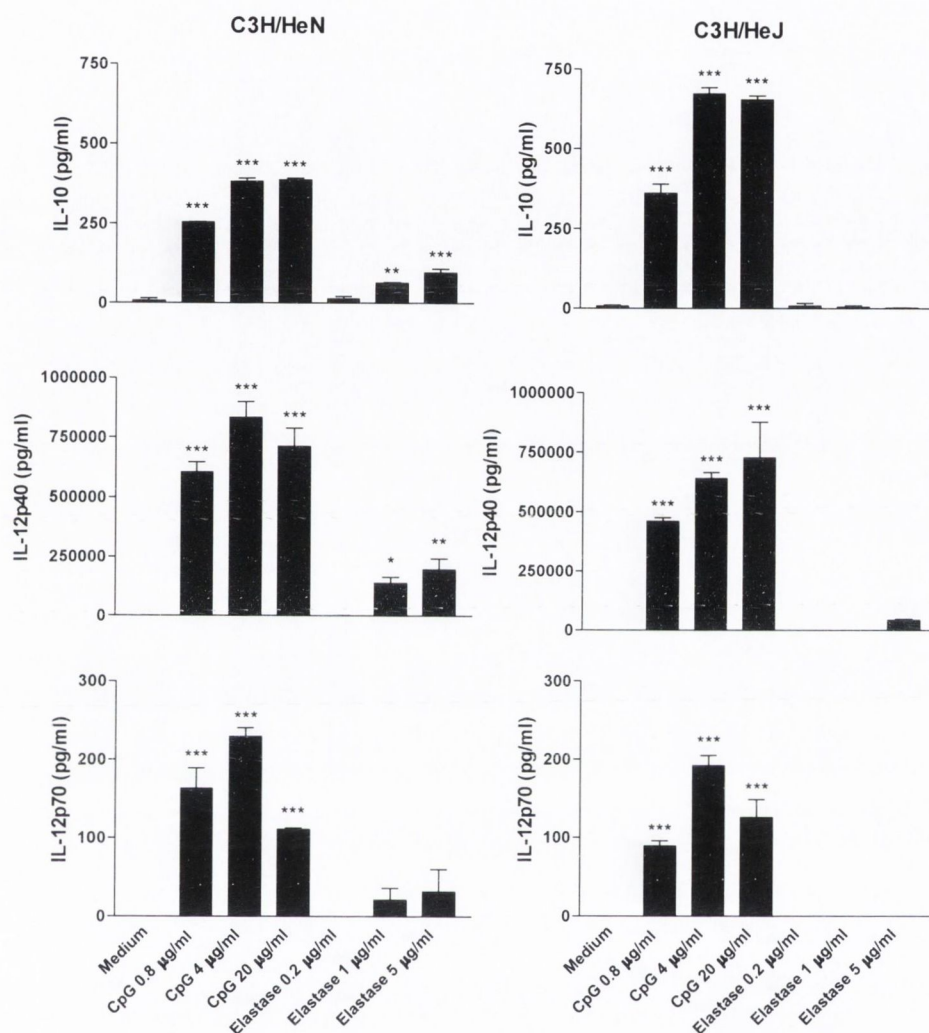


Figure 5.5: In comparison with CpG-ODN, elastase induces low concentrations of IL-10 or IL-12 from DC. BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with CpG (0.8, 4 or 20 µg/ml), elastase (0.2, 1 or 5 µg/ml) or with medium alone. Supernatants were removed after 24 h and tested for IL-10, IL-12p40 and IL-12p70 production by ELISA. Treatment versus medium alone: **, $P < 0.01$; ***, $P < 0.001$.

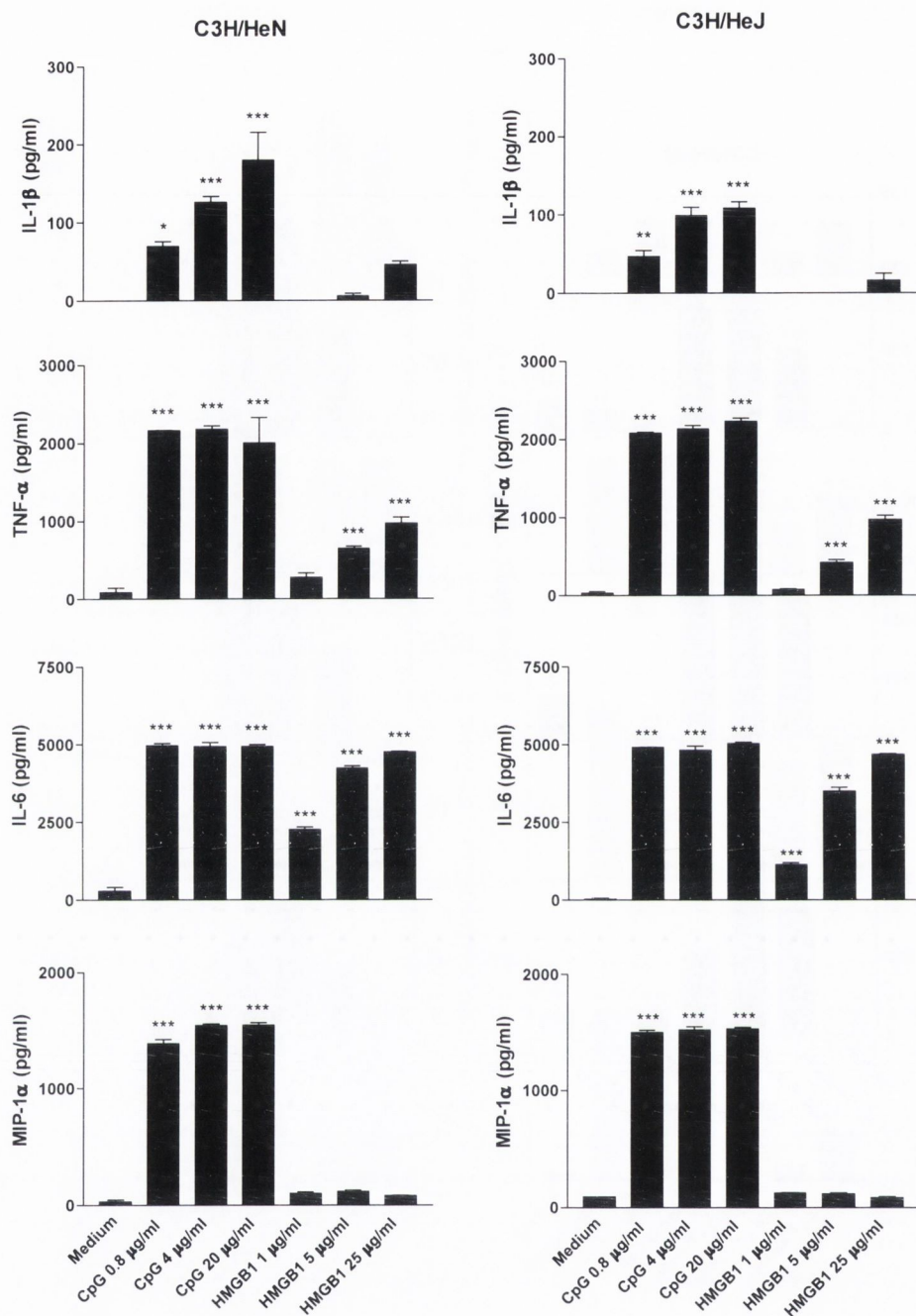


Figure 5.6: HMGB1 induces the production of TNF- α and IL-6 by DC in a TLR4 independent manner. BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with CpG (0.8, 4 or 20 μ g/ml), HMGB1 (1, 5 or 25 μ g/ml) or with medium alone. Supernatants were removed after 24 h and tested for IL-1 β , TNF- α , IL-6 and MIP-1 α production by ELISA. Treatment versus medium alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

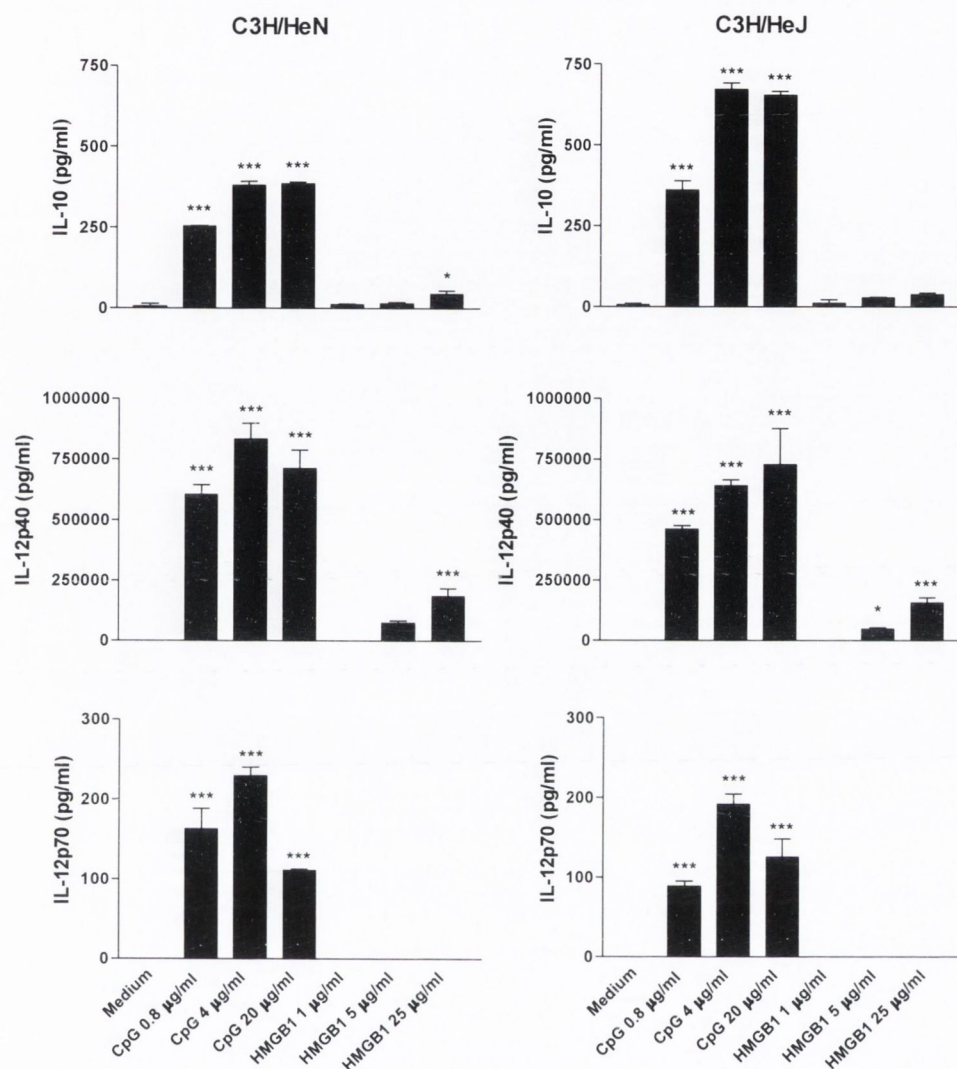


Figure 5.7: In comparison with CpG, HMGB1 induces the production of low concentrations of IL-10 and IL-12 from DC. BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with CpG (0.8, 4 or 20 μ g/ml), HMGB1 (1, 5 or 25 μ g/ml) or with medium alone. Supernatants were removed after 24 h and tested for IL-10, IL-12p40 and IL-12p70 production by ELISA. Treatment versus medium alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

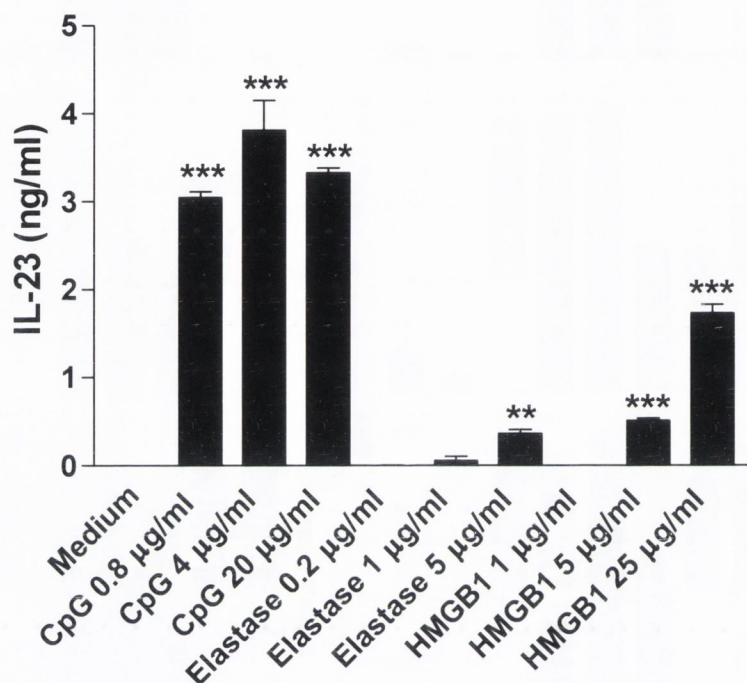


Figure 5.8: CpG, elastase and HMGB1 induce IL-23 production from DC. BMDC from C3H/HeJ mice (5×10^5 cells/ml) were stimulated with CpG (0.8, 4 or 20 µg/ml), elastase (0.2, 1 or 5 µg/ml), HMGB1 (1, 5 or 25 µg/ml) or with medium alone. Supernatants were removed after 24 h and tested for IL-23 production by ELISA. Treatment versus medium alone: **, $P < 0.01$; ***, $P < 0.001$.

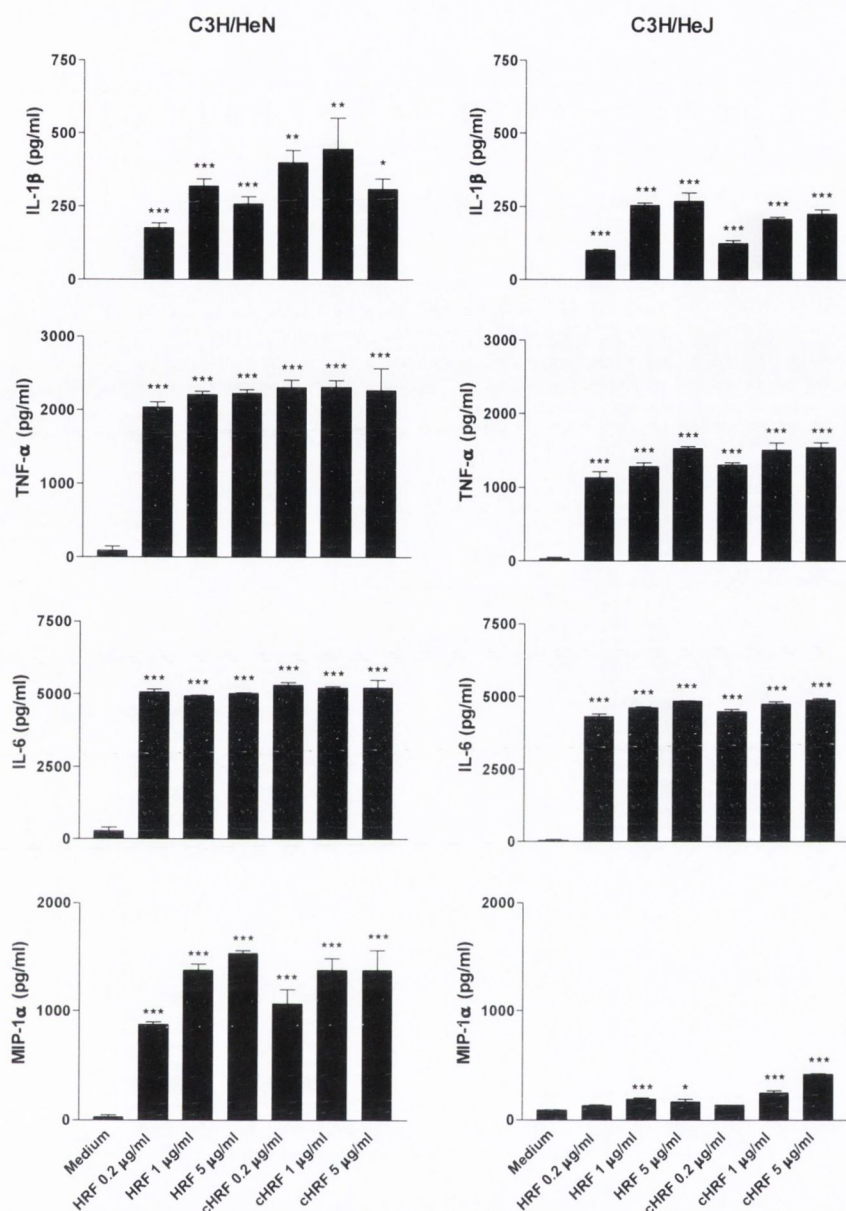


Figure 5.9: HRF in either its native or cleaved form strongly induces IL-6, TNF- α and IL-1 β production from DC. BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with CpG (0.8, 4 or 20 μ g/ml), HRF (0.2, 1 or 5 μ g/ml), cHRF (0.2, 1 or 5 μ g/ml) or with medium alone. Supernatants were removed after 24 h and tested for IL-1 β , TNF- α , IL-6 and MIP-1 α production by ELISA. Treatment versus medium alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

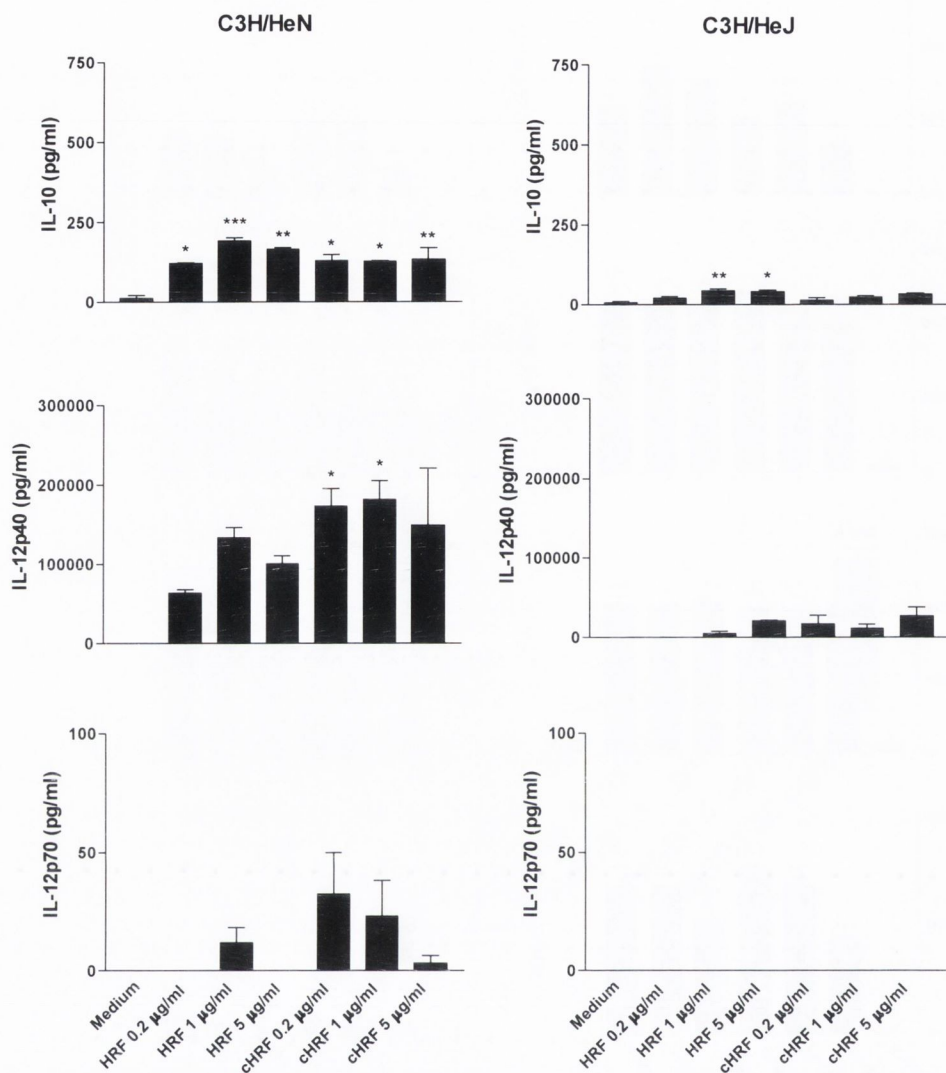


Figure 5.10: Neither histamine releasing factor nor the cleaved version of the protein induce secretion of IL-10 or IL-12p70. BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with CpG (0.8, 4 or 20 $\mu\text{g/ml}$), HRF (0.2, 1 or 5 $\mu\text{g/ml}$), cHRF (0.2, 1 or 5 $\mu\text{g/ml}$) or with medium alone. Supernatants were removed after 24 h and tested for IL-10, IL-12p40 and IL-12p70 production by ELISA. Treatment versus medium alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

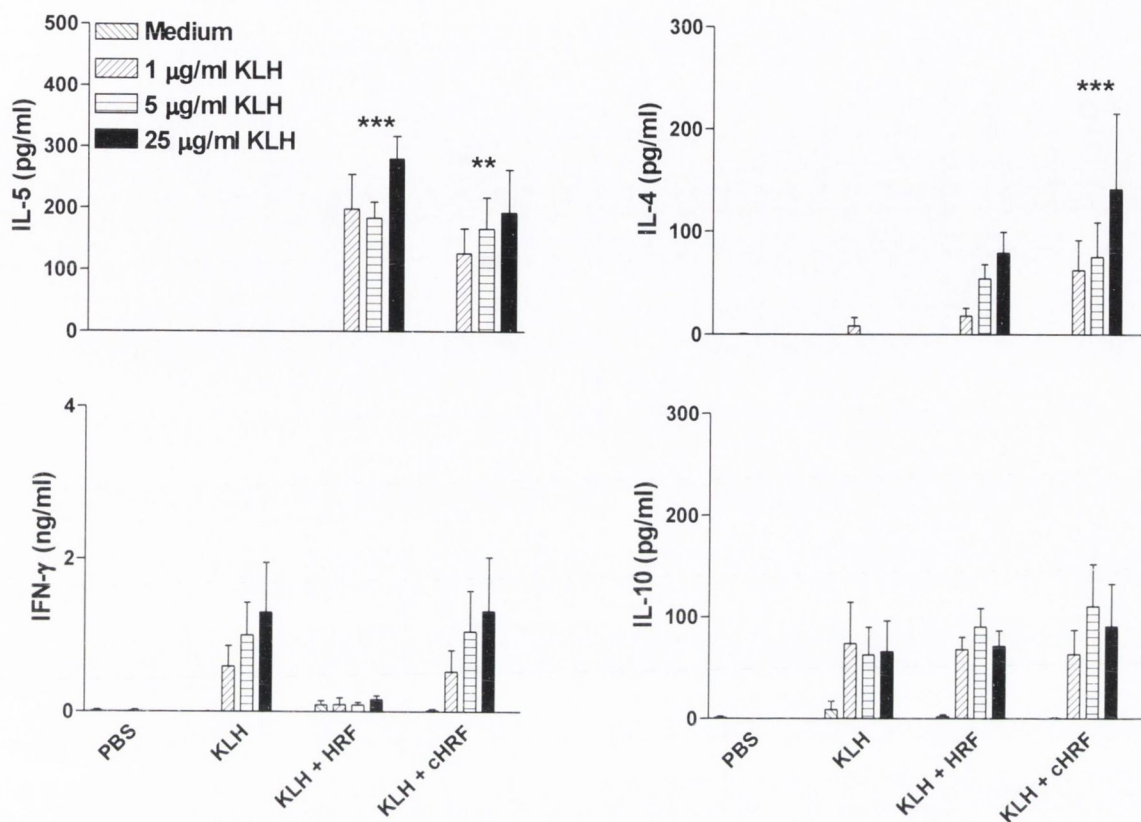


Figure 5.11: HRF and the cleaved version of the protein, cHRF, act as adjuvants enhancing Th2 responses to a co-administered antigen. BALB/c mice ($n = 5$) were immunised s.c. with PBS, KLH (10 μg), KLH and HRF (10 μg) or KLH and cHRF (10 μg). Mice were sacrificed after 7 d and spleen cells re-stimulated ex vivo with KLH (1, 5, 25 $\mu\text{g/ml}$). After 72 h supernatants were removed and tested for IL-4, IL-5, IL-10 and IFN- γ production by ELISA. KLH + adjuvant versus KLH alone: **, $P < 0.01$; ***, $P < 0.001$.

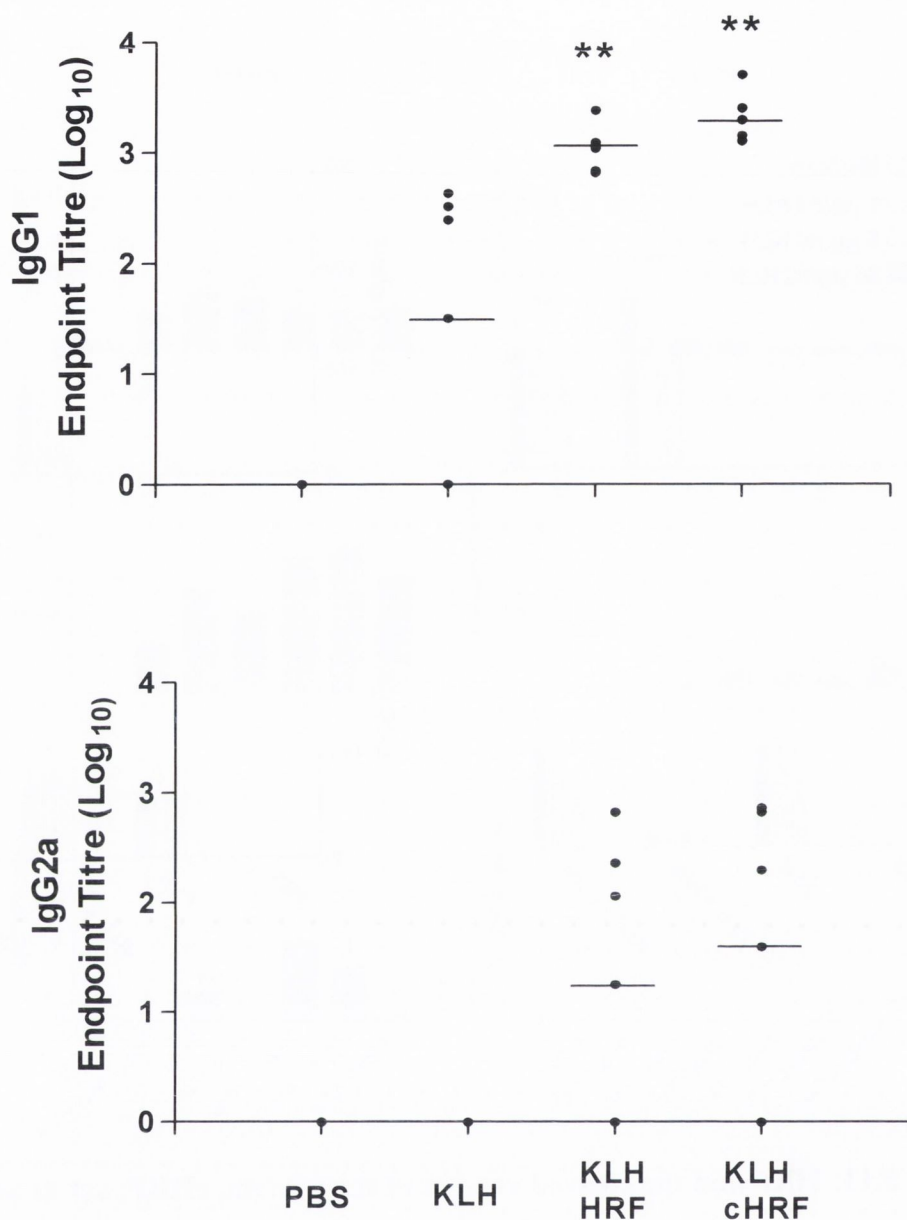


Figure 5.12: Injection of HRF or cHRF with KLH as a by-stander antigen enhanced antigen-specific IgG1 but not IgG2a antibody responses, consistent with enhancement of Th2 responses. BALB/c mice ($n = 5$) were immunised s.c. with PBS, KLH (10 μg), KLH and HRF (10 μg) or KLH and cHRF (10 μg). Mice were sacrificed after 7 d and KLH-specific serum antibody IgG1 and IgG2a determined. KLH + adjuvant versus KLH alone: **, $P < 0.01$.

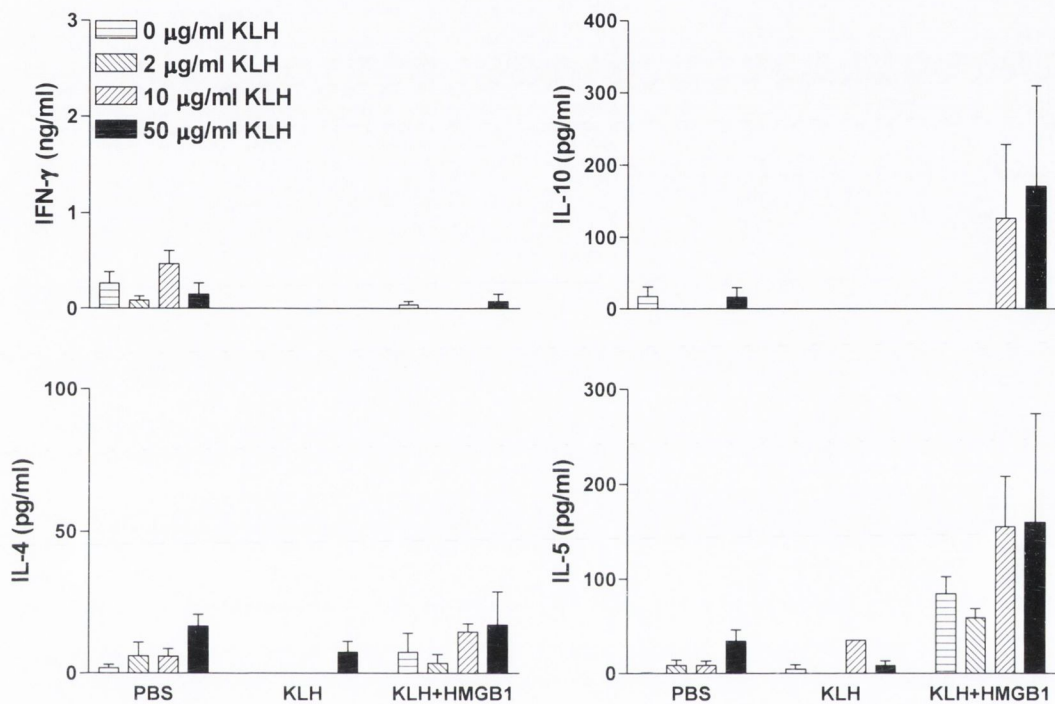


Figure 5.13: HMGB1 acts as an adjuvant to enhance antigen-specific IL-5 and IL-10 production to a co-administered antigen. C57BL/6 mice ($n = 5$) were immunised s.c. with PBS, KLH (10 μ g), KLH and HMGB1 (10 μ g). Mice were sacrificed after 7 d and lymph node cells re-stimulated ex vivo with KLH (2, 10, 50 μ g/ml). After 72 h supernatants were removed and tested for IL-4, IL-5, IL-10 and IFN- γ production by ELISA.

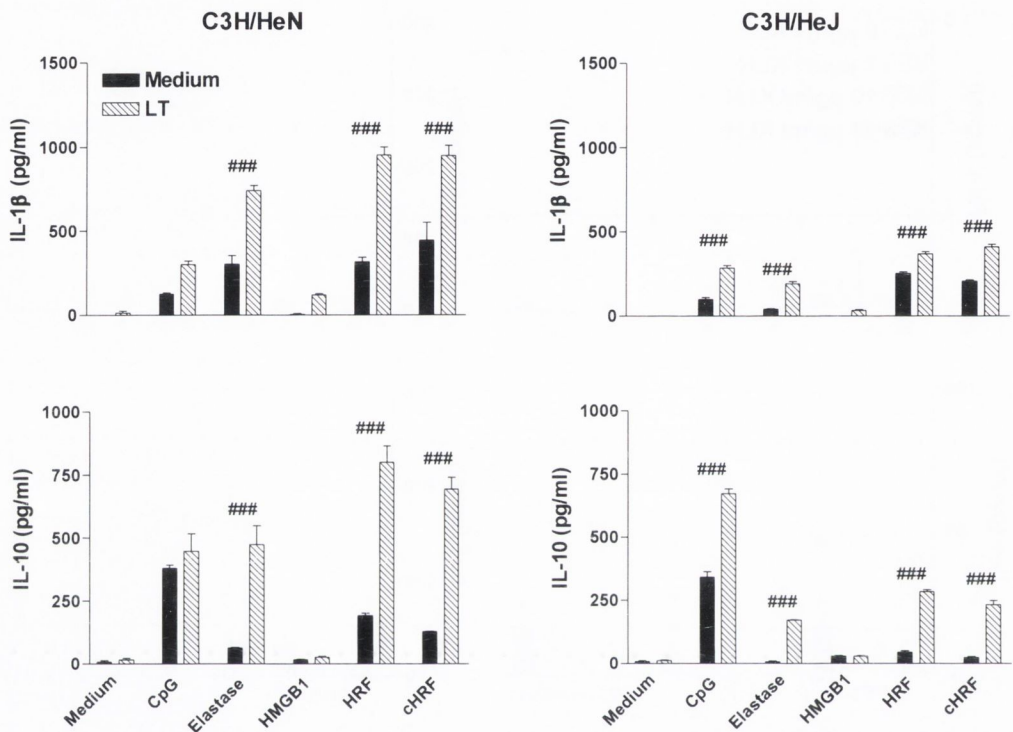


Figure 5.14: DAMPs as well as PAMPs can synergise with LT to induce IL-1 β and IL-10 production from DC. BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with LT (1 μ g/ml), CpG (4 μ g/ml), elastase (1 μ g/ml), HMGB1 (5 μ g/ml), HRF (1 μ g/ml) or cHRF (1 μ g/ml), LT followed 6 h later by CpG, elastase, HMGB1, HRF or cHRF or with medium alone. Supernatants were removed after 24 h and tested for IL-1 β and IL-10 production by ELISA. DAMP versus DAMP + LT: ###, $P < 0.001$.

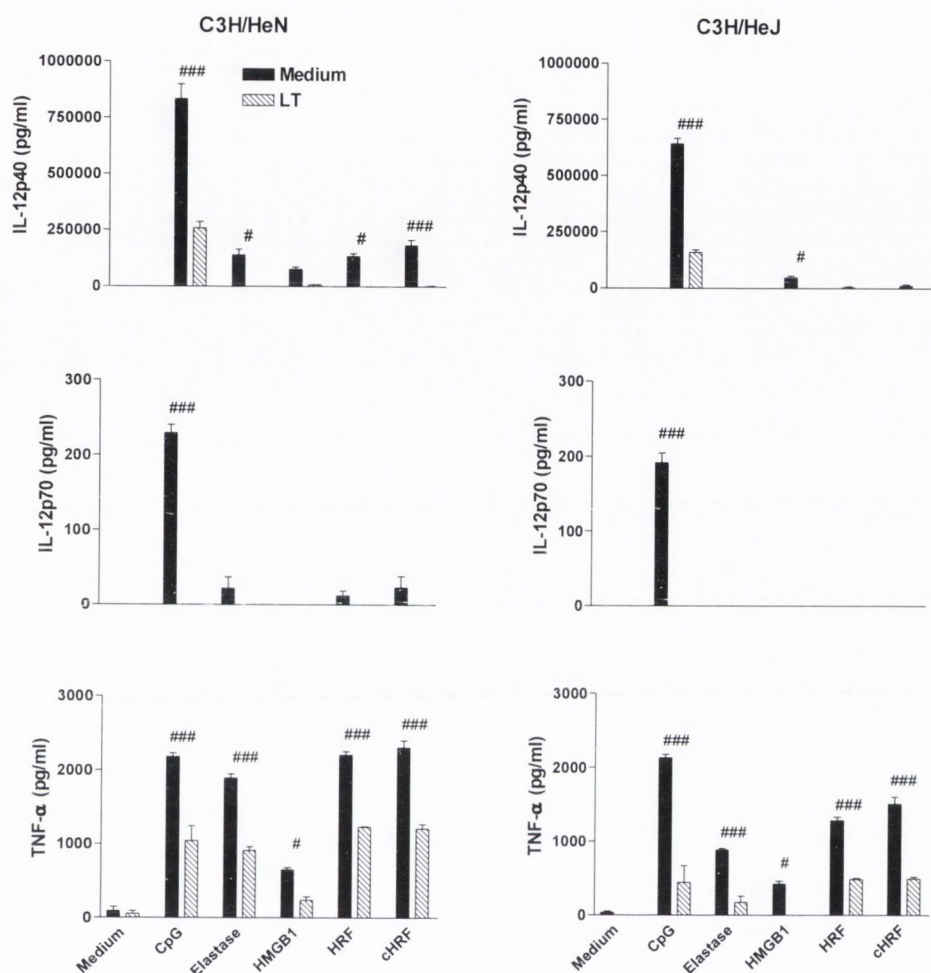


Figure 5.15: DAMP-induced TNF- α production from DC can be inhibited by LT.

BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with LT (1 μ g/ml), CpG (4 μ g/ml), elastase (1 μ g/ml), HMGB1 (5 μ g/ml), HRF (1 μ g/ml) or cHRF (1 μ g/ml), LT followed 6 h later by CpG, elastase, HMGB1, HRF or cHRF or with medium alone. Supernatants were removed after 24 h and tested for IL-12p40, IL-12p70 and TNF- α production by ELISA. DAMP versus DAMP + LT: #, $P < 0.05$; ###, $P < 0.001$.

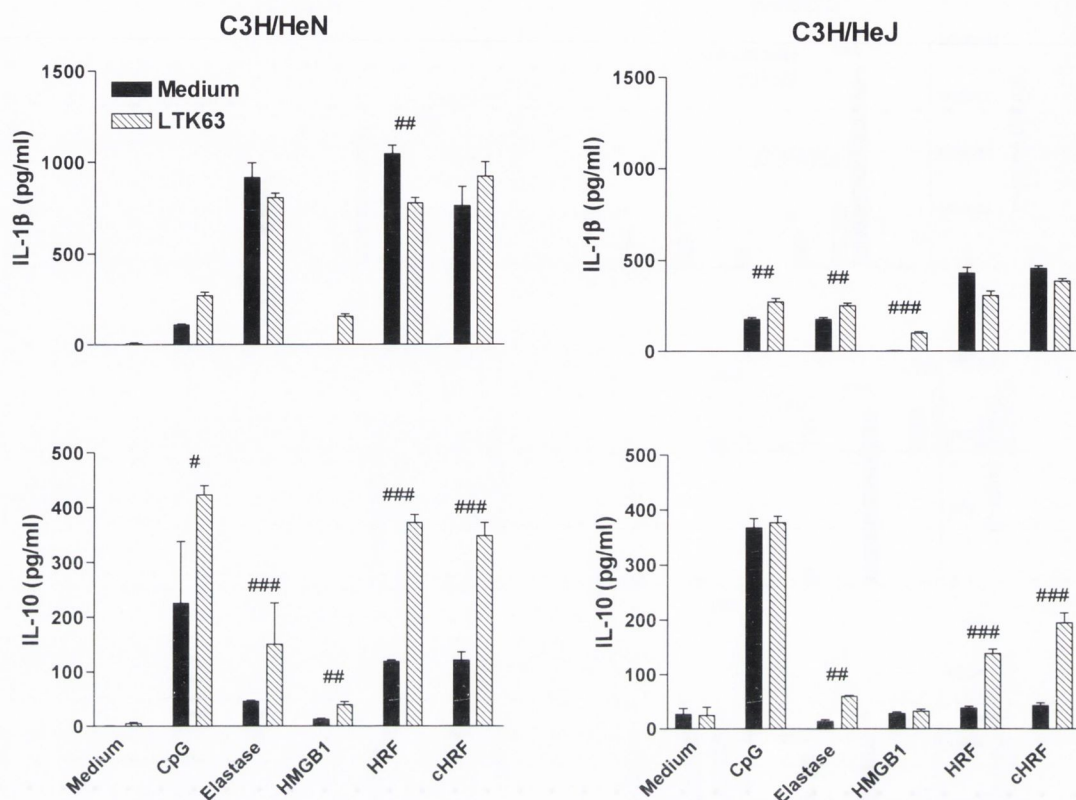


Figure 5.16: DAMPs can synergise with LTK63 to enhance IL-10 production from DC. BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with LTK63 (1 μ g/ml) CpG (4 μ g/ml), elastase (1 μ g/ml), HMGB1 (5 μ g/ml), HRF (1 μ g/ml) or cHRF (1 μ g/ml), LTK63 followed 6 h later by CpG, elastase, HMGB1, HRF or cHRF or with medium alone. Supernatants were removed after 24 h and tested for IL-1 β and IL-10 production by ELISA. DAMP versus DAMP + LTK63: #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$.

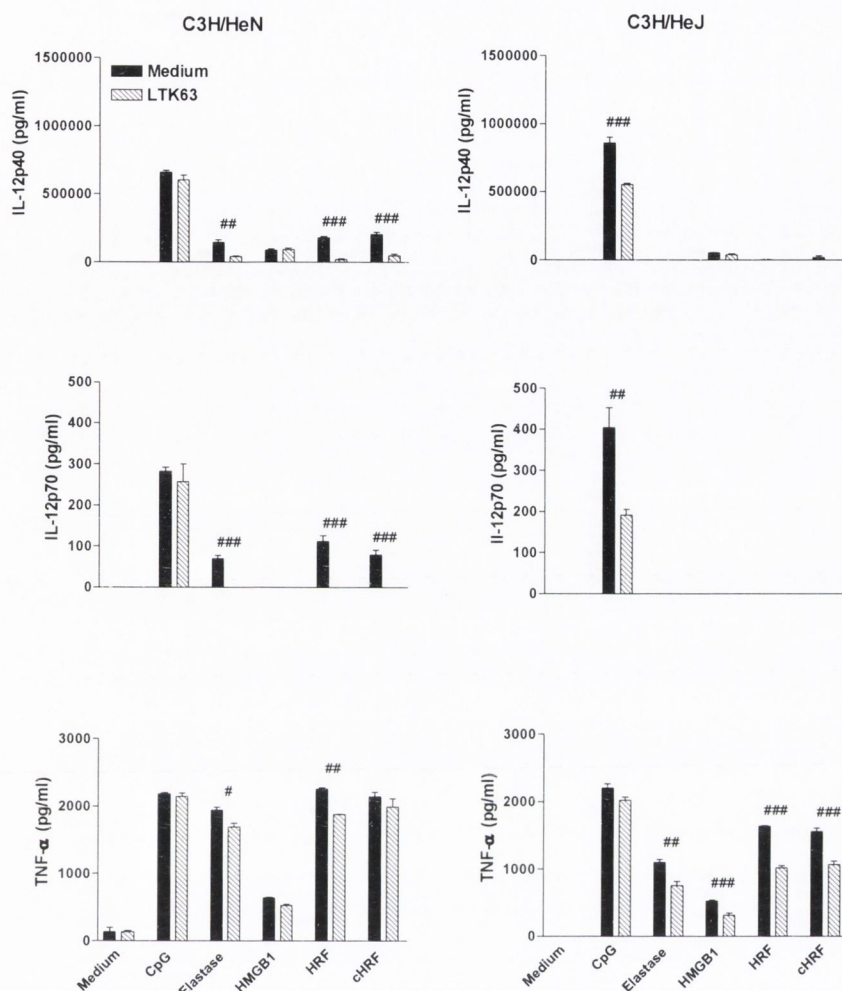
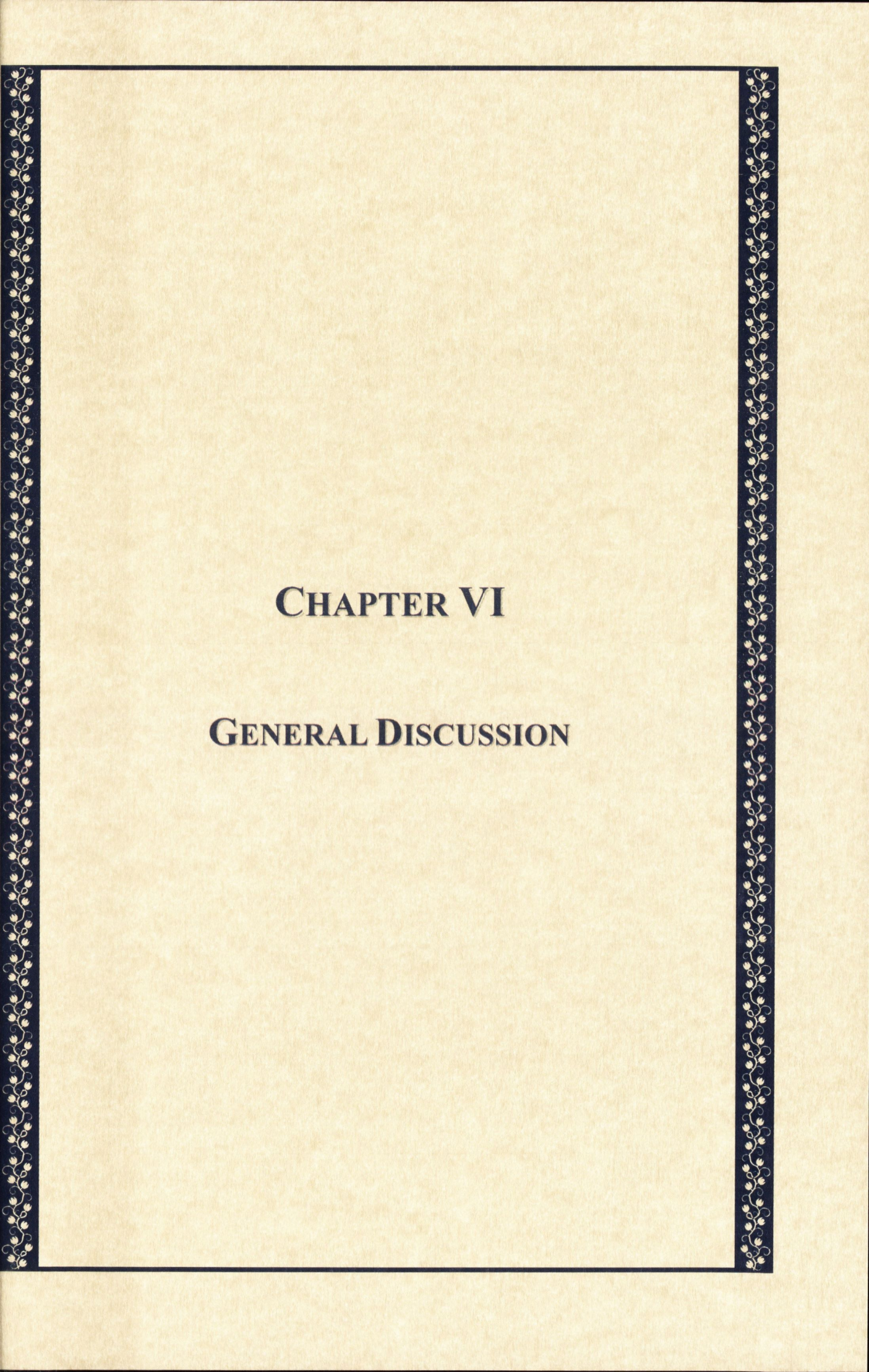


Figure 5.17: DAMP-stimulated TNF- α is inhibited by LTK63. BMDC from C3H/HeN and C3H/HeJ mice (5×10^5 cells/ml) were stimulated with LTK63 (1 μ g/ml), CpG (4 μ g/ml), elastase (1 μ g/ml), HMGB1 (5 μ g/ml), HRF (1 μ g/ml) or cHRF (1 μ g/ml), LTK63 followed 6 h later by CpG, elastase, HMGB1, HRF or cHRF or with medium alone. Supernatants were removed after 24 h and tested for IL-12p40, IL-12p70 and TNF- α production by ELISA. DAMP versus DAMP + LTK63: #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$.



CHAPTER VI

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

The immune system relies upon complex and multifaceted interplay between the innate and adaptive arms of the immune response. Understanding the interplay between the two arms of the immune response has helped to understand the role of the immune system in disease and has assisted in the development of new treatments for these diseases. One immunological intervention that has become one of the best approaches for effective control of infectious diseases has been the use of vaccination. In fact global vaccination programmes have eradicated, or are close to eradicating, diseases, such as smallpox and polio that have claimed the lives of millions of people in the past. However, the side effects associated with traditional whole cell vaccines, such as fever and seizures, as well as increases in antibiotic-resistant bacteria, combined with the emergence of new pathogens has resulted in a greater demand for a new generation of safer and more effective vaccines. One such advancement has been the development of acellular or subunit vaccines comprised of purified antigenic portions of the whole pathogen. These subunit vaccines are generally far less immunogenic than their whole cell counterparts. This is most probably due to the plethora of innate cell activators transferred with antigen in the case of the whole cell vaccines, which are not present in the subunit vaccines because of their purified nature. Therefore, these subunit vaccines require the inclusion of adjuvants, capable of enhancing the immunogenicity of weak subunit-antigen, ultimately leading to strong adaptive immune cell activation.

The AB₅ enterotoxins, LT and CT, have been shown to be potent adjuvants (Jiang *et al.*, 2003; McCluskie *et al.*, 2001; Rappuoli *et al.*, 1999; Williams *et al.*, 1999). However their clinical use is hampered by the toxicity of their enzymatic A

subunit (Rappuoli *et al.*, 1999; Williams *et al.*, 1999). An active enzyme is not, however a prerequisite for adjuvanticity given that mutant versions of LT and CT, with partial enzyme activity (LTR72, LTG192), enzymatically inactive (LTK63, CTF61) or recombinant B subunit (LTB, CTB) preparations can each provide adjuvant activity in vivo (Bregenholt *et al.*, 2003; Douce *et al.*, 1999; Giuliani *et al.*, 1998; Richards *et al.*, 2001; Yamamoto *et al.*, 1997; Yamamoto *et al.*, 1997). An active or partially active enzyme does, however, appear to be necessary for optimal responses and the induction of mixed Th1/Th2 responses (Williams *et al.*, 1999). Both LT and the partially enzymatically active LTR72, which retains approx 1% of the enzyme activity of the wild type toxin, generate a mixed Th1/Th2 response against a co-administered antigen. In contrast, immunisation with an antigen in the presence of an equivalent dose of the enzymatically inactive mutant LTK63 induced a polarised Th2 response in vivo (Douce *et al.*, 1999). These observations were confirmed by experiments performed in the present study. Indeed mutant enterotoxins are already being explored as safer adjuvants for use in vaccination, such as inducing protective immunity against infection with *Helicobacter pylori* and pneumococcal infections (Jakobsen *et al.*, 1999; Marchetti *et al.*, 1998).

The adjuvant properties of the enterotoxins rely on their activation and maturation of innate immune cells. However, from the previous studies it has been shown that enzyme activity is not essential for adjuvanticity. The ADP-ribosylation mutants, LTR72 and LTK63, therefore are a useful tool to dissect the role of enzyme activity and the non-toxic AB complex of LT on innate immune cell maturation. Experiments performed in this study found that LT enhances the expression of the B7 co-stimulatory family members CD80, CD86 and B7-H1 on DC, indicative of DC activation. It has previously been reported that B7-H1 negatively regulates T cell

responses and induced T cell apoptosis, in contrast to CD80 and CD86 (Dong *et al.*, 2002). However, this negative regulation appears to be confined to activated CD4⁺ and CD8⁺ T cell located in the periphery, and does not affect naïve T cells located in the lymphatic tissues (Wang *et al.*, 2003). Furthermore, B7-H1 has been shown to induce naïve T cell proliferation and IL-10 production (Dong *et al.*, 2002), which may partially explain the antigen-specific IL-10 induced following immunisation with LT and antigen. In contrast to the regulation of the B7 family members, LT inhibited expression of CD40 and ICAM-1, thus promoting DC activation into a semi-mature phenotype. The regulation of DC surface marker expression by bacterial enterotoxins has previously been attributed to an active enzyme through increased cAMP concentrations within the innate cells (Bagley *et al.*, 2002; Cong *et al.*, 1997). However, this study demonstrates that enzyme activity is not essential, as both the LTR72 and LTK63 mutants of LT, like the wild type toxin can modulate surface marker expression on DC. However, previous studies, which concluded that DC activation and maturation was dependent on enzyme activity, were based on experiments with the recombinant B subunit of CT. In contrast, experiments performed in the present study were based on inactivated whole toxins and not recombinant B subunit preparations. The demonstration of comparable regulation of surface marker expression by LT, LTR72 and LTK63 indicates that the non-toxic AB₅ complex is capable of modulating the activation of innate cells.

However, the enterotoxins alone did not induce complete DC maturation, which requires an increase in MHCII expression and cytokine production (Lavelle *et al.*, 2004; Lavelle *et al.*, 2003), in view of the fact that priming of adaptive responses requires antigen presentation, by APC that express co-stimulatory markers, and a source of regulatory cytokines (Pasare and Medzhitov, 2003), indicating that the

adjuvanticity associated with the enterotoxins may not be entirely due to their effect on DC. Adjuvanticity may, therefore, be linked to the previously reported observations that CT was capable of modulating innate cell activation, including cytokine production and surface marker expression in response to other stimuli (Gagliardi and De Magistris, 2003; Lavelle *et al.*, 2004; Lavelle *et al.*, 2003).

Experiments performed in this study demonstrate that LT, like CT, could modulate TLR agonist-induced innate cell activation. Furthermore, it was found that modulation of TLR agonist-induced cytokines by LT was not dependent on an active enzyme, and cAMP production, as both LTR72 and LTK63 were capable of modulating TLR agonist-induced DC cytokine production, including production of IL-10 and IL-12. IL-10 has been associated with the development of inducible Treg cells, which control both Th1 and Th2 responses (Higgins *et al.*, 2003; McGuirk *et al.*, 2002; O'Garra *et al.*, 2004), while IL-12 promotes Th1 type responses. This study found that enhancement of LPS-induced IL-10 by LT was not responsible for inhibition of LPS-induced IL-12, despite the fact that IL-10 has previously been reported to inhibit the Th1 polarising cytokine (Rahim *et al.*, 2005).

Although the enzyme inactive mutant LTK63 had similar modulatory effects to LT and LTR72, the kinetics of these effects were different. With the exception of the modulation of LPS-induced IL-1 β production, a longer pre-incubation period was required with LTK63 in order to demonstrate enhancement of LPS-induced IL-10 or inhibition of IL-12. IL-1 is itself a potent adjuvant when co-administered with a bystander antigen, enhancing antigen-specific IgG and IgA, comparable with that induced after immunisation with CT with antigen (Staats and Ennis, 1999). Experiments performed in the present study demonstrated that IL-1 is, at least partially, responsible for the adjuvanticity of LT and LTK63. The induction of KLH-

specific IFN- γ , IL-10, IL-17, IL-5 and IL-4, following immunisation with KLH in the presence of LT or LTK63, were all reduced in IL-1RI^{-/-} compared with wild type mice. Confirmation of the role of IL-1 in the development of all T_{IL-17} cells was provided by the demonstration that induction of KLH-specific IL-17 production by immunisation with KLH, LPS or LTK63 was significantly reduced in IL-1RI^{-/-} mice as was PMA and anti-CD3-induced IL-17 production. This result indicated that IL-1RI had a role in the development of all IL-17 producing T cells. The findings of this project, showed that mutants, with reduced or depleted enzyme activity, had comparable innate immune cell maturation as wild type LT, suggesting that cAMP induction is not the sole mechanism of adjuvant activity of LT.

IL-17 is a pathogenic cytokine associated with the development of autoimmune diseases (Komiyama *et al.*, 2006; Nakae *et al.*, 2003). The IL-12 family member IL-23 has already been shown to play a role in the development of these IL-17 producing T cells (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005). This study found that IL-1 and IL-23 synergised to promote IL-17 production by spleen cells from naïve mice. Indeed stimulation of CD3⁺, CD4⁺ and CD8⁺ T cells as well as whole spleen cells with IL-1 and IL-23 led to IL-17 production. CD4⁺ and CD8⁺ T cells required co-stimulation with anti-CD3 as well as the T cell growth factor IL-2 in order to produce IL-17 in response to IL-1 and IL-23. In contrast, CD3⁺ T cells did not require co-stimulation with anti-CD3 in order to produce significant concentrations of IL-17, although concentrations of IL-17 produced in the absence of anti-CD3 were significantly reduced compared with those produced in the presence of co-stimulation. These findings provide an alternative explanation for the effectiveness of the IL-1 antagonist, IL-1Ra, as a therapy for autoimmune disease. It had previously been assumed that IL-1Ra blocked the inflammatory effect of IL-1 downstream of T cell

activation. However, the present study demonstrates that IL-1Ra can block the development of T_{IL-17} cells. Indeed IL-1 and IL-23-induced IL-17 production from C57BL/6 spleen cells was inhibited by treatment with IL-1Ra. In contrast, blocking IFN- γ , with a neutralising antibody increased IL-1 and IL-23-induced IL-17 production, agreeing with recent reports, suggesting that IFN- γ can inhibit the development of T_{IL-17} cells (Murphy *et al.*, 2003).

TNF- α and IL-6 as well as the TLR4 and TLR2 agonists, LPS and Pam3Cys, were also capable of synergising with IL-23 for IL-17 production. However, induction of IL-17 by these factors, in combination with IL-23, was found to be IL-1RI dependent, suggesting that IL-1 mediated the effect of the TLR-agonists. IL-18, which is a close homologue of IL-1 α and IL-1 β , could also synergise with IL-23 to induce IL-17 production by spleen cells from wild type and IL-1RI^{-/-} mice. However, the concentration of IL-17 produced by spleen cells from IL-1RI^{-/-} mice was approximately half of that observed in spleen cells from C57BL/6 mice. This finding may explain why a small percentage of IL-1RI^{-/-} mice can develop EAE and why PMA and anti-CD3 stimulation of cells from IL-1RI^{-/-} mice results in the production of small concentrations of IL-17. This finding may also explain why IL-1 and IL-18 appear to have very similar roles to play in autoimmune diseases, and why blocking either IL-1 or IL-18 are effective treatments for certain autoimmune diseases (Kinoshita *et al.*, 2004; Verbsky and White, 2004). However, unlike the other pro-inflammatory cytokines examined, the more distant IL-1 family member IL-33 was unable to synergise with IL-23 for IL-17 production in the absence of anti-CD3 stimulation. This may be due to IL-33 signalling through its receptor ST2, which has been reported to inhibit TLR and IL-1 receptor signalling (Liew *et al.*, 2005).

Recent papers have implicated the Treg associated cytokine TGF- β as well as the pro-inflammatory cytokine, IL-6 in the induction of IL-17 production by T cells (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). The present study confirmed these findings, with TGF- β and IL-6 stimulation of spleen cells resulting in IL-17 production, but only in the presence of anti-CD3 and anti-CD28. Furthermore, TGF- β and IL-6-induced IL-17 production was found to be partially dependent upon IL-1RI. In the absence of co-stimulation, however, TGF- β and IL-6 were unable to induce IL-17 production from spleen cells, which contrasts the effect of IL-1 and IL-23, which induce IL-17 in the absence of anti-CD3 or anti-CD28. However, it should be noted that TGF- β does not affect the generation of human IL-17 producing T cells (Acosta-Rodriguez *et al.*, 2007). In contrast IL-1 and IL-23 were found to be responsible for the development of human IL-17 producing CD45RO⁺ T cells (Acosta-Rodriguez *et al.*, 2007; Wilson *et al.*, 2007). These results indicated that IL-1 and IL-23-induced IL-17 may be produced by unconventional T cells, such as from NK T cells or $\gamma\delta$ T cells, given that ligation of the CD3 complex is required for activation of conventional $\alpha\beta$ T cells. This argument was strengthened by the finding that, in the absence of anti-CD3 stimulation, IL-1 and IL-23 induced an IL-17 producing population of CD3⁺ T cells, which was neither CD4⁺ nor CD8 α ⁺. Upon further examination it was found that the IL-17 producing unconventional T cells were $\gamma\delta$ T cells. IL-17 production from $\gamma\delta$ T cells is in broad agreement with published reports, which indicate that infection with *E. coli* induces IL-17-producing $\gamma\delta$ T cells, which are essential for the control and elimination of the invading bacteria (Shibata *et al.*, 2007). However, IL-1 and IL-23-induced IL-17 production is not limited to $\gamma\delta$ T cells, given that stimulation of spleen cells from $\gamma\delta^{-/-}$ mice as well as CD3⁺ T cells depleted of $\gamma\delta$ T cells, with IL-1

and IL-23, induced significant concentrations of IL-17. However, this IL-17 production was only detected in the presence of anti-CD3 co-stimulation. This indicates that IL-1 and IL-23 can stimulate IL-17 production by conventional $\alpha\beta$ T cells, with TCR co-stimulation, as well as $\gamma\delta$ T cells in the absence of co-stimulation.

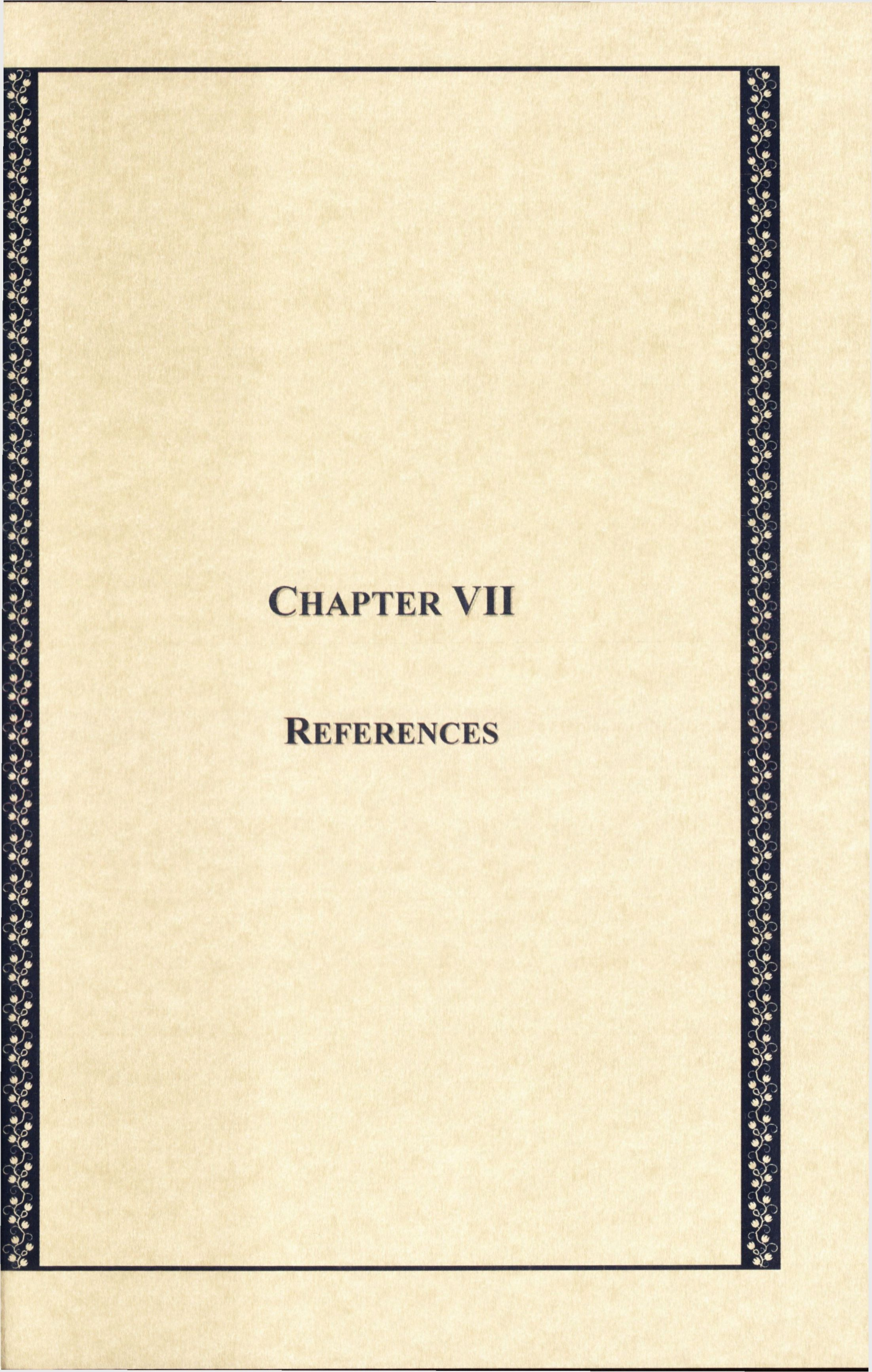
IL-17 has been associated with the development of certain autoimmune diseases. Inhibition or removal of IL-17, using neutralising antibodies or IL-17^{-/-} mice respectively, resulted in a reduction in the incidence of EAE (Komiyama *et al.*, 2006). IL-1RI^{-/-} mice also are significantly less susceptible to the development of autoimmune diseases, such as EAE. The role of IL-1 in EAE has been attributed to the pro-inflammatory nature of IL-1 precipitating neurodegeneration and inflammation, downstream of T cell activation. However, this study has found that the reduced incidence of EAE, in IL-1RI^{-/-} mice, correlates with a decrease in antigen-specific IL-17 producing T cells and reduced serum concentrations of IL-17. Furthermore, this study demonstrates that IL-1RI^{-/-} mice are capable of developing EAE following transfer of wild type MOG-specific T cells, suggesting that IL-1 is required for the activation of T cells that mediate EAE. This finding indicates that IL-1 is not essential for the neuro-inflammation, associated with EAE, and that there may be redundancy in pro-inflammatory cytokines responsible for the inflammation in EAE. The CNS inflammation in EAE may be mediated by TNF- α and IL-6 in the absence of IL-1.

The present study also demonstrated that $\gamma\delta$ T cells stimulated with IL-1 and IL-23 were capable of promoting IL-17 production by $\alpha\beta$ T cells, and that this was significantly enhanced in the presence of DC. Stimulation of DC with recombinant IL-17 stimulated production of IL-1 and IL-23. Furthermore stimulation of DC with the pro-inflammatory cytokines IL-1, TNF- α and IL-6 promoted IL-23 and IL-1

production. Collectively these findings suggest that $\gamma\delta$ T cells promote IL-17 production by $\alpha\beta$ T cells indicated by IL-1 and IL-23 production by DC. Thus DC may facilitate a positive feedback loop by which the $\gamma\delta$ T cells and $\alpha\beta$ T cells produce further IL-17.

Activation of innate immune cells is an essential step in the development of EAE, where mice immunised with MOG in combination with incomplete Freund's adjuvant (IFA) without mycobacterium, failed to develop EAE. However, supplementing IFA with, TLR agonists CpG or LPS, either alone or in combination, resulted in the development of EAE (Hansen *et al.*, 2006). However, a direct link between infection and development of multiple sclerosis in humans has never been established. It is therefore, possible that alternative immunostimulatory molecules may be responsible for the development of autoantigen-specific immune responses that lead to the development of MS. This study found that endogenous danger signals (DAMPs) such as elastase, HMGB1 and HRF were capable of DC activation. Furthermore, this effect was not due to contamination with known TLR agonists. The DAMPs examined in this study induced both IL-1 and IL-23 production from naïve DC, two cytokine already shown to promote the IL-17 production and consequently development of EAE. However, stimulation of DC with DAMPs did not generate the same cytokine profile as induced by PAMPs. IL-1, TNF- α and IL-6 production induced by DAMPs from DC was comparable to that induced following PAMP stimulation. However, unlike PAMPs, DAMPs were unable to induce the production of either IL-10 or IL-12 from DC. IL-6 has been shown to be a key cytokine in reversing the suppressive effects of Treg cells, thus allowing for effector T cell responses to progress (Pasare and Medzhitov, 2003). This suggests that DAMPs may be capable of priming adaptive immune responses that mediate autoimmune disease.

DC maturation and regulatory cytokine production is also required for the generation of antigen-specific T cells and successful vaccination. LT and LTK63 alone induce only partial maturation of DC. A second signal, such as that provided by a TLR-agonist is required to generate full DC maturation in vitro. However, when LT and LTK63 are used as adjuvants, especially for parenteral immunisation, they affect an immune response to co-administered antigen in the absence of TLR agonists or other exogenous activators of innate immunity. Therefore, when used in vivo the adjuvant effect of LT and LTK63 may be enhanced by a second signal from an endogenous activator of innate immune cells. The present study demonstrates that both LT and LTK63 modulated HMGB1, elastase and HRF-induced cytokine production, increasing DAMP-induced IL-1 and IL-10. Thus, DAMPs may provide a second stimulus for the enterotoxin induced maturation of DC in the absence of PAMPs.



CHAPTER VII

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7.1 REFERENCES

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