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'Regulation of Human T helper 17 cell Responses'

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Abstract

T helper 17 (Th17) cells potently produce interleukin (IL)-17, which is essential for Th17 cell-mediated pathogen clearance. Failure to regulate Th17 cells can increase Th17 cell numbers and IL-17 production, and is associated with autoimmune disease pathology. Therefore, understanding how Th17 cell responses are controlled may improve treatments in instances of Th17 cell dysregulation. Investigations in mice and humans have mainly studied the cytokine signals that determine Th17 cell responses. However, the strength of TCR signalling has previously been shown to be a further factor capable of determining effector T cell development. The central hypothesis of my thesis is, therefore, that the strength of TCR stimulation is also capable of regulating Th17 cell responses. I also investigated if T-cell density, a parameter often overlooked in investigations, can also affect Th17 cell responses. Cell density has been shown previously to be capable of modulating many parameters including the expression of certain Th17 cell-related transcription factors.

To assess the effect of T-cell stimulation strength on Th17 cell responses, human $CD4^+$ T-cells were activated with high or low strength stimuli administered by bead-bound antibodies which activate the TCR/CD3 complex and the costimulatory molecule CD28, or by monocyte-derived dendritic cells pulsed with decreasing superantigen concentrations. Experiments were performed in the presence of pro-Th17 cell cytokines IL-1β, TGFβ and IL-23. In both systems low strength TCR stimulation profoundly and significantly promoted Th17 cell responses, both proportionately and absolutely. The enhancement of Th17 cell responses by low TCR stimulation only occurred in the presence of co-stimulation through CD28. Furthermore, it was revealed by chromatin immunoprecipitation that low strength stimulation promoted Th17 cell responses by allowing binding of a Ca^{2+} regulated transcription factor NFATc1 to the *IL-17* promoter in a Ca^{2+} dependent manner.

To investigate how low strength T-cell stimulation might promote human Th17 cell responses *in vivo*, 20 healthy donors were genotyped for a single nucleotide polymorphism within the gene Protein Tyrosine Phosphatase-N22 (*PTPN22*), which is

highly associated with the autoimmune diseases type I diabetes and rheumatoid arthritis. *PTPN22* encodes a TCR signalling molecule, Lyp, which in minor allele carriers confers both reduced TCR and Ca^{2+} signalling. Culture of genotyped memory $CD4^+$ T-cells with anti-CD3/anti-CD28 stimulation and pro-Th17 cell cytokines revealed a trend indicating that the presence of the minor T allele promoted both IL-17 and IFN-γ production but decreased regulatory IL-10 secretion.

Regulation of Th17 cells by T-cell density was explored by culturing memory $CD4^+$ T-cells at decreasing T-cell densities in the presence of either pro-Th17 or pro-Th1 cell cytokines. Low T-cell densities profoundly promoted Th17 cell responses both proportionately and absolutely. No effect was observed on the IFNy response within cultures containing pro-Th1 cell cytokines, suggesting that T-cell density specifically affects Th17 cell responses. STAT3 activation, important for IL-17 expression, can be regulated by cell density. Analysis of STAT3 activation by western blot revealed higher STAT3 activation in low density cultured T-cells compared to high density cultured T-cells, which may provide an explanation for the increased Th17 cell responses observed.

The data within this thesis provide interesting and novel mechanisms by which human Th17 cell responses are regulated. I have demonstrated that Th17 cell responses are favoured by both low strength TCR stimulation and low T-cell density. These data highlight the diversity of factors capable of affecting Th17 cell responses *in vitro*; factors of which in the majority of studies have been overlooked.

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List of abbreviations

7-AAD	7-Aminoactinomycin D
ADCC	Antibody-dependent cell mediated cytotoxicity
AhR	Aryl Hydrocarbon Receptor
AICD	Activation induced cell death
Aire	Autoimmune regulator
ALK	Activin receptor-like kinase
AP-1	Activator protein-1
APC	Antigen presenting cells
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APL	Altered peptide ligand
ARAM	Antigen Recognition Activation Motifs
ATP	Adenosine Triphosphate
BAFF	B-cell activating Factor
BATF	Basic leucine zipper transcription factor
BCG	Bacillus Calmette-Guérin
BCL-6	B cell lymphoma-6
BCR	B-cell antigen receptor
BNC-2	Basonuclin
Breg	Regulatory B cell
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cDC	Conventional dendritic cells
Cdk	Cyclin dependent kinase
CDR	Complementarity determining regions
CFSE	Carboxyfluoroscein succinimidyl ester
ChIP	Chromatin Immunoprecipitation
ChiPseq	Chromatin Immunoprecipitation sequencing
CIA	Collagen-induced arthritis
CLIP	Class II-associated invariant chain peptide

c-Maf	Musculoaponeurotic fibrosarcoma oncogene homolog
CNS	Central nervous system
CRAC	Ca2+ release-activated Ca2+ channels
CREB	Cyclic-AMP- responsive-element-binding protein
CRS	Cytokine release syndrome
CSF	Cerebrospinal fluid
cSMAC	Central supra-molecular activation complex
cTEC	Cortical thymic epithelial cells
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
CysA	Cyclosporin A
DAG	Diacylglycerol
DC	Dendritic cell
DLL	Delta like ligand
DMSO	Dimethyl Sulphoxide
dSMAC	Distal supra-molecular activation complex
EAE	Experimental autoimmune encephomyelitis
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbant Assays
Erk	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box p3
FRC	Fibroblastic reticular cell
FSc	Forward scatter
GATA3	GATA-binding protein-3
GITR	Glucocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GSI	Gamma secretase inhibitor

GSK	Glycogen synthase kinase
GWAS	Genome wide association scan
HBSS	Hanks Balanced Salt Solution
HiD	High denstity culture T-cells
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
ICAM	Intercellular adhesion molecule
ICOS	Inducible T-cell co-stimulator
ICS	Intracellular cytokine staining
IDO	Indolamine 2-3-dioxygenase
IFNγ	Interferon-gamma
Ii	Invariant protein
IkB	Inhibitor of NF-κB
IL	Interleukin
IMDM	Iscoves Modified Dulbeccos Medium
IPEX	Immune dysregulation polyendocrinopathy, enteropathy, X linked
IRAK	IL-1 receptor associated kinase
IRF4	Interferon Regulatory Factor 4
IRS10	Iscoves Modified Dulbeccos Medium + Penicillin/Streptomycin, L-glumatime and Serum Replacement (10%)
ITAM	Immunoreceptor Tyrosine-based Activation Motifs
Itk	Inducible T-cell kinase
iTreg	Inducible regulatory T-cell
JAK	Janus Kinase
JNK	c-jun kinase
L e.g. CD62L	Ligand
LAP	Latency associated peptide
LAT	Linker for the activation of T-cells
LFA	Leucocyte Functional Antigen
LoD	Low density culture T-cells
LPS	Lipopolysaccharides
LRS	Leucocyte Reduction System

MACS	Magnetic activated cell sorting
MAPK	Mitogen-associated protein kinase
MHC	Major histocompatibility complex
MIP-2	Macrophage inflammatory protein-2
MoDC	Monocyte derived dendritic cell
MS	Multiple Sclerosis
mTEC	Medullary thymic epithelial cell
N-ICD	Notch-Intracellular domain
NFAT	Nuclear Factors of Activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NKT	Natural Killer T-cell
NOD	Nucleotide oligomerisation domain
NOD mice	Nonobsese diabetic mice
nTreg	Natural regulatory T-cell
OR	Odds ratio
PAMP	pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD1	Programmed death-1
pDC	Plasmacytoid DC
PE	Phycoerythrin
PGE2	Prostaglandin E2
PGN	Peptidoglycan
PK	Protein kinase
PLC- γ1	Phospholipase C-γ1
PMA	Phorbol 12-myristate 13-acetate
p-MHC	peptide-MHC
PRR	Pattern recognition receptors
pSMAC	Peripheral supra-molecular activation complexes
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene Fluoride

RA	Rheumatoid Arthritis
Rac1	Ras-related C3 botulinum toxin substrate 1
RAG	Recombination activating genes
RANK(L)	Receptor Activator of NFκB (Ligand)
RBC	Red Blood Cell
RF10	RPMI + Penicillin/Streptomycin, L-glutamine and FBS (10%)
RFLP	Restriction fragment length polymophism
RIG-1	Retinoic acid inducible gene I
RIR	RIG-I–like receptors
RNA	Ribonucleic acid
ROCK2	Rho-associated coiled coil containing protein kinase 2
RORa	RAR-related orphan receptor alpha
RORc	RAR-related orphan receptor C
RORyt	RAR-related orphan receptor gamma T
RPMI	Roswell Park Memorial Institute medium
RSS	Recombination signal sequences
Runx1	Runt-related transcription factor 1
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SEB	Staphylococcal enterotoxin B
SEFIR	SEF/IL-17R domain
SFK	Src Family Kinase
SH2	SRC homology domain 2
SHP2	SH2–domain-containing protein tyrosine phosphatase 2
SMAC	Supra-molecular activation complex
SNP	Single nucleotide polymorphism
Socs	Suppressor of cytokine signaling
SR	Serum Replacement
SSc	Side Scatter
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
TAP	Transporter associated with antigen processing

Tbet	T-box transcription factor
TBST	Tris buffered saline-Tween
T_{CM}	Central Memory T-cell
TCR	T-cell receptor
T_{EM}	Effector Memory T-cell
TEMED	N,N,N’,N’-tetramethylenediamine
T_{FH}	Follicular helper T-cell
TGF	Transforming growth factor
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
Th22	T helper 22
Th3	T helper 3
Th9	T helper 9
THi	High strength stimulated T-cell (1 anti-CD3/anti-CD28 bead : 1 T-cell)
TLo	Low strength stimulated T-cell (1 anti-CD3/anti-CD28 bead : 50 T-cells)
Th-POK	T-helper inducing-POZ krüppel factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TolDC	Tolergogenic dendritic cell
Tr1	Regulatory T-cell type 1 (IL-10 induced)
TRAF6	TNF Receptor Associated Factor 6
Treg	Regulatory T-cell
TSA	Tissue-specific self antigen
VDR	Vitamin D receptor
ZAP-70	Zeta-chain-associated protein kinase 70

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Publications

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1. Chapter 1: Introduction

T-cells are vital for instigating a successful adaptive immune response. They are capable of recognising virtually any invading pathogen and co-ordinating an appropriate immune response, resulting in the successful clearance of infection. Defects in T-cell responses can propagate persistent and damaging infections or result in chronic autoimmune diseases; highlighting the importance of T-cells, and the need for an appropriate response. By improving our understanding of the events that control the development and expansion of T-cells, new or improved therapies may be developed to 're-set' the immune system in individuals whose T-cell responses have become deregulated.

1.1 T-cells

The first line of defence confronting an invading pathogen is the innate immune response. The cells of the innate immune system are able to recognise and respond to pathogens in a non-specific manner. The innate immune response is also able to activate the highly specialised cells of the adaptive immune response which, unlike the innate response, are able to specifically recognise the pathogen and can provide long lasting immunity (1, 2). The adaptive immune system has two main lines of defence: The humoral and cellular responses mediated by B-cell lymphocytes and T-cell lymphocytes, respectively. T-cells were discovered in the 1950s by Gell and Benaceraf (3–5). The role of T-cells is to recognise both intra and extracellular pathogens and orchestrate their immune clearance. One of the main features distinguishing T-cells from B-cells is the way that they recognise antigen. T-cells recognise denatured protein antigens via their T cell receptor (TCR) whereas B-cells recognise native protein via membrane-bound immunoglobulin (referred to as B-cell receptors) that are specific for discontinuous peptide epitopes (6). T-cells can only recognise and bind denatured peptide antigen when the peptide is associated with multi-histocompatibility complex (MHC) molecules expressed on antigen presenting cells (APC). The T-cell recognition process is termed MHC restriction and was defined by Zinkernagel and Doherty (7). T-cell recognition and binding to peptide-MHC (pMHC) occurs through the TCR (8, 9) and T-cells may be divided into two populations depending on expression of the

heterodimers comprising the TCR either: αβ T-cells or γδ T-cells. Which TCR heterodimers are expressed is determined during T-cell development within the thymus. αβ T-cells may be further subdivided by expression of cell surface markers CD4 and CD8 whose expression is also determined during thymic development (1, 10).

1.1.1 T-cell Receptor

The TCR allows the T-cell to recognise peptide antigen, by specifically binding to pMHC. αβ T-cells have a TCR which is formed from herterodimeric α and β chains (11, 12). Each chain is composed of an N-terminal variable domain (Vα and Vβ) and constant domain (Cα and Cβ), and the chains are linked together via a disulphide bond. The α- and β-chains are anchored to the cell surface by a transmembrane region and each chain has a short cytoplasmic tail at the C-terminal end, which is thought to be incapable of signal transduction. To facilitate signal transduction the TCR associates with the CD3 complex (discussed in section 1.4.4). Each Vα and Vβ domain of the TCR consists of 3 hypervariable regions called complementarity determining regions (CDR), these CDR determine the peptide specificity and affinity of the TCR. Hypervariability within CDR produces a diverse population of TCR, allowing detection of a wide range of peptide antigens. TCR variation is produced during thymic development, by random recombination of genes encoding the α- and β-chains (Figure 1). Approximately 70 Variable (V) α segments and 52 Vβ segments exist. The α-chain is produced by random recombination of a Vα segment with one of 61 Junctional gene segments (Jα). The β chain is produced by recombination of one Vβ segment to one of two Diversity (D) segments (Dβ), this then undergoes recombination with one of 13 Jβ segments. The Vα and Vβ segments then undergo recombination with the constant (C) regions Cα and Cβ respectively. Recombination is mediated by the enzyme VDJ recombinase which recognises recombination signal sequences (RSS) that flank the gene segments (13). The recombinase enzymes are encoded by recombination activating genes (RAG) 1 and 2. The lymphocytes of $RAG^{-/-}$ mice are unable to produce functional TCR, and so the lymphocytes fail to survive thymic selection and therefore $RAG^{-/-}$ mice contain no mature T or B lymphocytes (14, 15). The absence of mature T and B lymphocytes makes $RAG^{-/-}$ mice a useful tool in immunological research particularly for studying tissue transplantation (16). TCR variability is further increased by random insertion of

nucleotides between the connection sites of VDJ gene segments. Theoretically 10^{15} different αβ TCR can be generated; however humans only contain 10^{12} T-cells. Peptide cross-reactivity is a characteristic of T-cell antigen recognition, allowing recognition of virtually any antigen from a limited number of T-cells (11, 13, 17).

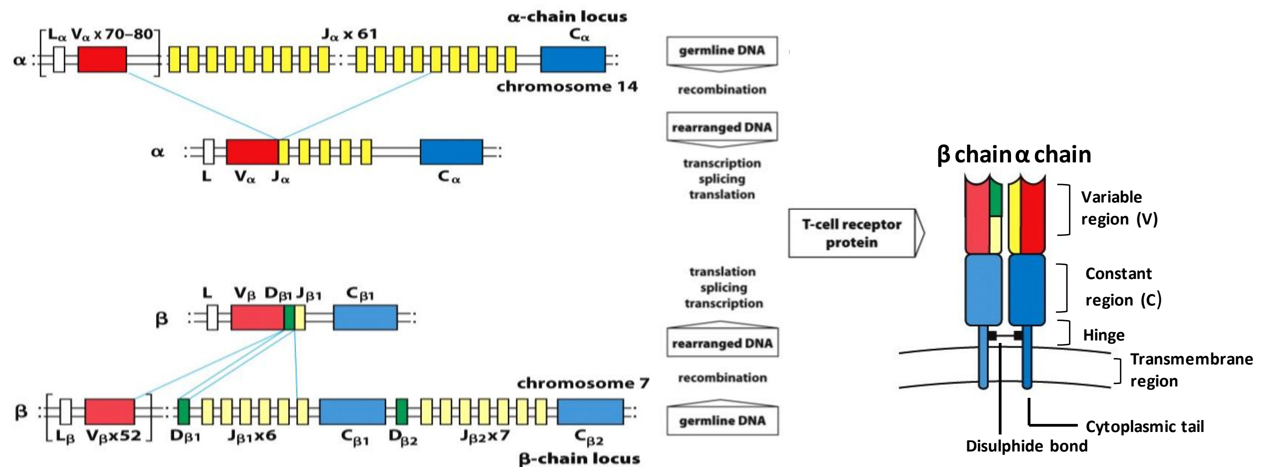


Figure 1. αβ T-cell receptor VDJ recombination. Recombination of the α- and β-chains of the TCR is mediated by enzyme VDJ recombinase encoded by the RAG genes. Transcription and splicing of the V (D), J, and C segments generates mRNA that is translated to yield the TCR α- and β-chain proteins. The α- and β-chains pair-up shortly after synthesis, resulting in the αβ TCR heterodimer. The figure is adapted from Immunobiology Janeway *et al*. (18)

1.1.2 T-cell Receptor Selection

It is important to have both a T-cell population capable of expressing a diverse array of TCRs, and a TCR repertoire that does not initiate immune responses against self. T-cell development and selection of appropriate TCRs occurs within the thymus (19). During thymic selection the αβTCR interacts with MHC molecules expressed by thymic stromal cells. The process of positive selection ensures that TCR recognition of antigen is MHC/Human Leukocyte Antigen (HLA) restricted. Positive selection occurs during the $CD4^+/CD8^+$ double positive stage of TCR development. T-cells expressing TCRs that bind to pMHC with low avidity, but with a sufficient strength to transduce TCR signals, are able to survive the positive selection checkpoint (20). T-cells capable of recognising pMHC turn off the genes encoding the TCR and increase TCR surface expression. In addition, TCR recognition of pMHC protects the T-cell from apoptosis and also stimulates the expression of chemokine receptors allowing T-cell trafficking to the medulla (21). T-cells which do not recognise pMHC will not receive low avidity signals and will therefore die by neglect through lack of TCR stimulation. The positive selection

process ensures that surviving T-cells are self-MHC restricted. The process of MHC/HLA restriction during positive selection contributes to T-cell mediated rejection of grafts in organ transplantation. Recipient T-cells bind to the non-self MHC/HLA molecules of the transplanted organ with high avidity eliciting an immune response.

Positive selection does not remove high affinity self-reactive T-cells, which may instigate immune responses against self in the periphery. In order to distinguish self-antigens from foreign antigens, T-cells also undergo negative selection, which is also termed central tolerance. Negative selection in the thymus causes T-cells expressing high avidity self-reactive TCRs to be eliminated by apoptotic clonal deletion (22). The negative selection process requires the exposure of the developing thymocytes to self-antigens, including antigens that have highly restricted tissue expression. The cells contributing to central tolerance in the thymus include: Medullary thymic epithelial cells (mTECs), bone marrow derived cells, DC and cortical thymic epithelial cells (cTECs) (23). DCs and mTECs significantly contribute to negative selection by expressing high levels of MHC and co-stimulatory molecules. In addition mTECs are capable of presenting tissue-specific self-antigen (TSA) due to the expression of the autoimmune regulator (Aire) protein, which is located in mTEC nuclei. Aire initiates TSA expression within mTECs, and presentation of Aire-induced TSAs to T-cells allows negative selection (24). T-cells with TCRs that recognise TSAs in the context of self-MHC are deleted. The TSA transcriptional start sites within mTec however differ from transcriptional start sites within the periphery, therefore peptides presented in the thymus may differ from peripheral peptides. The important role played by Aire in central tolerance was highlighted by the development of a multi-organ autoimmune disease in humans deficient in Aire, called Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (25). Aire deficiency leads to decreased TSA expression in the thymus, allowing the escape of self-reactive T-cells into the periphery. Although thymic DCs express vastly lower levels of Aire than mTECs, they are also important for negative selection (26). The DC subsets located within the thymus arise either from intra-thymic precursors or extra-thymically. The presentation of antigen by thymic DCs allows the deletion of self-reactive T-cells within the thymus and therefore contributes to negative selection. In addition antigen presentation by

thymic DCs is essential for the induction of thymic derived natural regulatory T-cell (nTregs) in both mouse and human sytems (23, 27). Furthermore recent investigations suggest that Aire regulates the transfer of TSAs to thymic DCs, allowing the indirect presentation of TSAs by thymic DCs to T-cells (28). In addition central tolerance can also be regulated by editing self-reactive TCR through further VDJ recombination (29, 30), which occurs extra-thymically and is dependent on RAG expression (31) .

1.1.3 CD4 and CD8 lineage commitment

During thymic development αβ T-cells determine their lineage commitment towards either a $CD4^+$ T-helper cell or $CD8^+$ cytotoxic T-cell. Committing the T-cell to express either CD4 or CD8 restricts the T-cell to the recognition of antigen in the context of either MHC class 2 or MHC class 1 molecules respectively. CD4 and CD8 are immunoglobulin family members and transmembrane glycoproteins that directly bind to MHC class II and MHC class 1 molecules, and play a role in stabilising the TCR:pMHC interaction. In addition CD4 and CD8 molecules can function within the TCR complex to augment TCR signalling by recruitment of Lck, a TCR signalling molecule (32, 33).

In addition to TCR selection, αβ T-cell development within the thymus also commits T-cells to either a $CD4^+$ or $CD8^+$ lineage, and the major developmental stages of the T-cells within the thymus can be distinguished by CD4 and CD8 expression. Early thymocytes are double negative for both CD4 and CD8 molecules. Thymocytes then proceed to the CD4/CD8 double positive stage of development which is the stage at which the αβ TCR is initially expressed. Finally T-cell lineage commitment results in two populations of single positive mature thymocytes expressing either $CD4^+$ or $CD8^+$ (34, 35). Therefore, during the double positive stage, the decision to express either CD4 or CD8 on the T-cell surface is made. Two models were originally proposed to explain how CD4 or CD8 lineage commitment is determined: the stochastic model or the instructive model. The stochastic model suggested that inappropriate co-receptor expression results in thymocyte elimination, whereas the instructive model proposed that CD4 or CD8 expression appropriately matches the MHC restriction of the TCR. More recently, another model has been proposed where CD4 or CD8 commitment is determined by TCR signal strength, rather than by expression of the co-receptors

themselves (35). This model, termed the quantitative instructive model, proposes that stronger and weaker signals lead to CD4 and CD8 commitment, respectively. More recently a transcription factor, T-helper inducing-POZ krüppel factor (Th-POK), has been found to regulate lineage commitment (36). This finding has led to the instructive model being favoured where Th-POK is regulated by TCR signal strength/duration leading to either CD4 or CD8 expression (34, 36).

1.1.4 Peripheral Tolerance

Only ~1-3% of thymocytes survive and exit the thymus (22). Within the thymus T-cells expressing high affinity self-reactive TCR are removed, however some T-cells expressing low affinity self-reactive TCRs are able to escape the thymus to populate secondary lymphoid organs. Peripheral tolerance mechanisms allow low affinity self reactive T-cells to be regulated (37). Immunological ignorance is one such mechanism. Physical anatomical barriers, such as the blood-brain barrier, can prevent potentially self-reactive TCR from recognising TSA (38). Naive T-cells, guided by CCR7 gradients, circulate from the blood to lymphoid organs where they scan DCs for foreign antigens. Failure to ligate TCRs results in the naive T-cell being circulated again through the lymphoid system. TSAs are expressed at low density within lymphoid organs, which is insufficient to activate self-reactive T-cells (39). The absence of TSA within the lymph system, maintains the naive T-cell in state of ignorance, preventing T-cell migration to tissues with high TSA density expression. More recently $CD45^-$ stromal cells have been observed to expess Aire and to cross-present TSA MHCI complexes within lymph nodes (40). Stromal TSA cross-presentation however occured in a non-immunogenic fashion, inducing tolerance in the self-reactive $CD8^+$ T-cells following TCR ligation.

A further tolerance mechanism protecting against self-reactive T-cells is clonal anergy. Anergy occurs when TCR ligation occurs in the absence of CD28 co-stimulation resulting in long term T-cell hyporesponsiveness characterised by repressed TCR signalling and Interleukin (IL)-2 production. Microbial derived ligands activate Toll like receptors (TLRs) expressed on DCs, which induce DC maturation. Maturation results in the up-regulation of CD80/CD86 molecule expression on the DC cell surface. CD80 and CD86 bind to CD28, inducing signalling, and prevent the induction of anergy. Self-

antigens are not associated with TLR activating ligands and therefore only TCR derived signals are provided preventing the T-cell from making an immunogenic response.

T-cells are also capable of activating cell intrinsic pathways to limit their own response. TSA are expressed at high density in peripheral tissues, and engagement with self-reactive TCR results in high avidity binding. High strength TCR signalling can induce the expression of FAS and FASL on the T-cell surface (41). When FASL interacts with FAS it triggers T-cell death causing deletion of the T-cell, termed activation induced cell death (AICD). Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is another molecule induced on the T-cell surface shortly after T-cell activation. CTLA-4 is a structural homologue of CD28 which binds to CD80/CD86 with higher affinity. CTLA-4 can inhibit TCR signalling by competing with CD28 for CD80/CD86 binding sites (29). Furthermore CTLA-4 has been demonstrated to trans-endocytose CD80/CD86 from the cell surface of APC to further inhibit CD28 signalling (42). In addition, ubiquitin ligases such as Grail, and CBL-B can also be up-regulated in response to strong TCR signals; these ligases interfere with TCR and CD28 signalling by tagging these receptors with ubiquitin (29, 43). Grail is capable of ubiquitinating a number of membrane associated TCR signalling components, including the TCR itself (44). Ubiquitination targets these TCR signalling molecules for proteasome degradation, which inhibits TCR signalling and leads to the induction of T-cell anergy. Central and peripheral tolerance mechanisms are sufficient to ensure immunity occurs in response to foreign antigen alone. Failure to regulate tolerance mechanisms however, can lead to the induction of debilitating autoimmune diseases.

1.2 Antigen Presentation

1.2.1 Major Histocompatibility Complex

T-cells are restricted to recognising antigen when presented by MHC molecules, known in humans as Human Leukocyte Antigen (HLA). In general, the MHC class II pathway processes and presents antigens from endocytosed pathogens present in vesicles, which are topologically extracellular antigens (45). Comparatively the MHC class I pathway processes and presents endogenously synthesised peptides such as those from viruses and certain bacteria (45). In addition the cross-presentation pathway

allows antigens from infected or abnormal cells to be acquired by DCs and presented by MHC class I molecules allowing $CD8^+$ T-cell priming with the addition of DC derived co-stimulation (46). However, the majority of peptides bound to both MHC class I and class II molecules are endogenous self-peptides which are thought to regulate T-cell homeostasis and activation in the periphery (47).

1.2.2 MHC Class I

MHC class I molecules (HLA A, B, C) are recognised by $CD8^+$ cytotoxic T-cells, whose function is to kill infected cells by releasing cytotoxins such as perforin and granzymes. MHC class I molecules present peptides from obligate intracellular pathogens that 'hijack' the cells biosynthetic machinery, such as viruses and certain intracellular bacteria and parasites (48). All nucleated cells may be subject to viral attack, thus nearly all cells express MHC class I. Red blood cells do not have a nucleus and are therefore MHC class I negative and so provide a site where infection can pass undetected, as occurs at the blood stage of infection with the parasite *Plasmodium falciparum* (49).

MHC class I is a heterodimer consisting of 4 extracellular domains α1, α2, α3, and β2 microglobulin. The α1 and α2 domains constitute the peptide binding sites that present degraded protein fragments (50). MHC class 1 molecules are assembled within the ER. Successful assembly requires peptide antigen to bind to the MHC class I molecule. Peptides are degraded within the cytosol by the multisubunit proteasome complex. Transporter associated with antigen processing (TAP), a heterodimer composed of subunits TAP1 and TAP2, transports proteasome degraded peptides from the cytosol to the endoplasmic reticulum. TAP is able to bind peptides with a degree of size and sequence specificity (51). TAP then transfers the peptide to the assembling MHC class I molecule (48, 50). Tapasin enhances peptide transfer by increasing TAP stability and facilitating peptide transfer to the assembling MHC class 1 molecules (52). Once peptide is loaded onto the MHC class I molecule, the resulting peptide-MHC (pMHC) is transported to the plasma membrane. The MHC class I binding domain is a groove with the sides formed by α-helices and the floor by 8 stranded β-pleats. Closed ends dictate that only peptides of 8-10 amino acids in length can bind (53). The

peptide-binding motif of the groove contains anchor residues which bind specific amino acids, and these are spaced by non-anchor residues which are less stringent in amino acid specificity. The groove allows a variety of peptides to bind, but conserved residues and closed ends limit the number of potential antigens capable of binding. This allows predictions to be made for peptides that can bind a given MHC class 1 molecule (53, 54).

1.2.3 MHC Class II

MHC class II molecules (HLA, DO, DP, DQ, & DR in humans) are recognised by $CD4^+$ T-cells. Unlike MHC class I molecules, MHC class II molecules present peptide from extracellular locations (55). Extracellular pathogens/antigens are internalised by phagocytosis or pinocytosis, and are then degraded by lysosomal proteases and the low pH within the vesicle (56). Class II molecules are formed by a heterodimer of membrane spanning α (consisting of α1 and α2 domains) and β (consisting of β1 and β2 domains) chains, very similar those domains comprising the MHC class I molecules. Like MHC class I, the MHC class II binding groove contains anchor residues. However, unlike MHC class I, MHC class II has an open ended peptide binding groove, formed by the α1 and β1 domains, allowing greater flexibility in peptide size than MHC class I (57). MHC class II molecules are assembled within the ER. As extracellular antigens enter the cell via endocytosis, MHC class II molecules bind peptides within the phagosome, rather than the ER (58). A chaperone protein called invariant protein (li) stabilises the class II heterodimer and prevents inappropriate binding of antigen to the class II binding groove. MHC αβ:li are transported through the trans golgi network, and may then traffic to the plasma membrane prior to being sorted into the endocytic pathway (58, 59). Endocytic organelles have a low pH allowing protein degradation. MHC αβ:li entry to endosomes allows li degradation by proteasomes, leaving behind a Class II-associated invariant chain peptide fragment termed CLIP. CLIP is removed by chaperone protein HLA-DM which directly binds to αβ:CLIP, and facilitates peptide sampling by the MHC class II binding grove (59, 60). Successful loading then results in pMHC class II expression on the APC surface. MHC class II expression is limited to a small group of cells termed 'professional' APC including macrophages, dendritic cells

(DC), and B-cells, all are capable of presenting exogenous antigen to $CD4^+$ T-cells in an MHC class II restricted manner.

1.2.4 Antigen presentation to T-cells

Naive T-cells are mainly primed within lymphoid tissues by professional APCs. Professional APCs are specialised for antigen presentation and T-cell activation, furthermore professional APCs can convey pathogen related information to the T-cells. Pathogen information is acquired by the APC in two forms: through the antigenic structure, and by the signals received from pattern recognition receptors (PRR) (61). Parasites, microbes and viruses contain evolutionarily conserved molecular patterns known as pathogen associated molecular patterns (PAMPs). PAMPs are present in various forms including lipopolysaccharide (LPS) and peptidoglycan (PGN) from bacteria, and double stranded RNA from viruses. PAMPs are recognised by a number of different PRRs, including TLRs, retinoic acid inducible gene I (RIG-I) –like receptors (RLRs), and nucleotide oligomerisation domain (NOD) like receptors (62). PRR are expressed on both hematopoietic and non-hematopoietic cells and provide innate recognition of pathogens. Activation of PRR initiates signalling pathways that allow the expression of innate response genes which allow professional APC to release pro-inflammatory chemokines and cytokines (63). For example PGN, a component of bacterial cell walls, binds to TLR2 (64). Activation of TLR2 results in activation of the Mitogen-associated protein Kinase (MAPK) and NfκB pathways which allow expression of pro-inflammatory genes. In addition caspase-1 is also activated which processes pro-IL-1β into active IL-1β for secretion.

Within the lymphoid tissues DCs/professional APCs can deliver the pathogen derived information that they acquired in the periphery to T-cells. Three main signals are delivered to the T-cell which can result in T-cell activation and the generation of an effector T-cell response. Signal 1 is delivered to naive T-cells which express a TCR specific for the antigenic peptide presented by MHC molecules. Signal 2 is provided by co-stimulatory molecules (CD80 and CD86) which can bind and signal through CD28 which is expressed on the T-cell surface. Signal 2 provides information about the pathogenic potential of the pathogen; indicated by the extent of DC maturation (65).

High CD80/CD86 molecule expression does not ensure T-cell activation; CTLA-4 can also bind to CD80/86 but initiates a signalling pathway that confers negative regulation of T-cell activation (65). The information received by the APC upon maturation also allows secretion of small quantities of cytokines (signal 3) which signal to direct the T-cell towards a specific effector T-cell response (66). The weak PRR-induced cytokine secretion is subsequently amplified by DC-T-cell cross talk through CD40/CD40L (63, 67). These signals act together to determine the development of distinct effector CD4^{+} T-cell phenotypes. Professional APC must therefore be capable of detecting the type of pathogen and conveying this information to T-cells in order to elicit the correct immune response, mediating pathogen clearance.

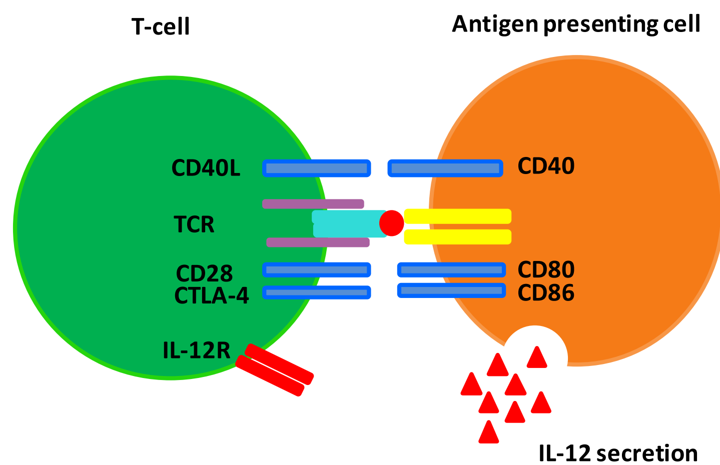


Figure 2. The interaction between T-cell and APC that results in T-cell activation. Signal 1 is provided by MHC class II presentation of antigen recognised by an antigen-specific TCR. Signal 2 is provided by co-stimulatory molecules (e.g. CD80/CD86) binding to CD28, though this interaction may be inhibited by CTLA4. Signal 3 is provided by polarising soluble or membrane bound molecules, for instance IL-12p70 secretion detected on the T-cell surface by IL-12R allowing a Th1 response (68).

1.3 Antigen-presenting cells

1.3.1 Dendritic Cells

In the 1970's Steinman and Cohn described a distinct population of large stellate cells that were highly capable at antigen presentation and T-cell stimulation, which they termed dendritic cells (DCs) (69, 70). DCs are able to sample tissue antigens and then migrate to lymphoid tissue to inform the adaptive immune system of dangers to the peripheral tissues (71). Immature DCs constantly endocytose material from the environment, however are poor at antigen presentation. DCs express a wide range of PRR on their cell surface, which allow DCs to make an innate immune response to

danger signals in the periphery. PRR triggering initiates signalling pathways such as the MyD88 and MAPK pathways, which result in 'DC maturation' (61, 63). DC maturation increases the cells' capability to process and present antigen in MHC class II molecules on the cell surface and to upregulate co-stimulatory molecules (e.g. CD80/86). In addition ligation of PRR activates distinct signalling pathways leading to secretion of different cytokines profiles depending the PRR molecules triggered (72). For example Dectin-1 agonist curdlan induces secretion of enhanced IL-1β and IL-23, where as TLR4 agonist LPS induced enhance IL-6 and IL-12-p70 secretion (73). Following PPR-mediated activation, DC stop endocytosing material from the microenvironment (63). Mature DCs are then able to mediate antigen-specific T-cell expansion and differentiation into appropriate effector T-cell responses. Interactions between DC and T-cells are not unidirectional. T-cells are able to signal to DC via CD40/CD40L interactions licensing the DC to prime $CD8^+$ cytotoxic T lymphocyte (CTL) responses, explaining the dependence of $CD8^+$ CTL on T helper cells (74).

DCs are widely distributed in low numbers within the body. There are a number of DC subtypes which are characterised based upon tissue location, migratory capacity and immune function. All DC subtypes are capable of taking up and presenting antigen. Two main classes of DC are found in mice: classical/conventional DC (cDC) and plasmacytoid DC (pDC) (71, 75, 76). cDCs are located in the lymphoid tissue, interface and connective tissues and may be further subdivided in to $CD8\alpha^-CD11b^+$ ($CD8\alpha^-$) or $CD8\alpha^+$ $CD11b^-$ ($CD8\alpha^+$) cDCs. $CD8a^-$ cDCs have a strong capacity to migrate to T-cell areas following TLR stimulation where they activate $CD4^+$ T-cells. The $CD8a^+$ DCs are particularly good at cross-presentation of antigen to $CD8^+$ T-cells, initiating CTL responses, and are also good at inducing Th1 cell responses (77). pDC are present in the spleen and lymphoid tissues but are also found to circulate in the peripheral blood. pDC can be activated via TLR engagement alone (78). TLR binding of CpG rich DNA motifs or double stranded DC initiates signalling pathways that activate pDC's allowing rapid production of large quantities of IFNa in response to viral infection (75).
However the relationship between mouse and human DC subsets is not clear; partially due to the low frequencies of DC in peripheral blood, which is the main experimental source of human immune cells (79). Human cDC are identified by $CD11c^+$ and $HLA\text{-}DR^+$,

whereas human pDC are CD304$^+$. CD8a is not expressed by human DC, and so three further markers are used to distinguish human cDC subsets CD16, CD141 and CD1b/c (80). More recently in humans the CD11c$^+$CD141$^+$ DC has been demonstrated to cross-present antigen and produce IL-12p70 similar to its murine CD8a$^+$ DC counterpart (81). A recent investigation has revealed that human peripheral blood DC are very similar to human splenic DC, supporting the use of blood DC to investigate human DC biology (79).

DCs are important at instigating immunity, however evidence now demonstrates that they are also capable of regulating T-cell responses; DCs with a regulatory capacity are termed tolerogenic DCs. Mature cDC can display tolerogenic function, secreting anti-inflammatory cytokines such as IL-10. Tolerogenic DC (tolDC) function can be induced during DC maturation if DC are activated by pathogen derived molecules in the presence of immune suppressive cytokines such as IL-10 and Transforming growth factor (TGF)-β (82). In addition tolerogenic DCs can be generated *in vitro* by treatment with pharmacological immunosuppressive drugs such as vitamin D3 and dexamethasone which suppress NfκB dependent maturation. TolDC offer a potential new therapy for the treatment of autoimmune diseases with experimental models highlighting the promise of tolDC cellular therapy (83) .

1.3.2 B-cells

B-cells are lymphocytes capable of both antigen presentation and secretion of antibodies that recognise and bind native protein antigen. Naive B cells are activated and able to capture antigen via their specific membrane bound B-cell antigen receptor (BCR). When Ag binds to the BCR the event triggers AgBCR internalisation and activation of the B-cell. B-cells are able to process and present the internalised antigen on MHC class II to CD4 T-cells; B-cells also upregulate CD86 on the cell surface providing signal 2 (84). Compared to DC, B-cells are considered poor APC. This is in part due to low numbers of circulating antigen specific B-cells and their absence from skin/mucosal sites (84). However, B-cells can contribute highly as APC, and are capable of inducing T-cell activation or tolerance (85), as highlighted by failures in T-cell responses in their absence (86, 87). In addition B-cell antigen presentation is

particularly efficient when antigen load is low. The BCR allows antigen specific uptake of antigen which permits much greater quantities of low abundance antigen to be processed and loaded onto MHC class II molecules (88). B-cells also secrete cytokines and are able to promote T-helper cell responses including Th1, T helper 2 (Th2) or regulatory T-cell phenotypes (89, 90). Furthermore B cells, like T-cells, may also be further subdivided based upon their cytokine secretion profile which determines their function (91). B-cells can produce cytokines including IL-12, Interferon-gamma (IFNγ) and Tumour necrosis factor (TNF)-α and B effector type two cells can secrete IL-4 and IL-13 (91, 92). In addition regulatory B cells (Bregs) are characterised by the secretion of IL-10, allowing Bregs to inhibit T-cell proliferation and effector cytokine production (91). B-cells can also play an important role in the pathogenesis of autoimmune diseases by producing autoantibodies which recognise self-proteins, and via production of inflammatory cytokines (88). B-cell depletion therapies (such as rituximab therapy) are used to treat auto-antibody associated autoimmune diseases such as Rheumatoid Arthritis (RA) (93). The mechanism by which the therapy acts is not fully understood but is thought to occur by either: directly removing the source of the auto-antibodies, removing the source of cytokine/chemokine secretion (which can alter the T-cell response), or by preventing priming of auto-reactive T-cells.

1.3.3 Macrophages & monocytes

Macrophages are highly phagocytic cells located in mainly non-lymphoid tissues and inflammatory sites where they are specialised to up take nectrotic or apoptotic material; macrophages can clear ~$2x10^{11}$ erythrocytes per day for host reuse (94). A number of different macrophage subtypes have been characterised based upon their tissue of residence e.g. kupffer cells in the liver or microglia in neuronal tissue. Macrophages are capable of antigen presentation and can also initiate pro- or anti-inflammatory responses depending on the means of activation (95). The classical view of macrophage activation is via dual TNFα and IFNγ/β signalling. This signalling requirement is often initiated via activation of certain TLRs/IL-1R resulting in endogenous TNFα and IFNγ/β production. In response to these signals macrophages are able to upregulate expression of both MHC class II and CD80/86 (94, 96). Macrophages can initiate Th1 and Th2 cell responses and have also been

demonstrated to have regulatory/tolerogenic function (96). In addition, classically activated macrophages have been demonstrated to express IL-6, IL-23 and IL-1β expression and could regulate T helper 17 (Th17) cell responses.

Monocytes are increasingly being acknowledged as being capable of determining the polarisation of T-cell immune responses (97). TLR-activated monocytes rather than DC are capable of initiating Th17 cell responses (98, 99). Monocytes are present at sites of infection and T-cell priming, allowing monocytes to influence T-cell responses via cell-cell contacts and cytokine secretion.

1.4 T-cell-APC interactions

1.4.1 T-cell - APC interactions within the lymph node

Antigen presentation to T-cells predominantly occurs within the lymph nodes. Many antigens enter the body in non-lymphoid organs, requiring migration of APC to the lymphoid organs. Migration occurs through changes in adhesive interactions and expression of chemokines such as Chemokine (C-C motif) Ligand-7 (CCL-7) (100). T-cells present within the lymph nodes are guided towards interactions with DC by the fibroblastic reticular cell (FRC) network which also provides survival factors including IL-7 (101). Following entry into the lymph node, antigen presenting DCs stably position themselves on to the FRC network allowing T-cells to rapidly sample antigens. In comparison to random distribution of DCs, the FRC is thought to increase T-cell-DC interactions. Upon finding a DC with a relevant antigen epitope, the T-cell binds and forms a longer interaction that lasts for several hours, studies show that after 24-30 hours T-cells disengage from DCs (100).

1.4.2 Immunological synapse

Formation of an immunological synapse allows cross-talk between the T-cell and APC, controlling downstream effects (102). Initial TCR-pMHC interactions have low affinity compared to antibody-antigen interactions, thus initial contacts are made by accessory molecules such as CD2 and Leucocyte Functional Antigen (LFA-1), whose ligands, LFA-3 and Intercellular adhesion molecule (ICAM)-1 respectively, are present on APC. These interactions bring the APC and T-cell together in close proximity. Non-specific TCR-

pMHC interactions result in dissociation of the two cells. Specific cross-linking of the TCR to pMHC results in the generation of TCR signals through Lck phosphorylation and calcium influx (103). Following TCR-pMHC ligation and initiation of TCR signalling, T-cell movement ceases. TCR signalling events also lead to changes in cytoskeletal arrangement and aggregation of cell surface receptors around the synapse between the TCR and APC (104). As the synapse matures, its composite molecules are organised into distinct areas called the supra-molecular activation complexes (SMAC) which may be viewed by confocal microscopy as a 'bulls-eye' structure (105). Multiple TCRs comprise the central region (cSMAC), whereas adhesion molecules such as LFA comprise the periphery (pSMAC) (103). A difference in the pattern of IS formation between differentiated effector T-cells has been observed (105, 106). Th1 cells can form a mature IS with a distinct cSMAC and pSMAC, whereas Th2 cells form multifocal IS. Furthermore the composition of the synapse differed between effector T-cell phenotypes, e.g. CD45 was excluded from the Th2 cell IS interface but was included in the Th1 cell IS interface.

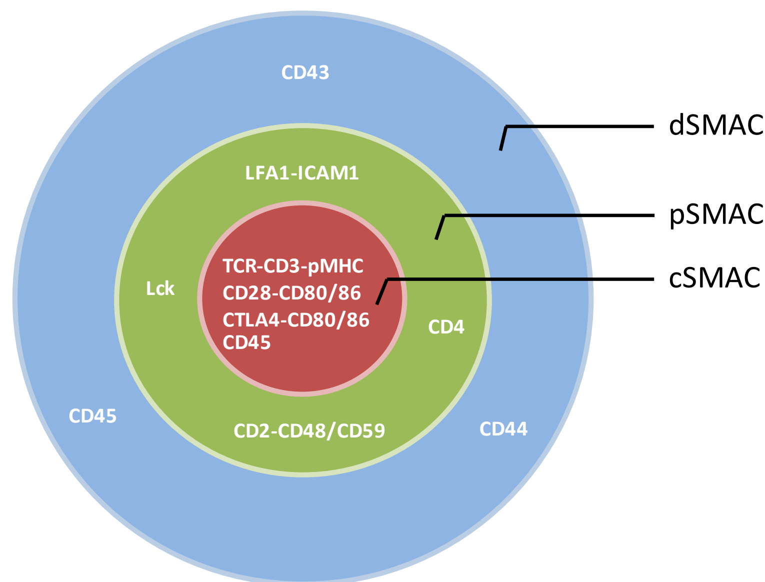


Figure 3. The Immune Synapse. The image demonstrates the 'bullseye' formation of the immune synapse as viewed face on. Figure adapted from Huppa & Davis Nature Reviews Immunology 2003 (107).

1.4.3 T-cell Receptor Complex

The TCR αβ chains span the T-cell cell membrane. The αβ chains have a positively charged transmembrane domain that is structurally unfavourable; they also lack signalling capabilities due to a short cytoplasmic region. To neutralise the positive

charge and allow downstream signalling, the αβ chains form a complex with CD3 molecules, including CD3 γ, δ, ε, and ζ, with a stoichiometry (TCRαβ, CD3γε, CD3δε and CD3ζζ) that neutralises the positive charge (108). The CD3 molecules cytoplasmic tails contain Immunoreceptor Tyrosine-based Activation Motifs (ITAM) and Antigen Recognition Activation Motifs (ARAM). Following TCR engagement these motifs are phosphorylated allowing activation of downstream signalling pathways that modulate gene expression within the nucleus (109, 110).

1.4.4 TCR signalling pathway

T-cells are able to detect a single pMHC ligand, however it requires >10 of these specific TCR-pMHC binding events to increase and maintain Ca^{2+} levels (107). In the absence of CD4 the initiation of TCR signalling requires a higher affinity TCR ligation than in the presence of CD4, between 25-30 pMHC complexes are required to be bound to increase Ca^{2+} levels. TCR recognition of pMHC and immune synapse formation results in Ca^{2+} signal transduction from the TCR to the nucleus, modulating gene expression and activating the T-cell to enable effector functions (107). TCR ligation results in activation of Src protein tyrosine kinases, Lck and Fyn, which phosphorylate ITAM motifs on CD3 molecules. Phosphorylation of CD3 molecule ITAM motifs in turn allows ζ-chain-associated protein-70 (Zap-70) recruitment; from this point a cascade of phosphorylation events occur allowing diversification of the TCR signal through a number of signalling pathways (111–113). Important targets of ZAP-70 are linker for the activation of T-cells (LAT), a transmembrane protein. LAT is able to recruit phospholipase C-γ1 (PLC- γ1) and is activated by inducible T-cell kinase (Itk). PCL-γ1 hydrolyses membrane lipid $PI(4,5)P_2$ into secondary messenger proteins diacylglycerol (DAG) and IP_3. DAG activates two major pathways Ras and protein kinase (PK)Cθ. Ras initiates MAPK pathway that leads to extracellular signal-regulated kinase 1 (Erk1). Erk1 activates activator protein-1 (AP-1), a transcription factor which forms a component of many transcriptional complexes. DAG binding and activation of PKCθ regulates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation. NF-κB is associated with inhibitor of NF-κB (IκB) in the cytosol of resting T-cells. T-cell activation results in PCKθ mediated activation of NF-κB through IκB

phosphorylation, allowing NF-κB nuclear translocation and transcriptional activity (112–114).

In addition to DAG, the secondary messenger molecule IP_3 is also generated by TCR-activated PLCγ1. IP_3 stimulates ER Ca^{2+} store release into the cytoplasm. Ca^{2+} store release in turn triggers sustained influx of extracellular Ca^{2+} through Ca^{2+} release-activated Ca^{2+} channels (CRAC) (115). Increased intracellular Ca^{2+} activates the Ca^{2+}-dependent enzyme calcineurin, a serine-threonine phosphatase that activates transcription factors including Nuclear Factor of Activated T-cells (NFAT), NF-κB, and Cyclic-AMP- responsive-element-binding protein (CREB) (112, 115).

An alternative TCR signalling pathway has also been described, which is independent of Lat and PLCγ1 (116). The alternative pathway results in MAPK p38 activation through direct phosphorylation by ZAP-70 (117). In addition the use of classical vs alternative pathway differs in naive and memory T-cells (118). Naive T-cells have low expression of PLCγ1 and therefore impaired signalling via the classical PLCγ1 pathway. Instead it is reported that naive T-cells signal via the alternative pathway, activating p38 through direct phosphorylation of MAPK by ZAP-70. Activation of p38 in naive T-cells via the alternative pathway results in the expression of vitamin D receptor (VDR), which in turn activates PLCγ1. Following priming memory T-cells are therefore able to signal via the classical PLCy1 pathway. The differential use of downstream TCR signalling pathways in naive and memory T-cells potentially explains the differences in their antigen response times (118).

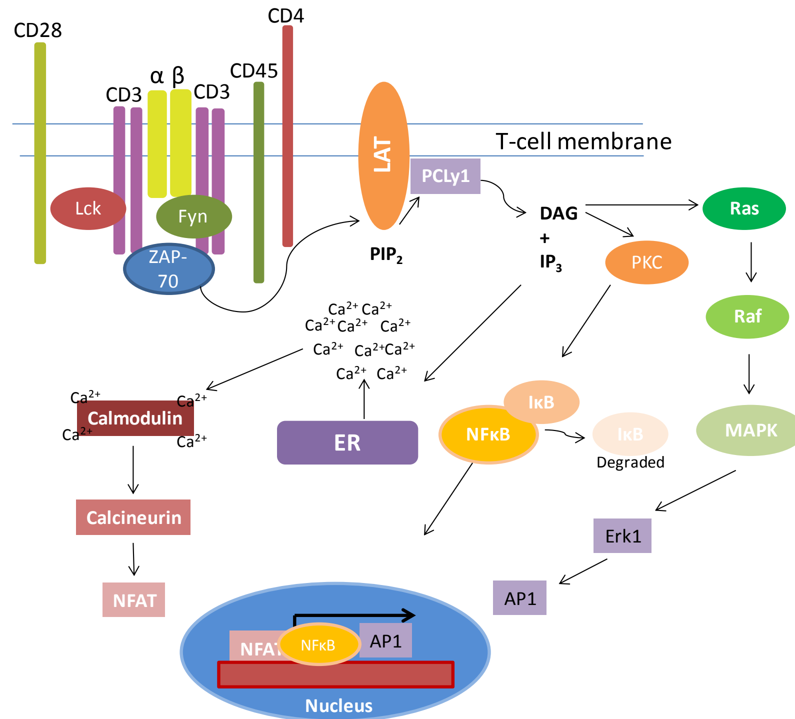


Figure 4. T-cell Receptor signalling pathway. Following TCR binding of pMHC the TCR signalling pathway is activated. Early signalling events include the activation of Src family kinases such as Lck, Fyn and Zap-70. Zap-70 target Lat which in turn recruits PCLγ1. Activated PCLγ1 hydrolyses PIP_2 to DAG and IP_3 which inturn initiates a number of signalling pathways including the NFκB, MAPK and the Ca^{2+}/NFAT pathways. The transcription factors activated by these pathways are then capable of altering T-cell gene expression within the nucleus.

1.4.5 CD28 signalling pathway

CD28 is a member of the immunoglobulin superfamily and is a homodimeric transmembrane protein expressed by T-cells. It is recruited to the cSMAC by the presence of PYAP motifs (119). CD28 interacts with CD80 (B7-1) and CD86 (B7.2) which are both expressed on APC following activation through TLRs (120). CD28, like the TCR, is capable of PI3K- and Itk-recruitment and Lck activation (121). TCR and CD28 signalling activates some of the same signalling pathways and so concerted TCR and CD28 ligation is thought to increase the duration and intensity of TCR signalling. In addition to increasing TCR sensitivity, CD28 ligation within the synapse also increases T-cell survival by inducing anti-apoptotic molecule Bcl-XL (122). Futhermore CD28 binding provides signal 2 which is required for T-cell activation and to prevent the induction of anergy (119).

CD28 binding to CD80/CD86 in addition to TCR:pMHC allows full TCR activation. The cytoplasmic domains of CD28 lack catalytic activity. However, tyrosine phosphorylation of YMNM binding motifs facilitates downstream signalling by providing binding sites for signalling molecules PI3K and GrB via their SH2 domains (123, 124). PI3K binding produces PIP_2 and PIP_3 lipids which indirectly activate PKCθ, PKB and PDK1; molecules capable of regulating pathways involved in protein synthesis, cell metabolism, and survival. The importance of Grb and PI3K pathways is highlighted by the interference of IL-2 expression resulting from mutations within the Y and M residues of YMNM motifs (120). Phosphorylation of guanine nucleotide exchange factor, Vav1, by Grb, leads to the activation of c-jun kinase (JNK). Vav1 induces Ca^{2+} release and enhances NFAT and AP-1 activation and NFAT mediated *il-2* transcription (125). Interestingly, Vav1 is able to activate Ras-related C3 botulinum toxin substrate 1 (Rac1) allowing remodelling of the actin cytoskeleton. Furthermore Vav1 allows clustering of the TCR/CD3 complex, indicating the importance of CD28/TCR cooperative effects. CD28 signalling also allows upregulation of immunoglobulin super-family and TNF-super family co-factors, which also contribute to signalling from the IS (126). Previous investigations have shown that CD28 signalling is Cyclosporin A (CysA)-resistant, whereas the TCR pathway is CysA-sensitive, indicating differences in the signalling pathways induced (127). However, more recent investigations have revealed that the CD28 signalling pathway is also CysA sensitive, and this finding is consistent with CD28-enhanced Ca^{2+} and NFAT induction (126, 128). CD28 signalling is inhibited by CTLA-4, which competes with CD28 for CD80/CD86 binding sites. CTLA-4 is also able to inhibit TCR signalling, by recruiting an intracellular phosphatase SHP2 (SH2–domain-containing protein tyrosine phosphatase 2) that is capable of dephosphorylating CD3 subunits. CTLA-4 is absent from naive T-cells, but mRNA expression is detected within a few hours of priming. Signalling via CTLA-4 is also capable of inhibiting cell cycle progression and preventing activation of transcription factors: NFκB, NFAT, and AP-1 (119, 124).

1.4.6 CD4 and CD8 contribution to T-cell signalling

CD4 and CD8 promote T-cell adhesion by binding to monomorphic regions of MHC class II and MHC class I, respectively. CD4/CD8 molecules are thought to act by cross-

linking the TCR: maximising TCR recruitment to the IS. More recent data demonstrate a predominant role of CD4/CD8 in delivering tyrosine kinase Lck to the IS to initiate TCR signalling (129). Interestingly Th2 cells have two-fold lower CD4 expression than Th1 cells, and CD4 is excluded from lipid rafts in Th2-APC. Furthermore Th2 and Th1 cells form morphologically distinct immune synapses (106). Lower CD4 expression together with CD4 exclusion from lipid rafts in Th2-APC interactions may explain why Th2 cells compared to Th1 cells have a reduced capacity to form mature IS (106, 130). Furthermore, Th2 cells also have a reduced capability to elevate Ca^{2+} signalling, which is thought to be due in part to reduced CD4 expression (130).

1.5 CD4$^+$ T-cells in the periphery

1.5.1 Naive and Memory T-cells

Antigen-inexperienced or ‘naive’ T-cells are released into the periphery following successful passage of a T-cell through the positive and negative selection criteria of the thymus. Naive T-cells circulate via the lymphatic system between the secondary lymphoid organs and the blood. Naive T-cells may also express receptors allowing migration to non-lymphoid tissue, including $\alpha_4\beta_7$ integrin which allows homing to the small intestine (131). Human naive T cells may be identified through expression of CD45RA, which is downregulated following antigen recognition, and expression of CD45RO (a CD45 isoform whose CD45RA, B and C exons have been spliced out) is upregulated on antigen experienced memory T-cells; these markers are used to separate human naive and memory CD4$^+$ T-cell populations.

Following antigen recognition, the expression of CD62L and CCR7 also increases on the T-cell surface allowing extravasation and lymphoid homing. Expression of CD62L and CCR7 also allows T-cells to migrate through high endothelial venules found in secondary lymphoid organs. The number of effector T-cells usually peaks a week into the immune response and ~90% of the effector T-cells die during the following week, leaving behind a long lived memory T-cell subset that is predominantly quiescent (132). Memory T-cells can be subdivided into Central Memory (T_{CM}) and Effector Memory (T_{EM}) T-cells (133). T_{EM} cells express CD62L, and also combinations of

chemokine receptors and adhesion molecules, which allow migration to sites of inflammation. T_{EM} cells can also rapidy produce effector cytokines within hours of TCR triggering (134). When T_{EM} cells are TCR stimulated under neutral conditions they retain the pattern of cytokine expression designated during priming, for example a Th1 or Th17 cell phenotype, however T_{EM} cells may produce a different cytokine profile if stimulated in the presence of an opposing polarising conditions (135).

T_{CM} cells are the predominant memory $CD4^+$ T-cell in peripheral blood and constitutively express secondary lymphoid homing receptor CCR7 along with CD62L, and so are enriched within the lymph nodes and tonsils (133). T_{CM} cells are very sensitive to antigenic stimulation and are less reliant on co-stimulation (135). Upon antigenic stimulation T_{CM} produce IL-2 and rapidly proliferate. T_{CM} cells however do not produce effector cytokines immediately after TCR stimulation, but following proliferation T_{CM} cells are capable of differentiating into effector T-cell phenotypes (133). Following TCR stimulation T_{CM} cells quickly loose CCR7 expression which marks the transition from T_{CM} to T_{EM} cell, and CCR7 expression is sufficient to separate the effector and central memory subsets (135).

1.6 Effector T-cell subsets

1.6.1 Regulatory T cells

Regulatory T-cells (Treg) critically function in the maintainance of peripheral tolerance: by regulating lymphocyte activation/expansion, and by preventing tissue destruction and inflammation (134). Treg cells have a suppressive action on the function of a range of immune cells including: $CD4^+$ T cell proliferation and effector cytokine production, $CD8^+$ T cell granule release, and B cell antibody production (136). Treg cells are also equipped to suppress inflammation via mechanisms that include: the production of inhibitory cytokines IL-10, TGF-β, and IL-35, cytolysis via secretion of Granzyme A or B and perforin, high CD25 expression causing death by IL-2 deprivation, and suppression of DC maturation via interaction of CTLA-4 and CD86 (137). Activation of Treg cells occurs in an antigen-specific manner; suppression occurs in a non-antigen specific manner via bystander effects on neighbouring effector T-cells. Furthermore, infectious

tolerance allows Treg cells to create a regulatory *milieu* favouring the outgrowth of Treg cells with distinct TCR specificity (138). *In vivo,* Treg cells exist as either naturally occurring Treg (nTreg) cells or inducible (or adaptive) Treg (iTreg) cells. A number of T-cells with regulatory properties have also been developed *in vitro* for use as cell therapies.

nTreg cells develop in the thymus during early foetal/neonatal T-cell development. The polyclonal nTreg cell population released to the periphery constitutes 5-10% of $CD4^+$ T-cells in mice and are characterised, in part, by expression of transcription factor Forkhead box p3 (Foxp3). The development of nTreg cells within the thymus occurs following high affinity TCR ligation of self-pMHC presented by $Aire^+$ mTec cells and accessory signalling via CD28 or CD40 which allows stable Foxp3 expression (139–141). The association of Foxp3 with nTreg cells was discovered as a result of identifying Foxp3 as the defective gene in the Scurfy mouse model; an X-linked recessive mutant with hyperactive $CD4^+$ T-cells and increased pro-inflammatory cytokine production (142, 143). nTreg cells fail to develop in Scurfy mice, causing a breakdown in peripheral immune tolerance. Human and murine *FOXP3* is highly conserved. *FOXP3* mutations in humans can result in the development of immune dysregulation polyendocrinopathy, enteropathy, X linked syndrome (IPEX) (144). Over 20 *FOXP3* mutations have been identified so far and the clinical outcome is determined by the location of the mutation. The most critical outcome is a severe autoimmunity which must be treated by a bone marrow transplantation (144, 145). Thymus-derived nTreg cells are $CD4^+$ and in addition to Foxp3, express high levels of CD25 (IL-2α), CTLA-4 and glucocorticoid-induced TNFR-related protein (GITR), all important to nTreg cell function (141). High CD25 expression is thought to be maintained by the frequent contacts with self-antigen (146, 147). High CD25 is functionally important to regulatory T-cell (Treg) cells by sequestering IL-2 preventing the proliferation of inflammatory effector T-cells populations. The importance of CD25 to human nTreg cell function is highlighted by the observation that a patient with CD25 deficiency developed pathology indistinguishable from IPEX (148).

Peripheral $CD4^+$ T-cells may also be induced or converted to a regulatory phenotype termed adaptive or inducible Treg (iTreg) cells. iTreg cells may be generated from $Foxp3^-$ naive or effector $CD4^+$ T-cells *in vitro* or within lymphoid tissue and are generally marked by upregulation of Foxp3 expression and CD25 (149, 150). In contrast to nTreg, iTreg cells usually have a more restricted TCR specificity (137). However, as nTreg and iTreg cells express similar markers, distinguishing the populations is not yet possible. Of the $CD4^+$ iTreg two main phenotypes are described: T helper 3 (Th3) cells are induced by TGF-β, and regulatory T-cell type 1 (Tr1) cells are induced by IL-10 (151). Th3 cells, commonly referred to as iTreg cells, are induced from $Foxp3^-CD4^+$ T-cells in the presence of TGF-β. High doses of ova induced oral tolerance via $CD4^+$ T-cell secretion of TGF-β ameliorating experimental tracheal eosinophilia (152). Tumours secreting TGF-β can suppress immunity by converting immature DC to a tolerogenic phenotype secreting TGF-β, and promoting the proliferation of Treg cells (153). Along with TGF-β, IL-2 is also important for the development of $Foxp3^+$ iTreg. IL-2 enhances Signal transducer and activator of transcription (STAT)-5 signalling and can act in concert with TCR stimulation to induce epigenetic modifications at the *Foxp3* locus that stabilise TGF-β-induced *Foxp3* expression (150). Tr1 cells are another subset of iTreg induced by IL-10 or IL-10-conditioned DC. Tr1 cells mediate their suppression via IL-10- and TGF-β-dependent mechanisms, although they are generally $Foxp3^-$ (154, 155). IL-27 alone or together with TGF-β has been demonstrated to induce IL-10-producing Tr1 cells (156). Furthermore, IL-27 allows the expansion of Tr1 cells via induction of c-Maf, leading to transactivation of IL-21 and Tr1 cell expansion (157). The adoptive transfer of Tr1 cells can suppress autoimmunity, graft vs host disease, and colitis (158). A loss of iTreg can be a characteristic of autoimmunity; depletion of Treg cells has been associated with inflammatory colitis, RA and diabetes. The balance between Treg cells and effector $CD4^+$ T cell populations appears important to immune regulation, and Treg may provide a promising therapeutic tool. *Ex vivo* generation and expansion of antigen specific Treg cells remains difficult. *In vitro* induction and expansion of iTreg allows generation of large numbers of Treg cells which may be administered for therapeutic purposes (159). Although cellular therapy with Treg cell is promising it is important that a delicate balance is achieved to prevent the development of infection and cancer (143).

1.6.2 T helper 1 (Th1) cells

Mossman and Coffman originally described Th1 and Th2 cells in 1986 based upon the observation of T-cells with distinct cytokine profiles/activity (160). Th1 cells are critical for co-ordinating the clearance of intracellular pathogens, including bacteria, such as *Listeria and Mycobacterium tuberulosis* and also viruses and parasites (161). Th1 cells are also associated with the delayed type hypersensitivity response (162). For a long time Th1 cells were considered to be the main drivers of autoimmune pathogenesis, however some of these data have been recently reviewed and re-interpreted since the discovery of Th17 cells and the role of IL-23 ((163, 164) (Discussed in section 1.7). The signature cytokine of Th1 cells is the pro-inflammatory cytokine interferon gamma (IFNγ), but Th1 cells also produce IL-2, IL-3, Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and TNF-β (165, 166). In addition, Th1 cells also provide help for B-cell production of complement fixing antibody IgG2a in mice or IgG1 in humans, mediating antibody-dependent cell mediated cytotoxicity (ADCC) (167, 168). Natural Killer (NK) cells and cytotoxic T- lymphocytes are also activated by Th1 cells (169). Like a number of effector T-cells, Th1 cells also produce IL-10 to limit tissue damage, and the levels of IFNy and IL-10 must be carefully regulated to minimise pathology whilst preventing persistent infection (170).

Th1 cell responses are induced by the cytokines IFNy, IL-12 and IL-18, which activate transcription factors required to express Th1 response genes. The cytokines required to initiate a Th1 cell response can be derived from innate immune cells. NK cells are an innate immune cell capable of producing IFNy as a result of DC derived IL-12 in combination with IL-18 or IL-15 (171, 172). Production of IFNy by the Th1 cell induces a positive feedback loop, maintaining NK cell production of IFNy, which reinforces the Th1 phenotype. Signalling via IFNy and the TCR induces the expression of IL-12Rβ2 on the cell surface of naive T-cells (173, 174). IL-12 is produced by DC activated with TLR3, TLR4, TLR7 and TLR9 either alone or in synergy (175). Binding of IL-12 and IFNy to their respective cytokine receptors activates Janus kinases (JAKs), which phosphorylate tyrosine residues on the intracellular cytokine receptor domains, creating binding sites for STAT proteins. Once bound STAT proteins are activated and dimerise, allowing transportation to the nucleus where they affect transcriptional regulation. Sustained

IL-12 signalling via IL-12Rβ2 in T-cells activates STAT4, which is further enhanced by IL-2 (176). IL-12 signalling via STAT4 in concert with TCR signalling upregulates expression of IL-18Rα (177). IL-18 functions in synergy with IL-12 to augment IFNγ production and to increase expression of IL-12Rβ2, potentiating the Th1 cell phenotype (178, 179).

In addition to STATs, T box expressed in T cells (Tbet) is an essential regulator of the Th1 cell phenotype. Tbet is induced independently of STAT4, via IFNγ-mediated activation of STAT1 (180). Tbet induces IFNγ production and represses *IL-4* expression. Furthermore induction of Tbet in Th2 cells induces a Th1 cell phenotype, Tbet is thus termed a master regulator of Th1 cell phenotype (181). Tbet in conjunction with Runx3 allows maximal *Ifnγ* expression, and silences *Il-4* expression (182). Furthermore, Tbet represses *Socs1* and *Socs3* expression by directly binding to their promoter regions (183). Tbet plays an important role in stabilising the Th1 cell phenotype by increasing *Ifnγ* expression, thereby initiating a positive feedback loop (184). In addition, Tbet is capable of inducing C-X-C chemokine receptor 3 (CXCR3) expression allowing migration of Th1 cells to sites of inflammation (185). Chemokine receptors do not strictly coordinate with T cell phenotype but CXCR3 and CCR5 are most strongly associated with the Th1 cell phenotype (186).

1.6.3 T helper 2 (Th2) cells

Th2 cells are critical to mediating the clearance of extracellular pathogens including helminths. A dysregulated Th2 cell response can result in atopy and asthma through the induction of IgE production (169). The function of Th2 cells is mediated by their effector cytokines IL-4, IL-5, IL-9, and IL-13 (187). IL-4 and IL-13 play a similar and key role in instructing IgE class switching in B cells, though IL-4 acts more potently on B-cell class switching than IL-13. IL-13 can also activate mast cells and eosinophils and induces airway MMP production. Secretion of IL-5 and IL-9 allows Th2 cell mediated eosinophil recruitment, and the production of mucins from epithelial cells respectively. IL-25 is also produced by Th2 cells and is a member of the IL-17 cytokine family (IL-17E). IL-25 enhances the production of Th2 cell cytokines and is capable of inducing CCL5- mediated eosinophil recruitment (161). Th2 cells tend to be characterised by

expression of chemokine receptors CCR3, CCR4, and CCR8, allowing localisation to mucosal tissue (186).

Th2 cell differentiation requires IL-4 signalling. There are no known stimuli that induce production of pro-Th2 factors such as IL-4, IL-9, or IL-25 by DC, suggesting other cellular sources may promote Th2 responses (188). Previous studies have demonstrated that membrane bound factors such as Inducible T-cell co-stimulator (ICOS) can regulate DC mediated Th2 clone expansion (189). Three more recent investigations have provided evidence that basophils, rather than DC, provide MHC class II dependent IL-4 to allow Th2 differentaition (190–192). Basophils were demonstrated to endocytose soluble ovalbumin, then process and present antigen both *in vitro* and *in vivo* (190). Depletion of basophils in response to *T.muris* impaired Th2 responses and the clearance of infection (191, 192). However the role of Basophils to drivers of Th2 cell responses has been refuted more recently (193, 194). Th2 cell cytokine responses were dramatically impaired by $CD11c^+$ cell depletion whereas basophil depletion resulted in only minor impairment. This has lead to a decription of a model whereby Th2 cytokine secretion in response in allergen antigens is initially mediated by DC and may be amplified by basophils. IL-4 binding to the IL-4R can induce STAT6 activation, enhancing the expression of transcription factors GATA3 and c-maf, thereby further increasing surface expression of IL-4R (169, 195). STAT6-deficient mice do not develop Th2 cell responses and overexpression of STAT6 in Th1 cells downregulates IL-12Rβ and increases expression of GATA3 and c-maf (195, 196). Together these experiments have demonstrated the importance of STAT6 to the development of Th2 cell responses.

GATA-3 is the master transcription factor of the Th2 cell lineage (197). Naive T-cells have low basal expression of GATA-3, but its expression is upregulated during Th2 cell development via IL-4-mediated STAT-6 activation (198). IL-4 signalling is sufficient to induce an up-regulation of GATA-3 mRNA, however expression of GATA3 is augmented by TCR activation which increases the translational stability of GATA-3 via the PI3K pathway (199). STAT6-independent mechanisms of GATA3 activation via Notch signalling have also been suggested to be essential for Th2 cell induction (197). *In vitro*

Th2 cell differentiation has been described to be impaired in the absence of physiological Notch signals. Th2 cells produce high levels of IL-4 that can reinforce GATA3 expression, providing a positive feedback loop and phenotype stability (200). GATA3 deletion prevents expression of IL-5 and IL-13, however IL-4 is still capable of being produced via transcription factor c-Maf (201, 202). GATA3 also acts by inducing an open chromatin conformational state within the *IL-4*, *IL-5*, and *IL-13* promoters that allows binding of transcription factors, and protein expression (197).

GATA3 inhibits Th1 cell differentiation and thus IFNγ production. Expression of GATA3 in Th1 cells is able to induce expression of Th2 cell response genes (203). IL-4 also exerts an antagonistic effect on Th1 cell development, however GATA-3 can inhibit Th1 cell development independently of IL-4 (204). T-cell activation allows the upregulation of IL-12Rβ2 on naive T-cells, which unlike IL-12Rβ1 is not constitutively expressed. Ectopic GATA-3 can supress expression of the IL-12Rβ2 and IL-12 responsiveness, preventing STAT4 activation and therefore Th1 cell responses (205). GATA3 is also capable of IFNγ inhibition by blocking Runx3 mediated IFNγ production. Mutual antagonism between Tbet and GATA3 has been described to increase the transcriptional imbalance promoting lineage divergence contributing to the stability of the Th1 and Th2 cell phenotypes (200).

1.6.4 T helper 22 (Th22) cells

IL-22 can be produced by Th17 cells in response to IL-23 signalling (206). IL-22 is, also expressed by Th1, NK and NKT cells, but to a lesser degree. More recently an IL-22$^+$ CD4$^+$ T-cell population has been described as an independent T-cell lineage termed Th22 cells (207). Th22 cells do not co-express IFNγ, IL-4, or IL-17. Approximately one third of *ex vivo* IL-22$^+$ cells from psoriatic or atopic dermatitis lesions were IL-22 single positive and a further third were dual positive for IL-22 and IL-17. IL-22 single positive cells were found to be enriched within the CCR10$^+$ CD4$^+$ fraction (207). In addition CCR4 is also expressed by Th22 cells (208). CCR10 and CCR4 allows migration towards their cognate chemokines expessed by skin tissues (208). The IL-22R is highly expressed in epithelial tissue, and Th22 cells are thought to play an important role in mediating innate epithelial and keratinocyte defence (207). Th22 cells are also proposed to be

important in wound healing. Furthermore, by synergising with factors including IL-17, TNFα, and Oncostatin M, IL-22 plays an important role in autoimmune diseases of the skin, including psoriasis (209, 210). IL-22 can have both pro- and anti-inflammatory effects *in vivo*; ascertaining the function of IL-22 is complicated as the surrounding cytokine *milieu* has a prominent role in dictating the immune outcome (211, 212).

Induction of Th22 cells arises via IL-1β, IL-6 and/or TNF-α signalling, which are factors also associated with Th17 cell induction (207, 213). Langerhans cells or dermal DC are also able to induce Th22 cells (214). Transcriptome analysis has revealed enhanced expression of transcription factors Basonuclin-2 (BNC-2) and FOXO-4 within Th22 cells, although it is currently unknown if and how these factors may be involved in Th22 cell polarisation (207). As expected expression of Retinoic acid related orphan receptor (ROR)-γt, Tbet and GATA-3 is reduced within Th22 cells. For Th22 cells to be defined as a distinct T-cell subset, a master transcription factor for Th22 cells must be identified. One proposed is the Aryl Hydrocarbon Receptor (AhR) transcription factor. AhR was established as important for Th17 cell induction increasing both IL-17 and IL-22 expression within mouse models (215). Interestingly, $AhR^{-/-}$ mice could still produce IL-17 but were unable to produce IL-22 (216). Contrary to these data it has been subsequently found that AhR ligands reduce IL-17 production but enhance the generation of Th22 cells (217). The broad expression of the AhR in T-cells may indicate that it is not a master transcription factor for the Th22 cell subset. More investigations are therefore required to further assess the factors controlling Th22 cell induction and regulation.

1.6.5 T helper 9 (Th9) cells

Th9 cells represent another potential T-cell subset, their description was based upon discrete production of IL-9 by murine $CD4^{+}$ T-cells (218). Th2 cells were originally described to secrete high levels of IL-9 in conjunction with IL-4, mediating immunity against helminth infection and allergy. It was later found that TGF-β could induce IL-9 production from $IL\text{-}17^{+}$ memory T-cells (219). However, in the presence of IL-4, TGF-β allowed Th2 cells to be converted to an IL-9-producing subset termed Th9 cells (218). Th9 cells were found to express high levels of IL-17RB (also expressed by naive and Th2

cells, but not Th1 or Th17 cells), which is the receptor for IL-25. The combination of IL-4 and TGF-β enhanced IL-9 production from Th9 cells, but IL-25 in concert with TGF-β is also capable of inducing IL-9 expression by Th9 cells (220). Transcription factor Interferon regulatory factor 4 (IRF4), also expressed in Th2 and Th17 cells, has recently been described to be crucial for IL-9 expression by 'Th9' cells (221). Chromatin immunoprecipitation demonstrates that IRF4 binds to the *Il-9* promoter and at present IRF4 seems a promising candidate as the master transcription factor for Th9 cells. *In vitro* generated T-cells are however heterogeneous. If T-cell subsets are not sorted by flow cytometry, the signature factors assigned to a subset may be representative of the heterogenous population as a whole, rather than the subset enriched within the total cell population (200, 221).

1.6.6 Follicular helper T cells (T_{FH})

T helper cells have long been recognised for their capability to 'help' in the activation and differentiation of B-cells; follicular helper T cells (T_{FH}) are now recognised as being specifically equipped to mediate B-cell help (222). T_{FH} cells continuously express CXCR5, the receptor for Chemokine (C-X-C motif) ligand-13 (CXCL13) which is secreted by follicular stromal cells, ensuring that T_{FH} cells reside permanently within B cell follicles. T_{FH} cell function is dependent on the transcription factors B cell lymphoma (BCL)-6 and c-maf, which allow expression of CXCR5, IL-21, ICOS and programmed death-1 (PD-1) (223, 224). Induction of B-cell lymphoma-6 (BCL-6) and c-maf has recently been demonstrated to be directly regulated by global transcriptional regulator in T-cells, BATF (225). BATF, an AP-1 family member, is essential in Th17 cell and T_{FH} development, but is dispensable to the development of Th1, Th2, B cells and DC (225). T_{FH} cells require the presence of IL-6 for induction of BATF and thus T_{FH} cell generation; many BATF-regulated genes are initially activated by IL-6 (225). T_{FH} cell induction and phenotype maintenance has also been demonstrated to require IL-21, but molecules including: IL-10, ICOS and PD-1 are also expressed by T_{FH} cells allowing B-cell expansion, differentiation and isotype switching (222). Naive T-cells capable of high affinity TCR interactions with pMHC, appear to be predominantly those cells selected to become T_{FH} cells (223). It is still not clear whether T_{FH} cells represent an independent subset, however they appear discrete from other T-cell subsets in their ability to

localise to B- cell follicles and in their capacity to express molecules which can provide B-cell help (226). Since the discovery of Th17 cells, there has been an increase in the number of potential new T-cell subsets present either *in vivo* or capable of being induced *in vitro*. This has occurred in part due to the advances in multicolour flow cytometry; it seems that the true complexity of T helper cells and their subsets is just beginning.

1.7 T helper 17 (Th17) cells

In the 2005 November issue of Nature Immunology two publications (one by Park *et al* and the other by Harrington *et al*) looked at two mouse models of autoimmunity, and described the existence of a distinct T helper cell subset capable of producing IL-17 (227, 228). These findings were based on *in vitro* and *in vivo* experiments indicating that Th1/Th2-independent cytokine and transcriptional programs gave rise to IL-17-producing $CD4^+$ T cells. Until these publications, due to difficulties in inducing substantial Th17 cell populations from naïve $CD4^+$ T cells, it was unclear if Th1 and Th17 cells arose from a common Th1 cell precursor (164). Park *et al* and Harrington *et al,* using IFNγ and IL-4 neutralising antibodies along with IL-23, resolved this in mouse models inducing distinct Th17 populations from naïve mouse $CD4^+$ T cells. Since these publications, a vast amount of research has been performed to ascertain the function, induction and maintenance of Th17 cells along with their roles in infection and autoimmune disease.

1.7.1 Th17 & infection

Th17 responses are important for the clearance of a number of pathogens. Hyper IgE syndrome is caused by a STAT3 mutation that prevents Th17 cell responses (229). Hyper IgE syndrome patients have a devastating susceptibility to *S. aureus, S. Pyogenes* and *C. albicans.* Mounting an appropriate Th17 response is key to clearance of *C. albicans* infection; reduced Th17 responses are found in chronic mucocutaneous candidiasis patients (230, 231). Furthermore, murine candidiasis models have demonstrated that either IL-23p19 or IL-17 deficiency results in severe candidiasis whereas IL-12p35 deficiency did not. Th17 cells may also have a role in the immune response against other fungal infections (232). For example neutralisation of IL-23 or

IL-17 increases the disease severity and fungal burden during *Pneumocystis carinii* infection. Th17 cells also mediate protection against bacterial infections. *Borrelia burgdorferi* and *Mycobacterium bovis* BCG peptides induce Th17 responses. *B. burgdorferi* causes Lyme Arthritis and is capable of inducing innate cell production of IL-6, IL-1β, and TGF-β via TLR2 activation. The cytokines induced as a result of TLR2 activation in turn induce Th17 cells within the joint synovium which can lead to Lyme Arthritis (232). Within IL-17R or IL-23p19 deficient mice the importance of Th17 cells for the clearance of *Kleibsiella pneumonia* infection in the lung is also clear (233, 234). In addition, IL-17 is also important for the clearance of *Helicobacter pylori* and *Salmonella* species (230). Th1 cell responses are induced by *Bordetella pertussis* infection and are thought to be protective (235), but more recent studies have demonstrated that Th17 cell responses are also important for clearance of B.pertussis infection (236). *B. pertussis* activates TLR4 signalling which stimulated DCs to secrete of IL-1β, IL-23 and TNFα, enhancing IL-17 expression from T-cells resulting in clearance of the *B.pertussis* infection. Together these studies highlight that the clearance of many pathogens, particularly *C.albicans* relies on a Th17 cell mediated response, with deficiencies in mounting a Th17 response associated with chronic infections.

1.7.2 Th17 function in autoimmunity

In the western world autoimmune diseases such as RA, Type I diabetes (T1D), and multiple sclerosis (MS) are a prevalent health problem that reduce the patients quality of life/life expectancy and also have a vast economic burden. Th1 cells were originally thought to mediate both experiment autoimmune encephalomyelitis (EAE) (a model of multiple sclerosis) and type II collagen-induced arthritis (CIA) (237). However, the discovery of Th17 cells altered our view of the role of Th1 cells in autoimmunity. Support for Th1-mediated autoimmune pathogenesis had in part stemmed from studies neutralising the p40 subunit of IL-12 and IL-12p40$^{-/-}$ mouse models (238, 239). These investigations suggested that IL-12, and therefore Th1 responses, promoted autoimmunity. IL-17 was understood to play an important role in the induction of inflammation prior to 2005 and the discovery of Th17 cells. Th17 cell responses are promoted by IL-23 and the IL-23 and IL-12 receptors share a common subunit called p40 which is required for both cytokines to signal. Discovering that the IL-23R specific

p19 subunit can pair with p40 to form the IL-23R, led to a re-evaluation of IL-12/p40 data (240). Subsequent studies found that $p19^{-/-}$ mice did not develop CIA whereas $p35^{-/-}$ (IL-12-specific subunit) did (163), indicating that immune responses requiring IL-23 may be more important to the development of CIA than those, such as Th1 cell responses which require IL-12 signalling. Although the role of Th1 and Th17 cells in the development of autoimmunity is still disputed in diseases such as RA. In addition, IL-23 can also drive the expansion of a pathogenic autoreactive T-cell population in EAE characterised by expression of IL-17A, IL-17F and IL-6 (Th17) (164, 241). The role of Th1 and Th17 cells in the development of autoimmunity is still disputed in diseases such as RA; however these data demonstrate that autoimmunity is not purely a Th1 cell mediated event.

1.7.3 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic and debilitating autoimmune disease that affects ~1% of the adult population (242). RA is characterised by the destruction of articular cartilage, progressive destructive joint inflammation, synovial hyperplasia, and in some cases systemic inflammation that affects organs including the lungs and heart (243). RA is a complex disease of unknown aetiology and a number of factors are associated with disease onset including genetic associations (244) and environmental factors such as smoking and obesity (245, 246). The importance of T-cells in the pathogenesis of RA, has been highlighted by the strong genetic association of T-cell related genes including: HLA, PTPN22, CD28 and CTLA-4 (247). Based largely upon data derived from mouse models RA was originally considered to be a Th1 cell mediated disease, although the severity of CIA increased in the absence of either IFNγ or the IFNγ receptors (237, 248). Prior to the discovery of Th17 cells, increased numbers of $IL\text{-}17^{+}CD3^{+}$ T-cells were observed within the joint synovium of RA patients (249). As described above antibodies blocking IL-23, which expand Th17 cell populations, decreased CIA pathogenesis (163). Enhanced levels of IL-17 have been observed in both the synovial fluid and serum of RA patients in comparison to healthy controls (250). Furthermore, the concentration of IL-17 is increased in RA synovial fluid compared to osteoarthritis (OA; a non-inflammatory arthritis) and RA peripheral blood (251). Although the concentrations of IL-17 found within the joint are low when compared to IL-1β and

TNFα, it is thought that IL-17 can enhance the effects of these and other cytokines (252). Within the joint Th17 cells exert their effects on a variety of cell types to cause joint destruction. Th17 but not Th1 cells induce the production of IL-6, IL-8, and destructive MMP1 and MMP3 from synovial fibroblasts. These effects were independent of TNFα, suggesting that IL-17 neutralisation alongside anti-TNF therapy may be beneficial for RA therapy (253). IL-17 also promotes inflammation by increasing angiogenesis and by enhancing monocyte and neutrophil cell migration/invasion (252, 254, 255). Monocytes and macrophages are induced by IL-17 to produce IL-6, IL-1β and TNFα along with COX-2, Prostaglandin E_2 ($PGE_{2)}$ and MMPs resulting in inflammation, tissue destruction and further Th17 cell differentiation (252, 254, 255). Chondrocytes and osteoblasts also produce IL-6 and IL-1β in response to IL-17 resulting in inflammation and further Th17 cell differentiation. Th17 cells are directly capable of mediating bone destruction and cartilage degradation (256). Th17 cells promote osteoclastogenesis directly by expressing Receptor Activator of NFκB Ligand (RANKL) and TNF or indirectly by inducing RANK expression by synovial fibroblasts, which result in synovial macrophage differentiation to bone resorbing osteoclasts (257, 258). IL-17 therefore promotes disease progression via inflammation and joint destruction. Moreover many of these disease progressing factors are inhibited by IFNγ including bone resorption and neutrophil migration (254, 257). However, although evidence demonstrates that IL-17 is a key mediator of inflammation in RA, more recent investigations have demonstrated that of the cells producing IL-17 within the RA synovium, T-cells constitute only a small proportion, and that the majority of IL-17 co-localised to mast cells (259, 260). However, these investigations have assessed IL-17 expressing cells in established RA, whether Th17 cells play a more important role in the pathogenesis of early RA requires further investigation.

1.7.4 Multiple Sclerosis

MS is a demyelinating disease of the central nervous system (CNS). Th1 and Th17 cells are both have been suggested to have a role in MS disease development. Patients exhibit different forms of the disease with varying pathologies, which are suggested to be due to the proportions of Th1 or Th17 cells at sites of inflammation (261). The number of Th17 cells was found to be significantly increased in the cerebrospinal fluid

(CSF) of relapsing remitting MS patients during relapse, and high levels of IL-17 were found within the CSF and plaques of these MS patients (261). Th17 cells can express CD146 allowing better attachment to the brain endothelium than Th1 cells. (262). Furthermore expression of CCR6 and CD6 by Th17 cells allows entry into the CNS in the EAE mouse model (263). As in RA, IL-17 is the main cause of Th17 pathogenicity in MS inducing secretion of inflammatory factors including IL-8, IL-6, TNFα, GM-CSF, and macrophage inflammatory protein-2 (MIP-2) by a wide range of cell types (261). Furthermore a main function of IL-17 in MS pathogenesis is the breakdown of the blood brain barrier enhancing the recruitment of neutrophils, monocytes and macrophages, leading to sustained myelin and axon damage (264, 265).

1.7.5 Diabetes

The role of Th17 cells has been less clear in autoimmune diabetes. Inhibition of IL-17 by neutralising anti-IL-17 treatment was found to prevent diabetes onset in NOD mice (266). Anti-IL-17 treatment ameliorates disease pathogenesis by decreasing islet inflammation and autoantibody formation. Interestingly the treatment increased the frequency of auto-antibody specific IL-17$^+$ T-cells, which may be why administration with recombinant IL-25 (which inhibits Th17 responses) was more effective at preventing diabetes onset than anti-IL-17 (266). Monocytes isolated from T1D patients were found to spontaneously secrete pro-Th17 cytokines IL-1β and IL-6, and a modest yet significant increase in the number of Th17 cells in T1D patients compared to healthy controls was observed (267). However, Th1 cells have an important role in diabetes onset. Transfer of autoreactive Th1 or Th17 cells into NOD/Servere combined immunodeficiency (SCID) mice demonstrated that anti-IFNγ treatment prevented disease induction whilst anti-IL-17 treatment did not. Th17 were found to convert to IFNγ producing Th1 cells following adoptive transfer (268). The role of Th17 cells in diabetes remains controversial, current data indicates that Th17 cells may play a role although Th1 cell responses remain important in diabetes disease pathology

1.7.6 Therapies against Th17 cells

Th17 cells have also been demonstrated to have a role in promoting the pathogenesis of inflammatory bowel disease, Crohn's disease and psoriasis (269). The therapeutic

targeting of Th17 cells is therefore promising for a number of autoimmune disease treatments. Therapies against Th17 in autoimmunity could either directly target IL-17 secretion by neutralisation, or by block the pathways that lead to Th17 cell development. A phase 1 trial using a humanised monoclonal antibody neutralising IL-17A has demonstrated positive results in RA (270). Monoclonal antibody blockade of the p40 subunit common to both IL-23 and IL-12 (and thus preventing both IL-12- and IL-23-mediated signalling) has had promising results in phase II trials in psoriatic arthritis (271). Furthermore, the anti-IL-1R antagonist anakinra has been successfully used to treat RA; one mechanism for this may be through decreasing numbers of Th17 cells (269). Anti-IL-17 therapy may also be powerful when used in combination with therapies which are already successful such as anti-TNFα (272). A highly specific ligand against RORγt and RORα has also been described in mice to inhibit Th17 generation and may be a future therapeutic tool (273).

1.8 Th17 effector molecules

1.8.1 Interleukin-17

Th17 cells are characterised by the expression of a number of phenotypic markers important for their function and localisation. Expression of a select group of cytokines and chemokines confers Th17-mediated effects. The *Il-17* gene previously known as CTLA-8 was discovered in 1993 (274). The IL-17 family of cytokines is composed of six members: IL-17A (was known as CTLA-8 now commonly known as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F (275). Both IL-17A and IL-17F are encoded on chromosome 6 and share the greatest level of homology (approximately 60%), and are structurally and functionally similar. IL-17 is secreted as a disulphide-linked glycoprotein homodimer of 25-30kDa. Both IL-17A and IL-17F are active as homodimers, but IL-17 is 10-fold more potent than IL-17F. Heterodimers of IL-17 and IL-17F have also been found, called IL-17A/F, which have an intermediate level of potency (276). It has been suggested that the IL-17A/F heterodimer may potentially represent the dominant composition of the IL-17 cytokine, but it does not appear to have a distinct function and all homo- and hetero-dimeric forms are capable of utilising the same receptor (277, 278). Initially IL-17 expression was identified in memory $CD4^+$

T cells, but expression of IL-17 is not confined to Th17 cells: $CD8^+$ T cells, natural killer (NK) cells, γδ T cells, mast cells and neutrophils are all capable of IL-17 production (260, 279). The IL-17 family members IL-17B, IL-17C, and IL-17D are largely produced by non-immune cells. IL-17B is highly expressed in human and murine spinal cord, and is also expressed, at a lower level, in a number of tissues including the heart and brain (280). IL-17D is widely expressed in skeletal muscle, adipose tissue and the brain (281). In contrast, expression of IL-17C is highly restricted to the adult prostate and fetal kidney. Th2 cells in addition to IL-4, IL-5 and IL-13 can also express IL-17E (IL-25) as an effector cytokine, but IL-25 is also expressed by other tissues (282).

IL-17 signals via the IL-17 receptor (IL-17R), which is a ubiquitously distributed type 1 transmembrane receptor. The IL-17 receptor family is comprised of five members: IL-17RA, -RB, -RC, -RD, and –RE (277). As with all cytokine receptors the IL-17R is multimeric, but like IL-17, the IL-17R lacks structural homology with other known cytokine receptors, which has contributed to the lack of understanding in the IL-17 signalling pathway. IL-17RA has an unusually large cytoplasmic tail, found capable of activating inflammatory events, such as NF-κB activation and IL-6 transcription, events typically associated with IL-1β and TNFα (277). In humans IL-17 binds with high affinity to IL-17RA, unlike IL-17A/F and IL-17F, which bind with intermediate and low affinities to this receptor subunit, respectively. However, all homo- and hetero-dimeric forms bind with comparable affinity to IL-17RC and this receptor subunit, therefore, could be a therapeutic target (283, 284). Transfection studies have revealed that IL-17RA and -RC expression are both required for IL-17 signalling and the IL-17R is most likely composed of at least two IL-17RA subunits and one IL-17RC subunit (285).

Understanding of IL-17 signal transduction has been limited, in part, by the lack of homology with other cytokine families, and has thus led to a ‘bottom up’ approach to revealing the pathway (277). Identification of IL-6 as an IL-17 target gene furthered the understanding of the IL-17 signalling pathway and its synergistic action with cytokines including IL-1β, IFNγ and TNFα (277). The IL-17R contains a functional domain similar to the Toll/IL-1R domain, termed SEF IL-17R (SEFIR). SEFIR is capable of recruiting adaptor molecule Act1, which is an activator of NFκB (286). The importance of Act-1 in

the IL-17 signalling pathway is highlighted within Act1-deficient cells which fail to respond to IL-17 (287). IL-17R activation via Act1 leads to activation of the transcription factor Nuclear Factor κB (NFκB) and MAPK pathways (288). TNF Receptor Associated Factor (TRAF) 6 is essential in the convergence of the NFκB and MAPK pathways, and is activated during IL-17 signalling. AP-1 binding sites are present in a number of IL-17 gene targets and some investigations suggest that IL-17 triggers activation of AP-1, whilst others suggest IL-17 is capable of only weak AP-1 induction and that during IL-17 signalling the AP-1 site within the *IL-6* promoter is dispensable (289). The transcription factors and genes activated as a result of IL-17 signalling are similar to those activated by the TLR and IL-1 signalling pathways (277). The IL-17 receptor family however bares little homology to such innate type receptors and so is unlikely to be a duplicate.

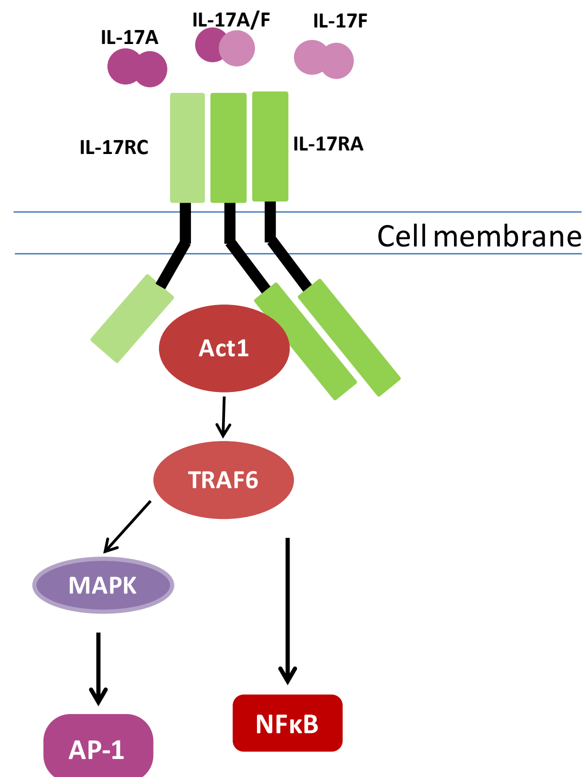


Figure 5. The IL-17 signalling pathway. Binding of Il-17A/IL-17F homo- or heterodimers to the IL-17R allows signalling via the NFκB and MAPK pathways. The IL-17R is throught to be composed of two IL-17RA subunits and one IL-17RC subunit.

1.8.2 Interleukin-21

IL-21 is an IL-2 family member, containing the common γ chain, and binds to a heterodimeric receptor consisting of an IL-21-specific receptor subunit and a common

γ-chain receptor. The IL-21 receptor is highly expressed on B-cells but is also expressed on T-cells and NK cells, conferring IL-21 responsiveness (290). Unlike other common γ chain receptor family members that signal via STAT5, IL-21 preferentially signals via STAT1 and STAT3. IL-21 drives *in vitro* T_H17 differentiation via an autocrine mechanism, regulated by STAT3 (291). IL-21 can also be produced in substantial amounts by Th17 cells, but is also produced by NK and Natural Killer T-cells (NKT) cells following exposure to mycobacterial antigens, providing a potential route of Th17 induction *in vivo* (292). IL-21 plays an important role in driving terminal differentiation of B-cells (293). In suboptimal IL-2/IL-15 conditions, IL-21 is capable of enhancing NK proliferation, and is also capable of driving $CD8^+$ T-cell proliferation (294). In addition IL-21 promotes inflammation by inducing expression of chemokine CXCL8 in macrophages which attracts neutrophils (294). Furthermore, IL-21 has been demonstrated to contribute to the pathogenesis of two autoimmune animal models of RA (295).

1.8.3 Interleukin-22

IL-22 is part of the IL-10 family of cytokines and has 80.8% homology to its murine counterpart. The IL-22 receptor is part of the cytokine receptor family class 2 consisting of two subunits, IL-22R1 and IL-10R2 (296). IL-22R1 expression is restricted to non-immune cells such as epithelia whereas IL-10R2 is ubiquitously expressed. A soluble IL-22 receptor called IL-22 binding protein also exists. IL-22 is able to activate STAT3 via the JAK/STAT pathway and IL-22 expression is upregulated by inflammatory mediators such as LPS, IL-1β and TNF-α (296). Although all inflammatory T helper effector lineages can produce IL-22, Th17 cells are a predominant source, with production 120-fold greater than by Th1 cells and 700-fold greater than by Th2 cells (206). IL-23 enhanced IL-22 production when Th17 cells were cultured in the presence of TGF-β and IL-6, however within an established Th17-differentiated cell population less than 10% express IL-22 (206). Recent investigations have revealed a population of IL-17-independent $IL-22^+$ cells, produced largely from $CCR10^+$ T-cells that also produce IL-10 with or without TNF-α. These cells have recently been termed Th22 cells (207), and are covered in greater detail in section 1.6.4. IL-22 is important for mucosal immunity, however it is also a major player in the chronic autoimmune skin disease

psoriasis (297). IL-22 can induce keratinocyte proliferation and the production of antimicrobial peptides, both of these factors are associated with psoriasis, further implicating a pathogenic role for Th22 cells which are capable of migrating to skin tissue (207, 298). Recently, it was shown that neutralising antibodies against IL-22 reduced pathology in a mouse model of psoriasis (299). IL-22 is also important in mediating host defence against bacteria: a protective effect of IL-22 has been demonstrated against bacterial pneumonia (299). Furthermore, it has been suggested that IL-22 induces the production of anti-microbial proteins by epithelial cells, which can bind to and kill gram$^-$ bacteria such as *K.pneumoniae* (300).

1.8.4 GM-CSF

Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) can be an indirect differentiating factor for Th17 cells and an effector cytokine. GM-CSF can be secreted in response to factors such as LPS and IL-1 by a number of cell types including macrophages, endothelial cells and T-cells (301). GM-CSF signals through its heteromeric receptor which is expressed by a variety of cells including monocytes, lymphocytes, granulocytes and endothelial cells (302). GM-CSF has been demonstrated to have an important role in the autoimmune pathogenesis in animal models such as EAE and CIA ((303, 304). Prior investigations have demonstrated that GM-CSF can indirectly promote T cell proliferation and Th1 responses by stimulating macrophages (304). More recent investigations have demonstrated that GM-CSF inhibits apoptosis and promotes Th17 cell development by inducing IL-6 secretion from TLR activated DC (305). Naive and effector CD4$^+$ T-cells do not express the GM-CSF receptor; therefore GM-CSF promotes IL-17 expression in T-cells via an indirect mechanism. APC secreted IL-23 enhances RORγt expression in T-cells which in turn can drive the production of GM-CSF from T-cells (306). The GM-CSF produced by T-cells positively feeds back to induce CD11c$^+$ DC to produce more IL-23 which signals to further indirectly promote Th17 cell responses. In addition IL-1β, in concert with IL-23, is also able to induce Th17 cell production of GM-CSF (307). GM-CSF is also produced by Th1 cells, however GM-CSF induced IL-23 secretion drives Th17 cell responses and inflammation in EAE (306, 307). An important role of Th17 cells is to recruit and activate neutrophils, and Th17-derived GM-CSF is especially important for mediating neutrophil activation

independently of IL-17 (308). Prior to the discovery of Th17 cells, the majority of IL-17^+ T-cells induced in response to *M.bovis* BCG were found to express GM-CSF although proportions of IL-17^+ and IL-17/GM-CSF^+ cells were low (309). However GM-CSF is not strictly viewed as a Th17 marker, but thought of as a marker of the Th17 cells pathogenic potential (310).

1.8.5 CCR6/CCL20

Chemokines are a family of chemotactic cytokines capable of inducing cell migration. Chemokine receptor expression determines the migratory capability of T-cells and therefore chemokine expression profiles can be used in addition to effector cytokines to define the immune function of memory T helper subsets. One of the most widely recognised chemokine receptors expressed on Th17 cells is CCR6 (311). All IL-17^+ memory T-cells isolated *ex vivo* are $CCR6^+$, although not all $CCR6^+$ T-cells are IL-17^+. CCR6 is also expressed on certain DC subsets, naive and memory B cells, NK and NKT cells, $CD25^{high}$ regulatory T-cells and on subsets of $CD45RO^+$ effector/memory T-cells (312–314). Unlike most chemokine receptors capable of binding a wide array of chemokines, CCR6 selectively binds CCL20 and β-defensins (315). CCR6 plays an important role in allowing T-cell migration to sites of inflammation or to the gut and small intestine, both tissues expressing CCL20 (316, 317). Th17 cells are also capable of producing substantial amounts of CCL20 in comparison to other T helper subsets. Joint synoviocytes also express CCL20 which allows recruitment of Th17 cells into the RA joint; CCR6 blockade in SKG mice (a spontaneous arthritis model caused by a mutation within ZAP-70 which alters thymic selection) suppressed disease onset and severity (318). *In vitro* CCR6 expression can be induced *de novo* from naive T-cells following TCR engagement in a pro-inflammatory cytokine *milieu* in combination with TGF-β. CCR6 expression is however transient and is lost during prolonged culture. Recently differential methylation of a non-coding region of the *CCR6* gene, which has transcriptional activity, has been described by Steinfelder *et al* (319). Pharmacological demethylation of this non-coding region led to stable CCR6 expression on the T-cells. A number of transcriptional regulators are suggested to be capable of binding to this region including ETS1, GATA, NFAT and CREB; although it appears that the region acts mainly as a methylation-sensitive enhancer allowing stable CCR6 expression. *C.albicans*

specific Th17 cells may be characterised by co-expression of CCR6 and CCR4 (320). However CCR6 and CCR4 can also be expressed by Foxp3^{+} T regulatory cells (321). Th17 cells also express a number of Th1- (CCR2, CXCR3, CCR5) and Th2- (CCR4) associated chemokine receptors (321). CCR6 is expressed by many non-Th17 cell subsets but at lower frequencies than Th17 cells (316). A number of chemokine trafficking receptors can be expressed by Th17 cells however CCR6 is expressed by all *ex vivo* Th17 cell subsets and is essential for Th17 cell migration in response to CCL20 and functional effects (316). These data demonstrate that chemokine receptor expression is not sufficient to define an effector T-cell subset and that it is important to assess additional phenotypic markers.

1.9 Regulation of Th17 cell responses

1.9.1 Regulation of murine Th17 responses

Studying *in vitro* the factors that regulate Th17 cell differentiation/response improves our understanding of how Th17 cells respond to different immune situations and may reveal how certain disease states, characterised by abnormal Th17 cell responses, arise. In addition understanding how Th17 cells are generated *in vitro* provides an initial tool for assessing the efficacy of treatments designed to reset the Th17 cell response. In mice one method to induce *in vitro* Th17 cell differentiation from naïve CD4^{+} T-cells results in 40-60% IL-17^{+} cells within the total T-cell population (322). The conditions employed to achieve this differentiation included TGF-β, IL-6, and TCR stimulation, along with neutralising antibodies against IFNγ and IL-4. However other groups have reported the induction of far lower proportions of Th17 cells, i.e. ranging from 5-20%. The variations in the capability of laboratories to induce Th17 cells have been attributed to subtle differences in the culture conditions employed including: the type of medium and serum used, and the genetic background of the mouse models. The culture medium Iscoves Modified Dulbeccos Medium (IMDM) was found to better support murine Th17 cell responses than the more commonly used medium, Roswell Park Memorial Institute medium (RPMI) (215). Compared to RPMI, IMDM contains a 4-times higher concentration of tryptophan which acts as a ligand for a proposed Th17 cell transcription factor, AhR. The serum used in culture media was also found to affect

in vitro Th17 cell differentiation. Foetal bovine serum (FBS) contains high concentrations of endogenous latent TGF-β (several nanograms per millilitre) (323). TGF-β is required for murine Th17 cell generation, however high endogenous concentrations of TGF-β in combination with exogenous TGF-β creates a TGF-β concentration which is inhibitory to Th17 cell induction (323). An alternative to FBS is serum replacement which does not contain endogenous TGF-β. The conditions for murine *in vitro* Th17 cell differentiation are generally defined as: IMDM media supplemented with serum replacement with the addition of TGF-β, IL-6, anti-IFNγ, anti-IL-4 (the neutralising antibodies are included in order to discourage development of Th1 cells and Th2 cells, respectively), and a TCR/CD28 activating stimulus (discussed in more detail in Chapter 2), though groups do differ in their use of cytokine cocktail.

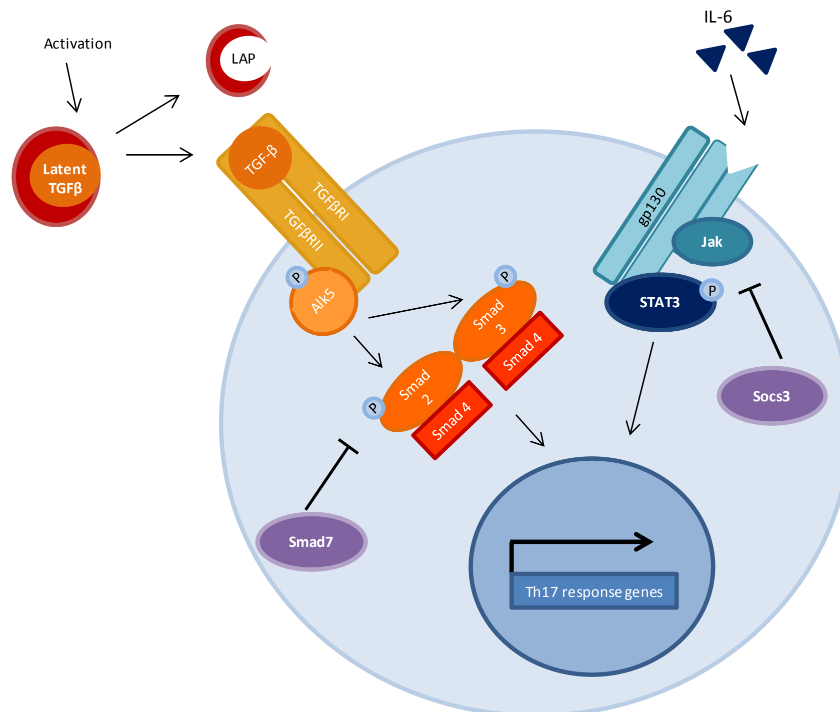


Figure 6. The induction of murine Th17 cells by IL-6 and TGF-β signalling. IL-6 binds to the IL-6 receptor which results in receptor dimerisation and STAT3 activation, active phosphorylated STAT3 is able to translocate to the nucleus to participate in the transcription of Th17 response genes. Socs3 is capable of blocking the STAT3 activation pathway and therefore inhibits Th17 responses. Latent TGF-β upon activation is cleaved from LAP and is capable of binding to the TGF-βR. TGF-βR signalling via Alk5 allows Smad activation and translocation to the nucleus but this event can be blocked by Smad7.

1.9.2 Induction of human Th17 cells by TGF-β

The addition of IL-6 and TGF-β to human naive T-cells does not result in the polarisation of high proportions/numbers of IL-17$^+$ cells. The requirement for TGF-β for the induction of Th17 cells in both mice and humans has been highly debated. Some studies reported that human Th17 cell differentiation can occur in the absence of TGF-β. For instance, Acosta-Rodriguez *et al* showed that IL-1β and IL-6 in the absence of TGF-β promoted Th17 cell induction (324) and Wilson *et al* found that a combination of IL-23 and IL-1β was sufficient for Th17 cell differentiation, again in the absence of TGF-β (325). Evans *et al* demonstrated that in cultures containing bulk CD4$^+$ T-cells and RMPI+10% FBS, that TGF -β (1-3ng/mL) had an inhibitory effect on Th17 cell responses (98). In contrast, many groups have reported that human Th17 cell generation does require TGF-β. Manel *et al* demonstrated that under serum-free the addition of exogenous TGF-β was required for the induction of the Th17 cell-specific transcription factor RORc (326). However, TGF-β can inhibit RORc activity, but addition of either: IL-1β, IL-6 and IL-21 or IL-23 was able to relieve TGF-β-mediated ROR inhibition. Volpe *et al* (327) and Yang *et al* (328) also confirmed that TGF-β is required for human Th17 cell induction when in combination with: IL-23, IL-1β and IL-6 (327) or IL-21 (328). As in mice, discrepancies between groups were attributed to high concentrations of endogenous TGF-β within FCS or human sera. Serum-free media, or media supplemented with serum replacement, which do not contain endogenous TGF-β are used for *in vitro* human Th17 cell differentiation. More recent data demonstrated that TGF-β indirectly favours Th17 cell responses by inhibiting Th1 cell responses. Compared to Th1 and Th2 cells, Th17 cells have lower expression of clusterin, higher bcl2 expression, and reduced apoptosis in the presence of TGF-β (329). Murine Th17 cells have also been induced from naive CD4$^+$ T-cells in the absence of TGF-β using IL-6, IL-23 and IL-1β. Interestingly Th17 cells generated in the presence of IL-23 but not TGF-β are observed to be more pathogenic in an EAE mouse model (330). Together these data indicate that IL-17 expression can be induced in the presence or absence of TGF-β, depending on the presence of other pro-Th17 cytokines. However the inclusion of TGF-β in the pro-Th17 cytokine *milieu* may further promote Th17 cell induction in an indirect manner by inhibiting Th1 cell and Th2 cell responses.

1.9.3 Induction of human Th17 cells by IL-1β

Investigations have assessed further cytokine combinations that might promote *in vitro* human Th17 cell induction. IL-1β is thought to be important for human Th17 cell responses. Early studies found that IL-1β was capable of promoting IL-17 expression in conjunction with IL-23 or in the presence of IL-6 (324, 325). TLR2 ligation on Langerhans cells results in the secretion of IL-1β which promotes Th17 cell responses (331). Interestingly Evans *et al* found IL-1β, TNFα, and IL-6 incapable of directly inducing Th17 cells (98). Instead TLR-activated monocytes were capable of Th17 cell induction in a cell contact-dependent manner though the precise mechanism was not revealed. Annunziato *et al* found IL-1β and IL-23 were sufficient to induce Th17 cell responses from $CD161^{+}$ $CD4^{+}$ $CD45RA^{+}$ T-cells (332). Expression of IL-1βRI has been found to correlate with IL-17 expression (333). The expression of IL-1βRI is thought to commit T-cells to produce IL-17 before TCR triggering. Although cord blood naive $CD4^{+}$ cells do not express the IL-1βRI, expression of this receptor is readily induced by IL-7, IL-15 and TGF-β. IL-17 expression by $IL\text{-}1βRI^{+}$ cells was enhanced by CD3/CD28 stimulation and IL-1β and further enhanced when in combination with IL-23, TGF-β, IL-6, and IL-21 (333). However $IL\text{-}1βR1^{-}$ cells can also produce IL-17 in response to TCR triggering, and interestingly in response to IL-1β. In the presence of IL-1β and IL-2, expression of Foxp3 is down regulated allowing $Foxp3^{+}$ Treg cells to be converted into $IL\text{-}17^{+}$ T-cells with diminished Treg cell suppressor function (334). In addition, inhibition of the IL-1β signalling pathway suppresses Th17 cell proliferation, further demonstrating the importance of IL-1β to Th17 cell responses (335). Increasingly the human and murine requirement for IL-1β does not appear to be as different as initially suggested. IL-1R expression also correlates with the Th17 cell phenotype in mice; $IL\text{-}1βR^{-/-}$ mice have diminished Th17 cell responses and decreased EAE disease severity. IL-1β was able to promote Th17 cell responses in the absence of TGF-β and can maintain the Th17 cell phenotype in the absence of TCR signalling (336). Moreover, murine fate mapping studies have demonstrated that IL-1βR expression may be used to identify ex-Th17 cells, which have switched to an IFNy-secreting phenotype (337).

1.9.4 Regulation of human Th17 cells by IL-23

The requirement for IL-23 was one of the factors that led to the discovery of Th17 cells as an independent subset (338). Like IL-6, IL-23 activates the transcription factor STAT3, which is important for the expression of Th17 cell response genes including RORc and IL-17A (339, 340). The IL-23R is expressed on memory CD45RA$^-$ T-cells rather than naive CD45RA$^+$ T-cells, and therefore IL-23 is most likely important for the maintenance, rather than induction, of Th17 cells. (325). In line with evidence suggesting that IL-23 acts as an amplifying/stabilising factor, IL-23 alone is unable to induce IL-17 production, but acts in synergy with other cytokines to promote Th17 cell responses. Th17 cell phenotype was promoted *in vitro* by the direct addition of IL-23 to CD3/CD28-activated human naive T-cells when in combination with either TGF-β and IL-6/ or IL-1β (341). IL-23 is also important for the conversion of Foxp3$^+$ Treg cells to a Th17 cell phenotype when in combination with IL-2, IL-β and TGF-β (342). Furthermore, in mice IL-23 promoted Th17 cell responses by inhibiting both Foxp3 and Tbet expression, though similar effects were also observed by a combination of TGF-β and IL-6 (343). IL-23 is produced by APC, not T-cells, in response to a wide array of pathogens and endogenous products, indicating the importance of Th17 cell responses in immunity. Pathogens capable of stimulating IL-23 production by APC include ***S. aureus***, ***S. pyrogenes***, ***T. gondii***, *B. pertussis,* ***K. Pneumonia,*** *C. albicans.* Pathogen derived products including PGN and **LPS** along with ATP, PGE_2, **CD40L** are also capable of inducing IL-23 (**bold** indicates that both IL-12 and IL-23 may be induced) (231, 338). Therefore, a number of studies have demonstrated that Th17 cell responses may be promoted by co-culture of APC that have been activated with factors capable of stimulating IL-23 secretion (338, 344–346). However, mutations inhibiting IL-12Rβ1 expression, the receptor binding subunit common to both IL-12 and IL-23, had less of an effect on reducing Th17 cell responses than a STAT3 mutation, suggesting that IL-23 is not essential for STAT3-dependent Th17 cell responses (347).

1.9.5 Induction of human Th17 cells by IL-21

IL-21 is another cytokine that has been proposed to be required for human Th17 cell development (348). IL-21, like IL-23 and IL-6, is capable of activating the STAT3 signalling pathway, which is important to the expression of a number of Th17 cell

response genes as mentioned above (339, 340, 349). Zhou *et al* demonstrated that IL-21 can be induced by IL-6 and is important for Th17 cell differentiation by activating an autocrine IL-21 feedback loop (348). Autocrine IL-21 is capable of up-regulating IL-23R expression, allowing synergistic IL-21 and IL-23 signalling and STAT3 activation, which in turn upregulates transcription of *Roryt* (348), confirmed by Wei *et al* (291). The IL-21 autocrine feedback loop has been demonstrated to require the transcription factor IRF4 (350). A requirement for IL-21 has also been demonstrated for human Th17 differentiation in combination with TGF-β as mentioned above (328). Korn *et al* demonstrated that IL-21 can induce an alternative pathway, preventing Foxp3$^+$ Treg induction in an IL-6-independent manner (351). However, Sonderegger *et al* found that IL-21 and IL-21R were not required for the differentiation of Th17 cells in mice, and demonstrated that IL-6, instead of IL-21, was sufficient to drive STAT3-mediated Th17 responses (352) Furthermore, IL-21 and IL-21R deficient mice were still capable of developing Th17 cell responses and developed comparable or even enhanced EAE disease severity when compared to wild-type mice (353). Overall it appears that IL-21 is dispensable for Th17 cell responses and can be replaced by IL-6 or IL-23 which also active the STAT3 pathway; however in the absence of these cytokines, Th17 cell responses may be induced or sustained by IL-21.

1.9.6 Further factors regulating human Th17 cell responses

Due to difficulties in inducing high proportions of IL-17$^+$ cells *in vitro*, particularly from naive CD4$^+$ T-cells, investigations have sought to find a 'factor X' to achieve this. Therefore a large number of additional factors have been assessed for their capability to promote *in vitro* human Th17 cell responses. Many groups have employed neutralising antibodies against IFNγ and IL-4 to prevent differentiation to either a Th1 cell or Th2 cell lineage, respectively. PGE_2 is a natural antagonist of IFNγ and has been demonstrated to have a positive effect on Th17 cell differentiation (346, 354, 355). PGE_2 is able to inhibit IL-12 and IFNγ expression and therefore is able to indirectly promote Th17 cell responses. PGE_2 is also capable of upregulating IL-1βR and IL-23R expression, amplifying IL-23 expression, and furthermore synergises with IL-23 and IL-1β to drive *RORc* expression (354, 356). More recently, TGF-β and IL-6 were shown to induce autocrine IL-9 production, which promoted Th17 cell responses (357). However,

IL-9 is negatively regulated by IL-23, and IL-9 is thought to enhance naturally occurring Foxp3$^+$ Treg functions (358). In addition, uric acid, a danger signal released from damaged cells, has been found to promote DC-driven Th17 cell responses in mice by inducing secretion of IL-1α/β, IL-6 and IL-18 (359). A further factor capable of promoting Th17 responses, via STAT3, is B-cell activating Factor (BAFF), a cytokine and a member of the TNF superfamily (360). Silencing of BAFF ameliorated CIA disease severity and diminished Th17 cell generation (361), whereas BAFF overexpression increased the number of Th17 cells and aggravated EAE disease severity (360). Together these investigations highlight the diversity of factors capable of regulating Th17 cell responses.

On the basis of data obtained through microarray assays it has been suggested that CD161 is a human Th17 cell surface marker and that CD161$^-$ cells are unable to differentiate to a Th17 cell phenotype (362). CD161 is the equivalent of murine NK1.1, a surface marker of NK cells (332). Cosmi *et al* suggested that in humans, IL-17 producing CD4$^+$ T cells originate from an NK-like CD4$^+$ precursor and that all IL-17-producing cells are contained within the CD161$^+$ fraction of CD4$^+$ T cells (362). Further research has demonstrated that umbilical cord blood naïve CD4$^+$ T cells expressing CD161$^+$ are capable of differentiating to a Th17 cell phenotype, whereas CD161$^-$CD4$^+$ T cells are unable to produce IL-17 (363). CD161$^+$ Th17 cells were proven not to be NKT cells as they have a broad TCR repertoire compared to NKT cells, which have a limited TCR repertoire. Additionally, antigen recognition by CD161$^+$ Th17 cells is MHC Class II-restricted whereas antigen recognition by NKT cells is CD1-restricted (363).

The application of murine *in vitro* differentiation conditions to human naïve CD4$^+$ T cells has proved unsuccessful in inducing large proportions of Th17 cells; combinations of TGFβ, IL-1β or IL-21, and IL-23 only marginally improves proportions of Th17 cells. It has been suggested that the presence of contaminating pre-activated T-cells within human Th17 cell cultures can have a strong inhibitory effect on naïve T-cell differentiation towards a Th17 cell phenotype (364). The presence of contaminating memory Th17 cells may also account for the small induction of Th17 cells from naive T-cells. Obtaining truly naïve CD4$^+$ T cells from humans is more difficult than in mice.

Humans are continually exposed to pathogens and naive T-cell selection relies on magnetic separation or flow cytometric cell sorting, which particularly with magnetic cell separation results in insufficiently pure populations. Mice can be housed in pathogen- free conditions allowing truly naïve $CD4^+$ T cells to be obtained more easily. Manel *et al* attempted to induce Th17 cell differentiation from antigen-inexperienced naive $CD4^+$ T-cells by using human naïve umbilical cord blood $CD4^+$ T cells (326). However, when culturing these cells in serum-free medium in the presence of TGF-β, IL-1β and either IL-6, IL-21 or IL-23, only 2-8% of cells differentiated into Th17 cells. The proportion of Th17 cells induced was found to be dependent on the concentration of TGF-β. However, this investigation further demonstrates that it is more difficult to achieve high percentages of Th17 cells from naive human T-cells *in vitro* compared to in murine naive T-cells. Nevertheless, the consensus at present is that the cytokine *milieu* for human Th17 cell responses requires IL-1β, TGF-β (if using a serum replacement) and a STAT3 activating cytokine such as IL-23 or IL-21.

In conclusion, it is generally accepted that a combination of TGF-β and IL-6 promotes substantial Th17 cell differentiation in certain mouse models. In contrast, for induction of human Th17 responses a combination of TGF-β, IL-1β, and IL-23 is required. However, the proportion of Th17 cells induced from human $CD4^+$ T-cells, particularly when differentiated from naive $CD4^+$ T-cells, remains very low. This has meant that the factor(s) required for optimal Th17 cells responses, particularly in humans, is still an area of investigation.

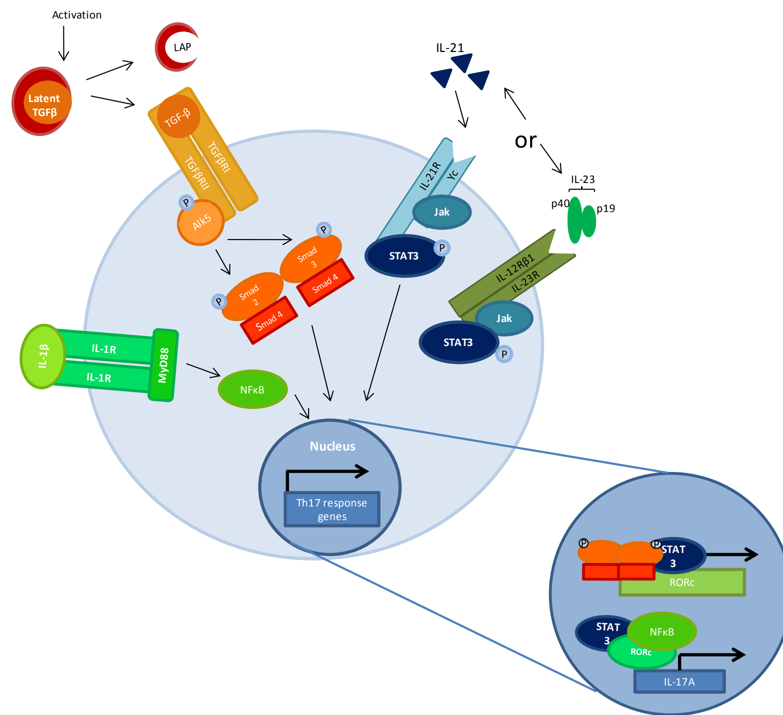


Figure 7. Summary of cytokine regulation of human Th17 cell induction. Activation of TGF-β results in cleavage of latent TGF-β from LAP and TGF-β binding to the TGF-βR. TGF-βR signalling via Alk5 allows Smad activation and translocation to the nucleus where Smads participate in *RORc* transcription and repression of IFNγ expression. STAT3 activation and translocation to the nucleus, as a result of either IL-23 or IL-21 signalling, also participates in *RORc* expression but also *IL-17* transcription. In addition signalling via IL-1β activates the NfκB pathway, which can also regulate Il-17 expression.

1.9.7 Th17 Transcriptional Regulation

The differentiation of Th1 cells and Th2 cells is dependent on cytokine signalling through the STAT family of transcription factors, which are capable of upregulating the Th1 cell- and Th2 cell-lineage specific transcription factors T-bet and GATA3, respectively (365). Characterisation of Th17 cells demonstrated that induction of Th17 cell-associated gene expression was not dependent on Th1/Th2 cell-associated transcription factors: STAT1, 4, 6, T-bet or GATA3 (366). A deficiency in STAT3 impairs Th17 cell differentiation and furthermore hyper-active STAT3 increases Th17 cell responses, demonstrating the importance of STAT3 signalling for Th17 cell development (348, 367). STAT3 has repressive effects on the transcriptional activity of both T-bet and Foxp3, which are required for Th1 and Treg cell development, respectively (367). Chromatin Immunopreciptation sequencing (ChIPseq) is a technique which allows the identification of protein associated DNA. Studies employing ChIPseq have demonstrated that STAT3 is capable of binding to a number of Th17-response genes including the *Il-17a* and *Il-17f* promoters. Although STAT3 is capable of binding

to the *Il-17* promoter, it is not considered the essential transcription factor for Th17 response gene expression (339). STAT3 overexpression in Rorγt$^{-/-}$ mice does not lead to *Il-17* transcription; whereas the overexpression of Rorγt does cause an increase in *Il-17* expression (though STAT3 is required for *Rorγt* expression) (348, 367). Therefore STAT3 is necessary but not sufficient for *Il-17* expression (368). Chen *et al* showed that a deficiency in SOCS3 (suppressor of cytokine signalling 3), which is a negative regulator of STAT3, greatly increases *Il-17* expression (369). Although SOCS3 is also induced by IL-21 and IL-6, TGF-β is capable of inhibiting SOCS3 expression, thereby prolonging STAT3 activation (370).

In 2006 Ivanov *et al* demonstrated that RORγt (RORC in humans), was the key transcription factor mediating differentiation of the Th17 lineage (371). RORγt and isoform RORγ1 are encoded by a single gene *Rorγ* (also known as *RORC*). Both isoforms have the same DNA binding domain. RORγt is preferentially expressed in thymic cells and Th17 cells, whereas RORγ1 is expressed in a variety of cells including muscle and kidney (372). RORγt-deficient mice have greatly reduced numbers of Th17 cells and secretion of IL-17, whereas R*orγt*-overexpression via retroviral transduction markedly increased both IL-17A and IL-17F production (371). Multiple RORγt binding sites have been found within the *Il-17* promoter (373); but the capability of RORγt binding to the *Il-17* promoter can be controlled by additional factors. For example, transcription factor Runt-related transcription factor 1 (Runx1) can interact with both RORγt and Foxp3 to regulate *Il-17* expression (374). Co-immunoprecipitation studies by Zhang *et al* demonstrated that Runx1 and RORγt directly interact to allow the expression of *Il-17A* and differentiation of Th17 cells. However, in the absence of pro-inflammatory cytokines Runx1 was able to interact with Foxp3, allowing FoxP3 to bind to RORγt and inhibit RORγt-mediated *Il-17* transcription. Conversely in the presence of pro-inflammatory cytokines Runx1 is able to bind RORγt, preventing Foxp3-mediated inhibition of RORγt and thus allowing *Il-17* transcription. Runx1 is therefore important to the differential regulation of Treg and Th17 cells. More recently, transcription factors c-Rel and RelA/p65, members of the NF-κB family, have been found to regulate RORγ and RORγt transcription, and promote Th17 differentiation (375). IL-1βR signalling activates the NF-κB signalling pathway, allowing IκBα phosphorylation which

inturn releases C-Rel and p65 for nuclear migration and *rorγ* and *rorα* expression. Within RORγt-deficient mice some Th17 cells remain indicating that further factors may be involved in Th17 cell responses (376). STAT3- dependent expression of RORα has also been found present in Th17 cells. As with RORγt, overexpression of *Rorα* increased Th17 cell differentiation, however RORα deficiency resulted in only a small decrease in IL-17 indicating a minor role for RORα in Th17 cell generation. Co-expression of RORγt and RORα led to enhanced Th17 cell differentiation whereas a double RORγt/RORα deficiency caused a global impairment of Th17 cell induction and protected mice from EAE.

Further transcription factors have also been demonstrated to regulate IL-17 expression and Th17 development. One such transcription factor thought to augment Th17 cell development is the ligand dependent transcription factor AhR, a type 1 nuclear receptor. AhR is expressed by both Th17 and Treg cells; however expression is higher in Th17 cells (215). Mice deficient in AhR were observed to have reduced Th17 cell induction along with a milder pathology of EAE (215). The culture medium IMDM contains natural agonists of AhR, which offers better support for the development of Th17 cells than the standard culture media RPMI which has only a low Th17 cell polarising effect (215). It is not fully understood how AhR regulates Th17 cell responses, but some evidence suggests that AhR is capable of preventing STAT1 activation, which is inhibitory to Th17 cell development (377). However, more recent data indicates that AhR may be more importantly involved in the expression of *Il-22* from Th22 cells than for *Il-17* expression from Th17 cells as AhR agonists inhibited *Il-17* expression but promoted *Il-22* (216, 217). More recently Interferon regulatory factor-4 (IRF4) has also been identified as a transcription factor important for Th17 cell development (378). IRF4 is vital for the development of Th2 cells but in its absence Th17 cells also fail to develop. *Irf4*$^{-/-}$ T helper cells have reduced expression of RORγt and increased expression of Foxp3. In murine T-cells activated in the presence of pro-Th17 cytokines, Rho-associated coiled coil containing protein kinase 2 (ROCK2) induced phosphorylation of IRF4 which caused an increase in the expression of both *Il-17* and *Il-21*. Furthermore, IRF4 is one of few factors that link the Th17 and Th2 cell phenotypes (368). The AP-1 family member basic leucine zipper activating transcription factor

(BATF) is expressed in Th1, Th2, Treg and Th17 cells (379). However, in *Batf*$^{-/-}$ mice, which were resistant to EAE only *IL-17* expression was reduced. TCR signalling can also regulate *IL-17* transcription, by activating NFATc1, a member of the NFAT transcription factor family, dicussed in more detail in Chapter 3 (368).

1.9.8 T-Cell Plasticity

As described in section 1.6 the Th1 and Th2 cell lineages were discovered by Mossman and Coffman in 1986 who at the time commented that their research ‘raises the question of T-cell diversity’, and they predicted that ‘it is quite possible that other T-cell types exist *in vivo* (160, 380). Until recently CD4$^+$ effector T cells (CD4$^+$CD45RO$^+$) have continued to be grouped into distinct effector lineages, termed the Th1/Th2 cell paradigm. This paradigm was based on the activation of discrete cytokine pathways in the naive T-cell, which resulted in the upregulation of unique transcription factor and cytokine profiles that mediate distinct immune responses. In recent years, as predicted by Mossman and Coffman, this paradigm has been overturned to include a wider range of lineages. One of the most surprising and interesting findings during the last couple of years has been the evidence suggesting that the effector lineages are not terminally differentiated. Effector memory T cells have a high potential for phenotype switching by modulating cytokine expression and therefore subset/function.

Discovery of the Th17 cell subset has led to a broader understanding of T-cell plasticity. It was quickly established in both mice and humans that Th17 cells could acquire the capability to produce IFNγ and switch off IL-17 production. Th17 cells highly express a part of the IL-12R, IL-12Rβ2, which is the receptor subunit not shared with the IL-23R, conferring Th17 cell responsiveness to IL-12. In the absence of TGF-β but in the presence of IL-12, T-cells are capable of switching from a Th17 to a Th1 cell phenotype, which is dependent on the activation of transcription factors STAT4 and T-bet (311, 381, 382). Inducible Treg cells were also found to be capable of switching towards a Th17 cell phenotype and vice versa (373, 374, 383). TGF-β induces both RORγt and Foxp3 expression in T-cells, and plasticity between these two subsets has been demonstrated to be controlled by the presence/absence of pro-inflammatory

cytokines. In the absence of IL-6, Foxp3 expression is induced by TGF-β and binds directly to RORγt preventing *Il-17* transcription, conversely, the presence of IL-6 prevents TGF-β-induced Foxp3 expression, allowing RORγt binding, resulting in Th17 cell induction. Furthermore, SOCS1 is highly expressed in Treg cells and loss of SOCS1 leads to the loss of Foxp3 expression and the conversion of Treg cells to Th1 cells characterised by high IFNγ expression. The CNS2 methylation status of *Foxp3* is important to the maintenance of Foxp3 expression. In the absence of SOCS1, ex-Foxp3$^+$ T-cells were characterised by IFNγ-induced methylation of the CNS2 region (384). Switching of more stable T helper cell phenotypes, such as Th2 cells, to another subset has also been demonstrated. For instance, in response viral infection Th2 cells can switch to a hybrid Th2/Th1 cell phenotype capable of producing both IL-4 and IFNγ (385). The hybrid Th2/Th1 cell was induced by viral infection which induced type 1 and 2 IFNs and IL-12. The presence of type 1 and type 2 interferons and IL-12 in conjunction with TCR stimulation activated STAT1 and STAT4 to allow stable T-bet expression alongside GATA3, allowing the T-cell to produce both IL-4 and IFNγ. Together these investigations have revealed that, at least *in vitro*, effector T-cells, not limited to Th17 cells, can acquire the different immunogenic capabilities to the phenotype expressed *ex vivo*. This switch is dependent on the *in vitro* culture conditions (an overview of T-cell plasticity is provided in Figure 8).

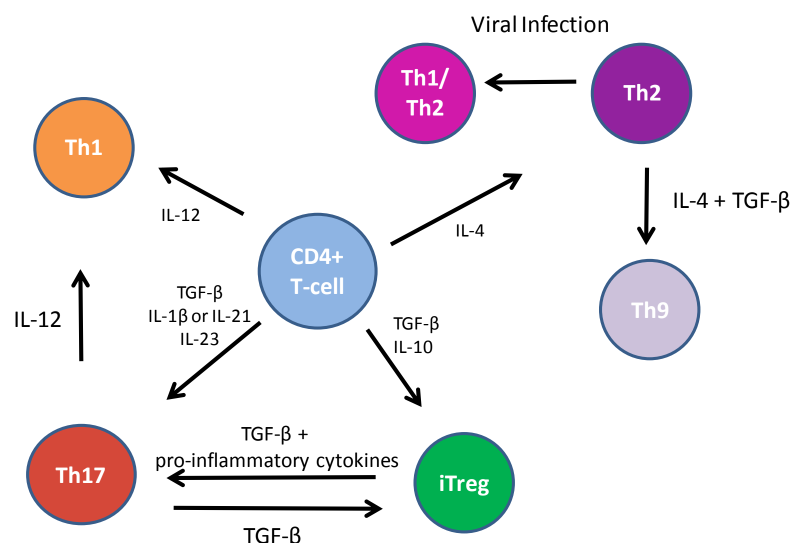


Figure 8. CD4$^+$ T-cell plasticity. T helper cells retain a degree of plasticity which allows the effector phenotype to be changed depending on the surrounding micro-environment and the cells capacity to respond to these factors. Currently the literature indicated that certain phenotype switches occur more readily than others. The arrows indicate the direction of phenotype change and the cytokine factors presently understood to be required to facilitate this.

An influential paper by Hirota *et al* has extended our understanding of T-cell phenotype plasticity by fate-mapping Th17 cells in a mouse model, assessing Th17 cell plasticity *in vivo* (386). By using a knock in mouse strain with a gene encoding a Cre recombinase in the *Il-17a* locus. The investigators track cells by eYFP expression which occurs when *Il-17* expression is initiated and eYFP expression is maintained. Maintainance of eYFP expression allows cells which are or have produced IL-17 to be tracked, regardless of their current production of IL-17. A number of interesting findings resulted from this investigation. Under chronic conditions, T-cells that were Th17 cells rapidly switched to produce IFNγ. The Th17 to Th1 cell phenotype switch only occurred upon the downregulation of CCR6; this event allowed the expression of the IL-12Rβ2 and T-bet. In comparison, Th17 cell responses occurred within 3 days of an acute infection response to *C. albicans*, but by day 5 eYFP$^+$ and therefore IL-17$^+$ cells were largely quiescent. The quiescent state induced was explained by the anti-inflammatory *milieu* produced from APC in the infected skin which downregulated inflammatory gene expression. Therefore acute inflammation is cleared by decreased expression of IL-17 rather than the switch to another effector phenotype as observed in the chronic immune response. As observed *in vitro*, the capacity for T-cells to switch phenotype was controlled by the cytokine *milieu*. Indeed microenvironments such as those created within the synovial joint during RA have been demonstrated to contribute to phenotype switching, allowing unstable Th17 cells to convert to Th17/1 or Th1 cells maintaining the presence of proinflammatory effector T-cells in the joint.

Although current data indicates that effector T-cells are capable of making a number of phenotypic transitions, certain transitions do not appear to be made (e.g. Th2 to Th17 or Treg, or Th1 to Treg), thus raising the question of what controls commitment vs flexibility. Current investigations in the T helper cell field are trying to elucidate the factors controlling which transitions are made and why. It has been suggested that certain phenotypes offer increased stability than others and therefore once the cell is in a stable state it is unlikely to return to an unstable state. The absence of self-reinforcing transcription circuitry in Th17 cells and Treg cells is thought to increase the instability of the phenotypes and therefore contributes to their increased capacity to switch phenotype (200). In addition, Th17 cells retain responsiveness to IL-12 which is

in contrast to Th2 cells which rapidly lose expression of IL-12R, becoming more refractory to Th1 cell phenotype switching (200). Due to the involution of the adult thymus, it may be that the immune system evolved to allow memory T-cells to respond to multiple infections by switching phenotype (387). The discovery of T-cell plasticity has complicated investigations seeking to determine the factors required for certain T helper cell responses. It now seems relevant to assess the capability of both naive and memory T-cells to differentiate towards a Th17 cell phenotype.

1.10 T-cell regulation by strength of stimulation

T-cell survival in the thymus is dictated by the TCR receiving either 'strong' or 'weak' signals that result in positive or negative selection, and life or death of the cell. Although cytokines play an important role, peripheral T-cell fate is also controlled by the strength of TCR binding to pMHC. The affinity and avidity of signals received by the TCR at the immune synapse can determine the proliferation, differentiation and death of the T-cell. Overall stimulation 'strength' may be determined by a variety of factors including: the rate of TCR triggering (determined by both antigen concentration and affinity); the availability of co-stimulation, and the duration of T-cell-APC interactions (388).

1.10.1 Stimulation strength and naive/memory T-cell activation

Depending on the T-cell maturation state different TCR signalling thresholds are required to activate the T-cell (389). Memory T-cells have been observed to require formation of approximately 100 pMHC:TCR complexes to trigger detectable IL-2 secretion whereas naive T-cells have a greater TCR triggering threshold and so require ~15 fold more pMHC to secrete a similar concentration of IL-2. TCR signalling thresholds in naive and memory T-cells can be reduced via CD28 signalling (390). Differences in the triggering thresholds of naive and effector T-cells are thought to be also controlled by variations in adhesion molecules and CD45 isoform expression, which together contribute to overall TCR engagement and downstream signalling thresholds (391, 392). More recent evidence indicates different TCR signalling pathways are induced in naive T-cells compared to memory T-cells (393). Naive T-cell activation induced the Erk signalling pathway, which had inhibitory effects on Ca^{2+}

mobilisation. Conversely, memory T-cell activation induced the MAPK p38 pathway, which did not attenuate Ca^{2+} mobilisation. Together these data may explain the weaker and slower response of naive T-cells compared to memory T-cells. Professional APCs are capable of lowering the signalling thresholds required to activate naive T-cells, as professional APC have higher cell surface expression of co-stimulatory and adhesion molecules (388). Non-professional APC, despite similar TCR engagement, are unable to reduce the TCR signalling threshold, as they do not express sufficient co-stimulatory/adhesion molecules and so are unable to activate naive T-cells.

Different experimental approaches can be used to modulate the rate of TCR triggering. Altering the antigen dose allows quantitative assessment of T-cell stimulation requirements capable of determining effector T-cell phenotypes. In the 1970's investigations first began assessing the effect of stimulation strength upon antibody/humoral (Th2 mediated) responses compared to cellular (Th1 mediated) responses (394). High antigen doses were found to induce an antibody response whereas low doses of antigen induced a cellular response, providing the first evidence that the strength of TCR binding can modulate the immune response. Further studies have demonstrated the effect of different antigen doses on Th1 cell and Th2 cells responses (395, 396). High doses of bacterial flaggelin or *Trichuris muris* were found to favour Th2 cell responses whereas low doses favoured Th1 cell responses *in vivo*. However, data in this field are conflicting, *in vitro* investigations assessing dust mite allergen dose responses in allergic donors found that Th2 cell responses were induced by low allergen doses (0.003-0.01 µg/mL), whereas high doses (10-30 µg/mL) induced little IL-4 (397). These results were however, highly dependent on the nature of the antigen used, as antigens themselves are capable of modulating APC phenotype through TLR ligation and cytokine secretion, and there are also variations in antigen processing and epitope presentation (398–400). In contrast, increasing the immunising dose of a peptide antigen such as collagen IV enhanced Th1 cell responses compared to Th2. Furthermore, Th2 responses were induced by low dose collagen IV immunisation, and similar findings were observed with an MHC peptide analog (401, 402). Although conflicting data were also presented using antigenic peptide dose models, differences are most likely explained by different dosing ranges and/or the

different antigenic peptides used. Overall the data indicates that low strength stimulation through low antigen dose favours Th2 cell responses, whereas high strength favours Th1 cell development (403, 404).

A further mechanism to modulate TCR-signal strength is via altered peptide ligands (APLs) which can be used to modulate the affinity/quality of the pMHC:TCR interaction. APLs are immunogenic peptides altered at the amino acid residues that participate in TCR binding, resulting in production of peptides with different TCR binding affinities. The presence of endogenous APL potentially makes these models relevant to the *in vivo* situation (405). APLs have been a useful tool to assess the effect of TCR-pMHC affinity upon T-cell effector phenotype (406). Use of a moth cytochrome c APL, 500-1000-times less potent than wild-type peptide (as assessed by reduced proliferation), demonstrated that the wild-type peptide yielded Th1 cell responses, whereas the weaker stimulating APL resulted in a mixed Th1/Th2 cell response. The addition of IL-2, which overcame the difference in proliferation, did not overcome the Th2 cell response induced by the APL (402). A different APL reduced the binding affinity to I-A^s but enhanced binding to I-A^b (407). *In vivo* priming of $CD4^+$ T-cells in low affinity I-A^s mice resulted in Th2 cell responses whereas priming in high affinity I-A^b mice resulted in Th1 cell responses. These data were confirmed in a further study using a myelin basic protein APL (402, 408). Differences in MHC peptide binding affinity are most likely to confer a difference in TCR signal strength by priming T-cells with a high vs low density of pMHC, resulting in the promotion of Th1 cell vs Th2 cell phenotypes, respectively. These data provided the first evidence that TCR-pMHC binding affinities can provide T-cells with signals that determine can the effector phenotype (407).

Changing the duration of T-cell-APC interactions can also modulate TCR signal strength (392). Interactions of short duration are insufficient to stimulate naive T-cell responses, as the required signalling threshold for IL-2 receptor expression is not provided and proliferatiion is not induced. However, prolonged TCR-APC interaction of naive T-cells enhances IL-2R expression and induces proliferation. Effector $CD4^+$ T-cells have been demonstrated to have different requirements for the duration of TCR signalling; one hour is sufficient for IL-2R expression and proliferation, whereas prolonged

interactions can be inhibitory (392). In addition, the effector phenotypes may also be dictated by the duration of TCR signalling (409). A short (24h) duration of TCR stimulation, induced a Th1 cell population proportionally comprising >70% of the total population (phenotype was assessed at day 7). By comparison Th2 cell responses from naive T-cells required a duration of 96h TCR stimulation to induce a Th2 population proportionally comprising >60% of the total population. This was explained by requirement of Th2 cells for simultaneous TCR and IL-4 receptor signalling, due to demethylation of the *Il-4* promoter requiring prolonged TCR stimulation. In comparison demethylation of the *Ifnγ* promoter occurs rapidly and does not require prolonged TCR stimulation, allowing *Ifnγ* expression after a short duration of TCR stimulation. Interestingly, in the absence of skewing cytokines, naive T-cells, activated for periods in excess of 72 hours, developed 'spontaneous' Th1 cell and Th2 cell responses, most likely because of endogenous cytokine production. In addition, long duration of T-cell interaction with a high affinity APL resulted in Th1 cell responses whereas low affinity APL over a moderate duration favoured Th2 cell responses, providing evidence for how variation in TCR signalling combine to affect T cell phenotype (410).

The presence and quality of co-stimulation can also contribute to modulation of overall TCR signal strength. CD28 signalling has the capability to lower TCR signalling thresholds and also has an affect on Th1 cell and Th2 cell responses (411). Disruption of CD28-CD80 interactions, via CTLA4Ig addition during priming, was found to be capable of blocking IL-4 expression but did not affect IFNγ expression. CD28 contributes to stimulation strength in combination with TCR stimulation. Low strength T-cell activation using an APL in concert with CD28 stimulation augmented IL-4 expression whereas CD28 co-stimulation could not overcome the inhibitory effect of high strength TCR signalling delivered by a wild-type peptide. Conversely, CD28 only augmented *Ifnγ* expression in conjunction with high doses of wild-type peptide (412). Interestingly, CD28 blockade, which abolished IL-4 production *in vitro,* could be reversed in the presence of IL-2. These data highlight the importance of separating the requirement of CD28 stimulation from that of IL-2, which is also induced as a result of CD28 signalling (413). Although data are limited, discrete effects of CD80 and CD86,

the CD28 binding molecules, have also been described. Treatment of murine naive T-cells with αCD80 promoted Th1 cell responses whereas αCD86 promoted Th2 cell responses (414). Moreover, neutralisation of CD80 can lead to disease attenuation in a BSA-antigen induced model of arthritis by enhancing IL-4 responses (415).

Clearly T-cell effector fate in the context of Th1 cell and Th2 cell phenotypes is affected by the overall strength of stimulation received, and the various means by which this may occur are depicted in Figure 9. How stimulation strength signals are interpreted and interact with the cytokine signalling pathways is still poorly understood. Furthermore, the majority of investigations have only assessed the effect of stimulation strength upon Th1 and Th2 cell responses, how newly identified subsets, such as Th17, are regulated by stimulation strength is poorly understood.

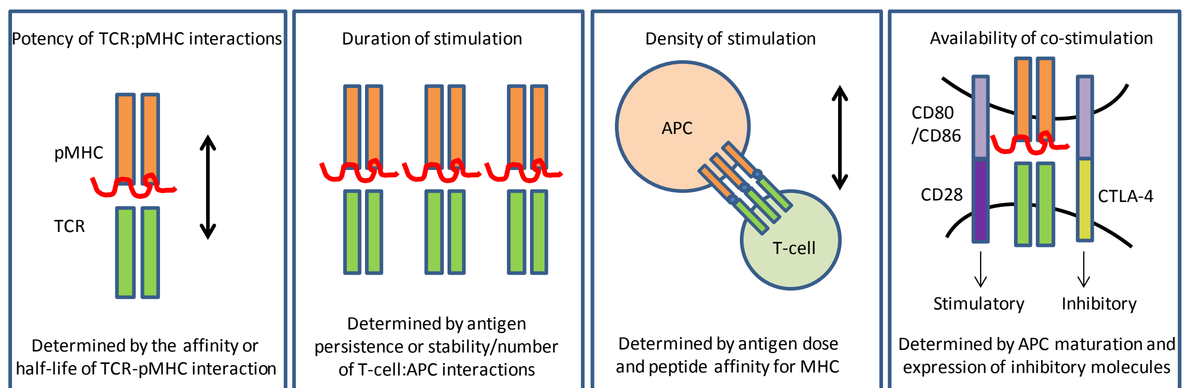


Figure 9. The variables that can influence the quality and quantity of T-cell stimulation strength. A concert of factors contribute to the overall strength of T-cell stimulation *in vivo* including the potency of TCR:pMHC binding, the duration or half life of the interaction, the number of interacting molecules and the availability of co-stimulatory molecules that are capable of lowering the signalling threshold, adapted from Corse *et al* (416).

1.11 Hypothesis

I hypothesised that human Th17 cell responses would be promoted by high strength TCR stimulation. This hypothesis was based upon experiments perfomed using murine T-cells which have revealed that Th17 cell responses may be promoted by high strength TCR stimulation. Having addressed the role of stimulation strength upon human Th17 cell responses, and disproving the above hypothesis, I next hypothesised that reduced TCR signalling conferred by the PTPN22 genetic polymorphism would promote human Th17 cell responses. Finally I hypothesised that *in vitro* T-cell culture density will modulate the human Th17 cell response. This is based upon the impact that T-cell density can have on factors including: cell-cell contacts, autocrine/paracrine cytokine signalling, and the regulation of transcription factors such as STAT3 and AhR. Within this thesis I therefore aimed to assess the effect of factors other than cytokine signalling, that may be capable of modulating human Th17 cell responses *in vitro*.

The aims of this thesis are to:

1. Investigate the effect of TCR stimulation strength on Th17 cell responses and to determine the mechanism by which this operates
2. Assess if Th17 cell responses are modulated by the PTPN22 genetic polymorphism which alters TCR stimulation strength and is associated with Rheumatoid Arthritis
3. Investigate if T-cell density is a parameter capable of modulating human Th17 cell responses.

2 Chapter 2. Regulation of human Th17 cell responses by stimulation strength

2.1 Introduction

In recent years our understanding of the $CD4^+$ T helper cell family has been dramatically increased. The Th1/Th2 cell dichotomy has been reviewed since the description of new effector T-cell phenotypes, including Th17 cells. Furthermore, it is apparent that rather than differentiating into terminal lineages, $CD4^+$ T-cells retain the capability to switch phenotype, termed T helper cell plasticity (200). Th17 cells are important for mediating the clearance of pathogens such as *C.albicans* (231). However, IL-17 contributes to tissue inflammation and evidence indicates that dysregulation of Th17 cells can initiate organ specific autoimmune disease pathologies, including MS and RA (417–419). Understanding how Th17 cell responses are regulated is important for improving treatments where Th17 cells contribute to disease pathology. The majority of research assessing the regulation of Th17 cell responses has focussed on the cytokine *milieu* required. However, T helper cell phenotype is determined by 3 signals: Signal 1 is provided by the 'strength' of TCR interactions with pMHC; signal 2 is provided by costimulation through CD28, and signal 3 is dependent on the cytokine *milieu* secreted by APC and the surrounding environment. Although investigations have furthered our understanding of human Th17 cell regulation, the proportions of Th17 cells induced in human *in vitro* cell cultures remain far lower than those induced in mouse *in vitro* cell cultures. This general inability to induce large numbers of Th17 cells in human *in vitro* systems may indicate that there are further factors, other than the cytokines already established, that are capable of regulating human Th17 cell responses. Previous investigations have demonstrated that Th1 and Th2 cells are regulated not only by the cytokine *milieu* (signal 3) but also by the strength of T-cell stimulation (signals 1 and 2). Therefore, establishing how human Th17 cells are regulated by stimulation strength may provide novel insights into Th17 cell regulation,

increase our understanding of the Th17 cell response in disease, and potentially improve therapies and treatments against Th17 cells.

2.1.1 Th17 and stimulation strength

Investigations within mouse models have begun to explore if and how Th17 cell responses are regulated by TCR activation strength. A recent investigation by Bouguermouh *et al* (420) using murine T-cells revealed that both naive and memory $CD4^+$ T-cell development towards a Th17 cell phenotype required a high strength TCR stimulation, which was delivered via plate bound anti-CD3 (10ug/ml) (421). Interestingly, the T-cell maturation state determined the requirement for anti-CD28 co-stimulation in Th17 cell induction. CD28 co-stimulation was found to significantly decrease murine naive $CD4^+$ T-cell differentiation towards a Th17 cell phenotype; however, CD28 co-stimulation did not decrease the induction of Th17 cells from memory T-cells. Concentrations of anti-CD28 as low as 0.06 µg/mL were capable of suppressing Th17 cell development from murine naive T-cells. However, the addition of CTLA4-Ig, which interferes with the interaction between CD28 and CD80/86, only marginally augmented Th17 cell proportions and IL-17 secretion from human naive $CD4^+$ T-cells. These data indicate that CD28 co-stimulation may be less inhibitory to human Th17 cell responses than it is in mice. These data also potentially indicate a difference between the T-cell stimulation strength requirements of human and mouse T-cells for the generation of Th17 cells. A further study has revealed that $CD28^{-/-}$ mice have decreased Th17 cell generation when compared to wild-type mice, and that the addition of CTLA-Ig decreased Th17 cell induction, contradicting the above study by Bouguermouh *et al* (420). Furthermore, blocking of CTLA-4 resulted in an increased Th17 cell response and potentiated the disease severity in experimental autoimmune myocarditis, a Th17 cell-mediated disease model. Thus, the role of CD28 co-stimulation in regulating Th17 development remains unclear. More recently, it was demonstrated that the expression of IL-17A and -17F is differentially controlled by TCR signalling. Using inducible T-cell $kinase^{-/-}$ ($Itk^{-/-}$) mice, TCR signalling was reduced, demonstrated by decreased phospholipase-Cγ phosphorylation and Ca^{2+} mobilisation. It was revealed that $Itk^{-/-}$ mice have reduced IL-17 expression despite normal expression of RORγt.

Interestingly, increasing the dose of anti-CD3 stimulation preferentially induced the expression of IL-17A over -17F (422).

Together these investigations reveal that murine Th17 cell responses can be regulated by the strength of T-cell activation signals. However, these investigations are limited and contradictory, and further investigations are required to clarify the role of T-cell stimulation strength in the induction of mouse Th17 cell responses. As the cytokine signalling requirements for mouse and human Th17 cell induction have already been demonstrated to differ, it is important to assess, if and how, T-cell stimulation strength affects human Th17 cell responses (363). The aim of this Chapter is therefore to assess in human T-cells if T-cell stimulation strength can modulate the Th17 response.

2.2 Aims

The aims of Chapter 2 were to:

1. Assess how human Th17 cell responses were affected by variations in stimulation strength administered by anti-CD3/anti-CD28
2. Characterise high and low strength stimulated cells for the expression of RORc and cytokines associated with Th17 and non-Th17 cells
3. Assess the proliferation and viability of high and low strength stimulated cells
4. Investigate if the difference in Th17 cell response between high and low strength stimulated T-cells were both proportional and absolute
5. Assess the effect of the duration of stimulation on Th17 cell responses
6. Compare how stimulation strength affects memory and naive T-cell Th17 cell responses
7. Investigate how modulating stimulation strength with a more physiological stimulus affects Th17 cell responses
8. Assess if high strength stimulation via TCR/CD3 or CD28 causes the modulation in Th17 cell responses

2.3 Experimental approach

To address the hypothesis that high strength T-cell stimulation would promote human Th17 cell responses I compared the response of human $CD4^+$ T-cells cultured with different ratios of anti-CD3/anti-CD28 beads. Anti-CD3/anti-CD28 antibody mediated activation of human cells is a commonly used technique which does not rely upon antigen specificity. Antibody bound beads were favoured over the use of immobilised plate bound anti-CD3 and soluble CD28 as it was considered that maintaining antibody mobility may better mimic the APC:T-cell interaction. Based upon the literature human Th17 cell responses were shown to be promoted by IL-1β, IL-23, and TGF-β at 10 ng/mL, I therefore cultured T-cells under with this combination and concentration of cytokines for a proTh17 culture system.

Unless otherwise stated, unfractionated $CD4^+$ T-cells were isolated by magnetic separation to a purity of >90% (Figure 92). $CD4^+$ T-cells were stimulated at a high strength stimulus of 1 bead:1 $CD4^+$ T-cell or a low strength stimulus of 1 bead:50 $CD4^+$ T-cells in the presence of pro-Th17 cytokines IL-1β, IL-23, and TGF-β each at a concentration of 10ng/mL. The culture media was IMDM containing 5% serum replacement; each well of a 24 well plate contained a total culture volume of 1 mL containing $1x10^6$ cells. At day 3 of culture 10 u/mL of IL-2 and 10 ng/mL of IL-23 were given to each culture well. The kinetics of splitting the different stimulation strength cultures is outlined below Figure 10. The basis for splitting/refreshing T-cell cultures was determined by lightening of the media colour and the amount of space that the cells had. The effect of T-cell stimulation strength upon Th17 cell responses was assessed at Day 6, unless otherwise stated. At day 6 T-cells were restimulated with PMA/Ionomycin for one hour and then cultured in the presence of Brefeldin A for a further 4 hours. Intracellular IL-17 and IFNy expression was determined by the gating strategy outlined below Figure 11.

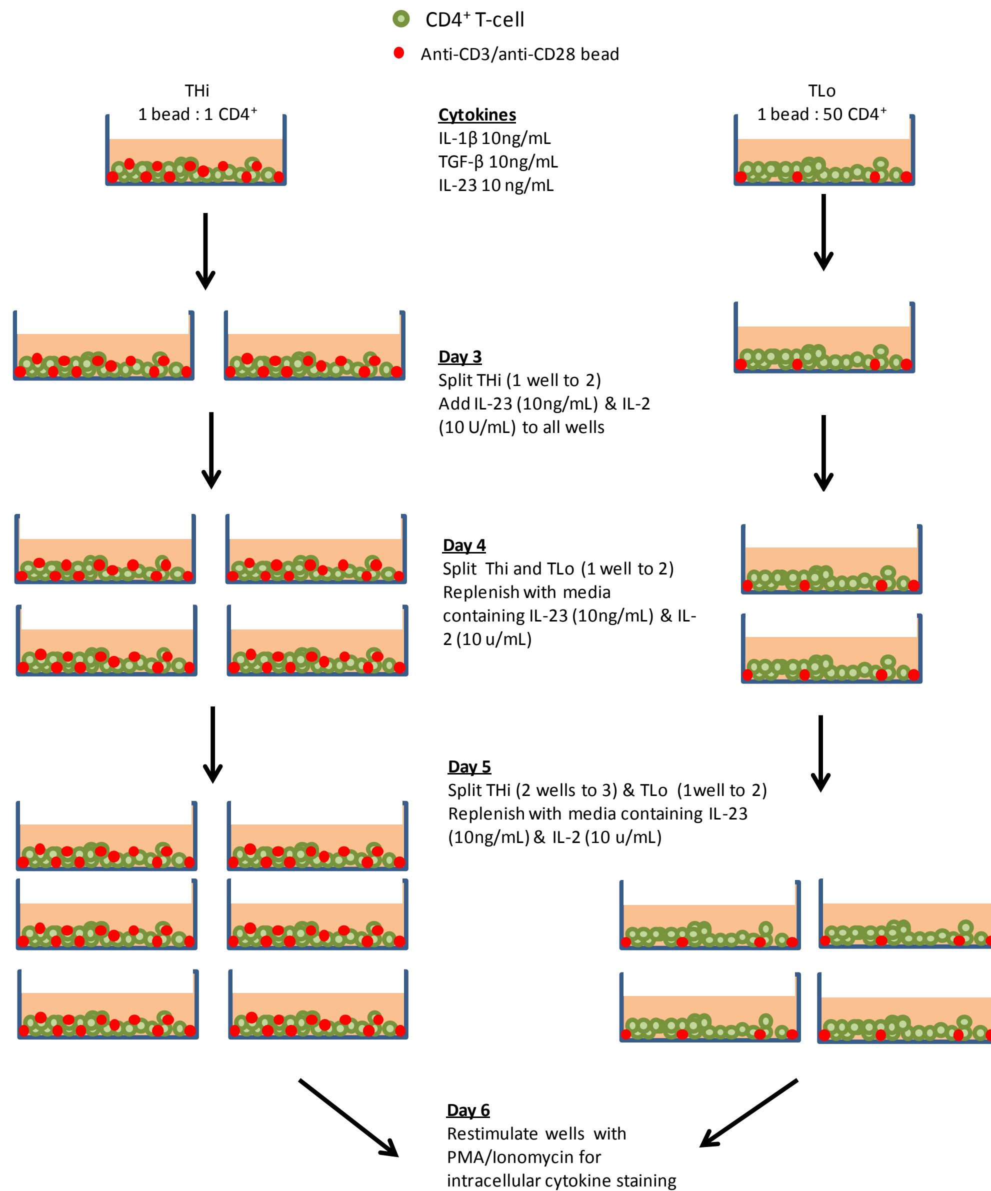


Figure 10. The THi/TLo culture system. 1 x 10^6 $CD4^+$ T-cells were cultured in IMDM with 10% serum replacement in a 1mL volume on a 24 well plate. T-cells were cultured with either $1x10^6$ (THi) or $0.02x10^6$ (TLo anti-CD3/antiCD28 beads in the presence of proTh17 cytokines IL-1β, IL-23 and TGF-β. T-cells were incubated at 37˚C 5% CO_2 for 6 days. During the 6 day culture cells were split as indicated above. Splitting of wells was based upon lightening of the media and a high cell confluency within the well. Day 4 and 5 media was replenished to a volume of 1 mL with IMDM + 10% serum replacement containing 10ng/mL of IL-23 and 10 U/mL of IL-2. At day 6 T-cells were restimulated with PMA/Ionomycin as described in section 7.5.2 and results were analysed as indicated in Figure 11.

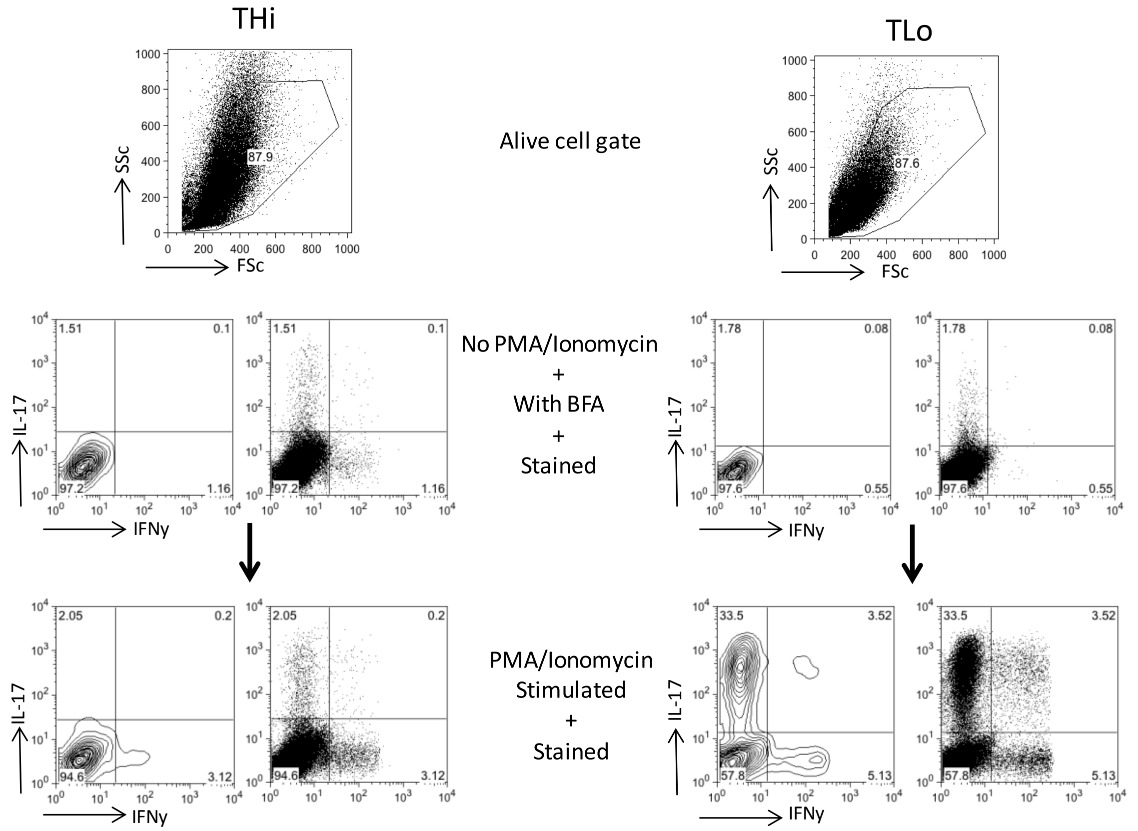


Figure 11. THi/TLo gating strategy. The lymphocyte population was gated on using FSC/SSc. The negative quadrant gates for cytokine expression were set on the BFA-alone treated cells providing the background staining for each stimulated population. These quadrants were then used to set the quadrants for the PMA/Ionomycin stimulated cells.

2.4 Results

2.4.1 Ex vivo proportion of IL-17 and IFNy expressing cells

To assess the effect of stimulation strength on Th17 cell responses, total (unfractionated) CD4$^+$ T-cells were used. Total CD4$^+$ T-cells were used because generating Th17 cell responses from naive human T-cells is difficult to perform and results in very low proportions of Th17 cell induction, as discussed in Chapter 1. By using total CD4$^+$ T-cells prior investigation have demonstrated that greater proportions of Th17 cells may be generated and so using total CD4$^+$ T-cells provides a better initial model for assessing how stimulation strength affects Th17 cell repsonses. Total CD4$^+$ T-cells contain both naive and memory T-cells and therefore potentially already contain a proportion of Th17 cells within the memory CD4$^+$ T-cell fraction. Investigations generally compare the Th17 and Th1 cell responses as measured by production of their signature cytokines, IL-17 and IFNy, respectively. Therefore, to determine the proportion of *ex vivo* Th1 and Th17 cells comprised within the starting population, freshly isolated CD4$^+$ T-cells were stimulated for 4 hours with Phorbol 12-myristate 13-acetate (PMA)/Ionomycin and stained for intracellular IL-17 and IFNy. The results in Figure 12 show that the proportion of IL-17$^+$ cells in *ex vivo* CD4$^+$ T-cells was ~0.5%, a finding also reported by Evans *et al* (98). The *ex vivo* Th1 proportion was found to be substantially higher at just under 10%.

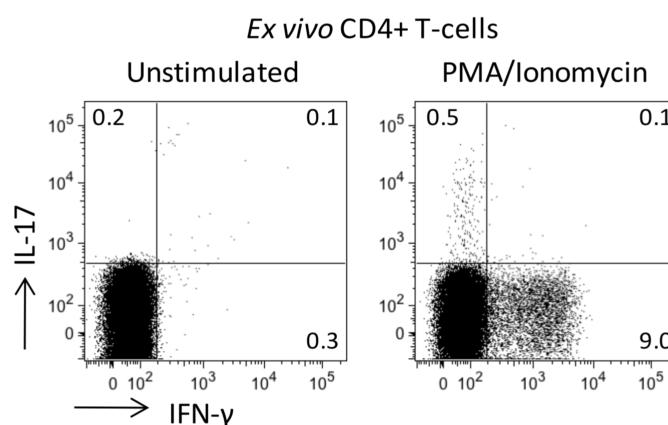


Figure 12. Proportions of IL-17- and IFNy-producing cells present in the *ex vivo* CD4$^+$ T-cell population. Freshly isolated CD4$^+$ T-cells were rested for 1 hour and subsequently stimulated with PMA/Ionomycin for 4 hours, the last 3 hours in the presence of Brefeldin A. IL-17 and IFNy expression was assessed by intracellular cytokine staining followed by flow cytometry. Data are representative of 3 independent experiments.

2.4.2 Titration of stimulation strength

The effect of stimulation strength on Th17 cell responses was investigated by culturing CD4+ T-cells in the presence of decreasing numbers of anti-CD3/anti-CD28 expander beads. The magnetic expander beads are pre-conjugated with anti-CD3 and anti-CD28 antibodies, which allow for T-cell activation in a non-antigen-specific manner. The manufacturer recommends to use a bead:T-cell ratio of 1:1. Cultures were performed in the presence of pro-Th17 cell cytokines; IL-1β, TGF-β, and IL-23. Following 6 days of culture, CD4+ T-cells were restimulated for 5 hours with PMA/Ionomycin, and IL-17 and IFNγ production was assessed by intracellular cytokine staining (ICS) and flow cytometry.

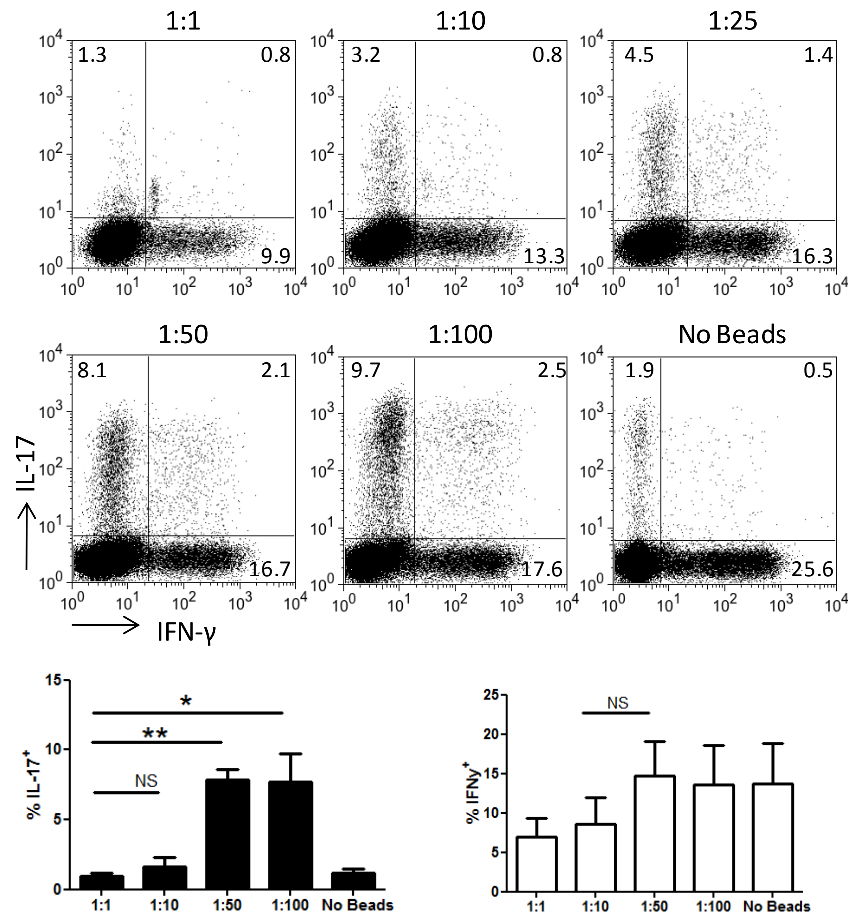


Figure 13. Reduced anti-CD3/anti-CD28 stimulation promotes Th17 cell responses. Human CD4+ T cells were activated with decreasing ratios of anti-CD3/anti-CD28 expander beads to CD4+ T cells: 1:1, 1:10, 1:25, 1:50, and 1:100. As a control T cells were cultured in the absence of anti-CD3/anti-CD28 expander beads (No Beads). After 6 days, T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Plots are representative of 3 independent experiments and the histograms below demonstrate the percentage of cells expressing IL-17 (black bars) or IFNγ (white bars) from the 3 independent experiments. Error bars represent SEM. P values calculated using a paired T-test; *p=<0.05 **p=<0.01.

The difference in stimulation strength was clearly visible by the difference in T-cell clustering and blasting, both events which mark T-cell activation. T-cells with a low strength stimulus took a longer time to form clusters and blasts than T-cells stimulated with a higher strength stimulus. Figure 13 demonstrates that as the stimulation strength decreased the proportion of IL-17$^+$ cells increased. An ~6-fold increase in IL-17 expression was observed when the bead:T-cell ratio decreases from 1:1 to 1:50. Below a 1:50 bead:T-cell ratio the effect of low strength stimulation reaches a plateau; no further marked increase was observed at a 1:100 bead:T-cell ratio. Interestingly, although the proportion of IFN-y$^+$ cells also increased as the stimulation strength decreased, the increase observed was less marked than that of the IL-17$^+$ population. Only a 1.7-fold increase in IFNγ$^+$ cells was observed when the bead:T-cell ratio decreased from 1:1 to 1:50. A similar effect is also observed in the IL-17/IFN-y dual positive cell population which increased by ~2.6 fold as stimulation strength decreased.

The experiment was repeated >20 times focussing on a high strength stimulus of a 1:1 (THi) bead:T-cell ratio compared to a low strength stimulus of a 1:50 (TLo) bead:T-cell ratio, where a striking effect of stimulation strength on IL-17 expression was observed. Figure 14 confirms data in Figure 13, demonstrating that Th17 cell responses are significantly increased by TLo stimulation when compared to THi stimulation. Furthermore, TLo stimulation also significantly increases the proportion of IFNy$^+$ and IL-17$^+$IFNy$^+$ dual positive cells compared to THi stimulation, but as observed in Figure 13 the increase within these latter populations is less marked than that of the IL-17$^+$ fraction. A high level of donor-to-donor variability occurs in T-cell cytokine responsiveness and these may be due to differences in the proportions of memory versus naive T-cells in donors. Further experiments compared stimulation strength via the THi vs TLo stimulation strength model: THi is defined as T-cell activation at a ratio of 1 bead:1 T-cell, TLo is defined as T-cell activation at a ratio of 1 bead: 50 T-cells both stimulations are performed in the presence of pro-Th17 cell cytokines unless otherwise stated.

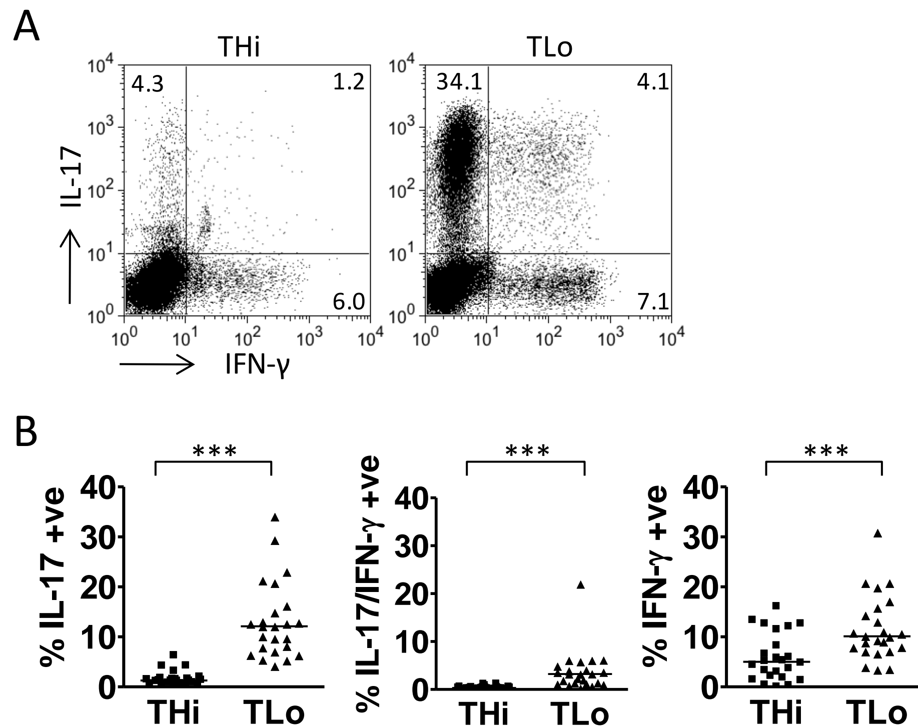


Figure 14. TLo stimulation promotes Th17 cell responses. Human CD4$^+$ T cells were activated with anti-CD3/anti-CD28 expander beads under THi (1:1 bead:cell ratio) or TLo (1:50 bead:cell ratio) conditions. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin. the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry. (A) Is a representative plot of 24 independent experiments, (B) percentages of single and double producers n=24; horizontal bars represent the median value; error bars represent SEM; p-values calculated using Wilcoxon test, ***$p<0.0001$. Effectivity of pairing was analysed using Spearman one tailed approximation; IL-17 $p=0.0067$, IL-17/IFNy $p=0.0192$, and IFNy $p=0.0048$, each graph demonstrates effective pairing.

Previous investigations in mice have revealed that Th2 cell responses are also favoured by low strength stimulation (403, 406). Therefore, to assess if TLo stimulation would also induce high proportions of the Th2 cell signature cytokine IL-4, CD4$^+$ T-cells following THi or TLo stimulation were examined for the expression of intracellular IL-4 at day 6. Figure 15 reveals that although TLo stimulation favours IL-4$^+$ cell responses compared to THi stimulation, only a low proportion of cells are IL-4$^+$, most likely due to the absence of pro-Th2 cell cytokines. It is interesting to note that whilst a moderate proportion of cells are IL-17$^+$/IFNy$^+$ dual positive, a IL-17$^+$/IL-4$^+$ dual positive population is absent, and may indicate reduced plasticity between the Th17 and Th2 cell phenotypes as compared to Th17 and Th1 cells.

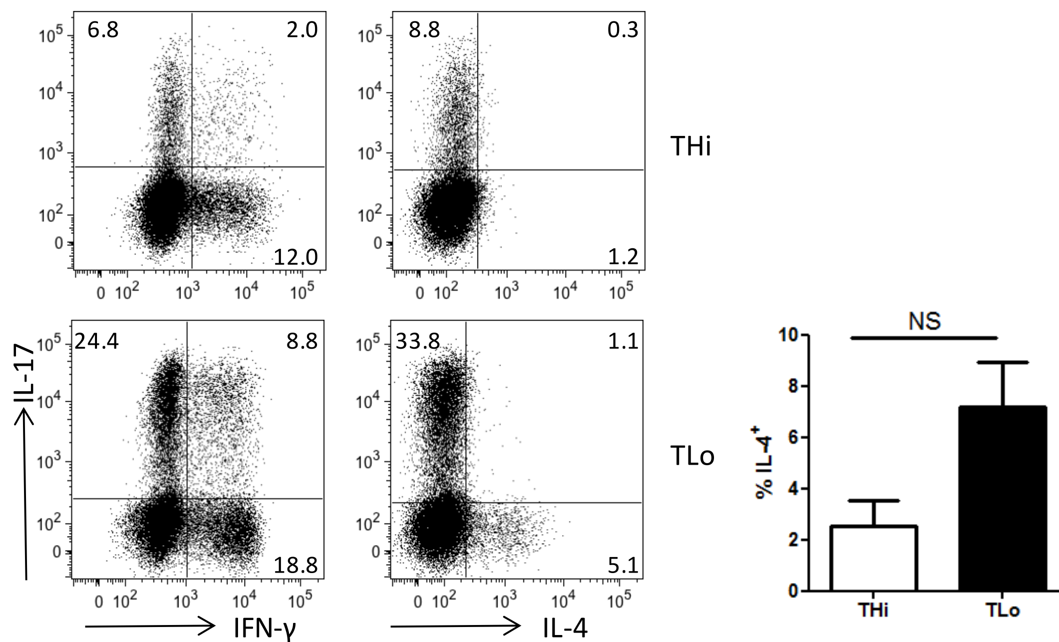


Figure 15. TLo stimulation does not promote large proportions of IL-4$^+$ cells. CD4$^+$ T-cells were stimulated under THi or TLo conditions. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin. the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Plots are representative of 3 independent experiments andhe histogram adjacent demonstrates the percentage of IL-4 expressing cells from the 3 independent experiments. Error bars represent SEM, P value calculated using paired T-test, NS= not signficant.

2.4.3 Cytokine secretion

Differences in cytokine secretion capabilities of THi and TLo cells were investigated by Enzyme Linked Immunosorbant Assay (ELISA). At day 6, THi and TLo cells were washed, counted and resuspended at $1x10^6$ cells/ml. Because TCR/CD3 activation may lead to a temporary downregulation of the surface TCR/CD3 complex (423), T cells were stimulated for 24 h with PMA and ionomycin. These reagents bypass the TCR but activate the same TCR signalling pathways (i.e. PKC and Ca^{2+}; (424)). Culture supernatants were assessed for IL-17 and IFNy by ELISA. Interestingly, although a large increase in IL-17 secretion is seen in TLo cell compared to THi cell supernatants, the concentration of IFNy is comparable in both THi and TLo cultures (Figure 16). The IFNy secretion differs from the intracellular IFNy staining data, however the IL-17 secretion and intracellular data match. TLo stimulation resulted in the secretion of greater concentrations of IL-17 than IFNy. Overall both ELISA and intracellular staining indicate that TLo stimulation results in a greater IL-17 response compared to THi stimulation, despite both conditions receiving a pro-Th17 cell cytokine *milieu*.

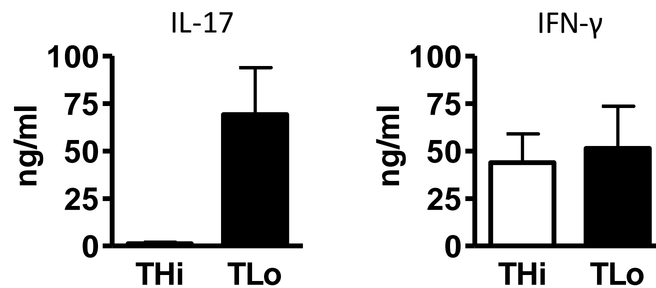


Figure 16. TLo stimulation promotes IL-17 secretion. Human $CD4^+$ T cells were stimulated under THi or TLo conditions. After 6 days T-cells were washed, replated at $1x10^6$ cells/well and restimulated with PMA/Ionomycin. After 24 h cell-free supernatants were harvested and levels of IL-17 and IFN-γ were measured by ELISA; error bars represent SEM, N=3.

2.4.4 Requirement for pro-Th17 cytokines

The signalling pathways activated via CD3/TCR and co-stimulatory molecule CD28 can determine the T-cell phenotype, along with the cytokine milieu. In order to assess if the addition of pro-Th17 cell cytokines, (IL-1β, TGF-β, and IL-23) affects Th17 cell responses, THi and TLo stimulated cells were cultured in the presence or absence of pro-Th17 cell cytokines and intracellular IL-17 and IFNy production was determined at day 6. In the absence of pro-Th17 cell cytokines the proportion of $IL\text{-}17^+$ cells in both THi and TLo cells remained low, but was marginally increased in TLo compared to THi cells (Figure 17). However, the absence of pro-Th17 cell cytokines substantially increases the proportion of $IFNy^+$ cells in TLo but not THi cultures. The pro-Th17 cell cytokines most likely act in part to prevent the expansion of the pre-existing Th1 cell population, but THi stimulation alone may further suppress IFNy expression by potentially inducing a regulatory rather than an effector cell phenotype (explored further in Chapter 3) (425). In the presence of pro-Th17 cell cytokines the proportion of $IL\text{-}17^+$ cells in TLo stimulated cell cultures was markedly increased compared to THi stimulated cell cultures. These data demonstrate that both the strength of stimulus and cytokine *milieu* contribute to the effector phenotype; furthermore, these data contradict prior suggestions that the cytokine *milieu* is able to negate the effects of stimulation strength (67).

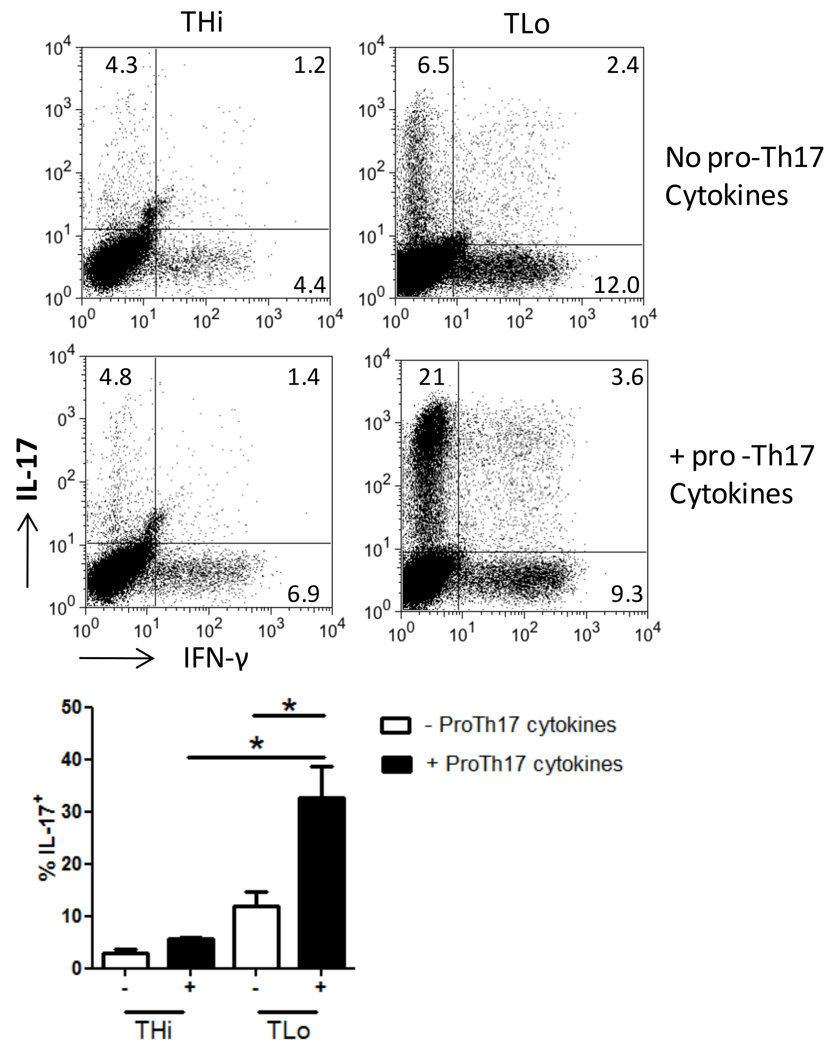


Figure 17. Promotion of Th17 responses by TLo stimulation requires pro-Th17 cell cytokines. Human CD4+ T cells were stimulated under THi or TLo conditions in the presence (top) or absence (bottom) of pro-Th17 cell cytokines IL-1β, IL-23 and TGF-β. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Plots are representative of 3 independent experiments and the histogram below demonstrates the percentage of IL-17 expressing cells from the 3 independent experiments. Error bars represent SEM. P values calculated using a paired T-test; *p=<0.05.

2.5 Further characterisation of THi & TLo cells

2.5.1 RORc

IL-17 is the predominant marker, and mediator of Th17 cell effects, however further factors are also used to characterise the Th17 cell phenotype. Transcription factor RORγt (RORc in humans) is required for transcription of *Il-17* and has been identified as the Th17 cell-defining transcription factor (371). THi and TLo cells were assessed by flow cytometry for the expression of RORc and also for intracellular cytokines, IL-17

and IFNγ. Figure 18 demonstrates that both THi and TLo cells express RORc and the majority of IL-17^{+} TLo cells were also positive for RORc. Interestingly a large proportion of cells present in both THi and TLo cultures are RORc^{+}/IL-17^{-}.

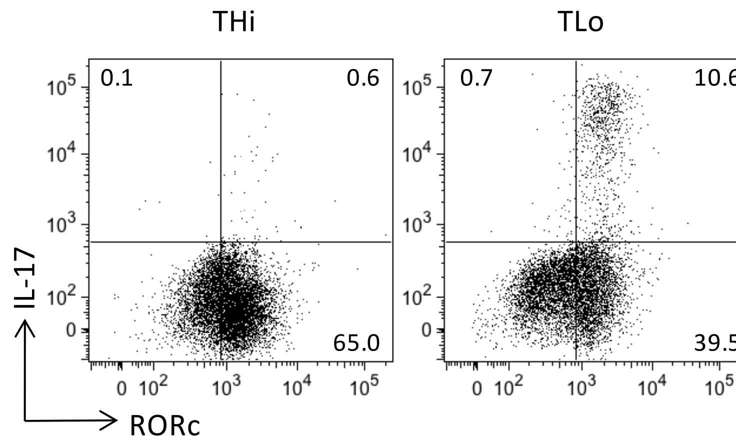


Figure 18. IL-17-producing T-cells express RORc. CD4^{+} T-cells were cultured under THi or TLo conditions. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and RORc was determined by intracellular staining followed by flow cytometry. Plots are representative of 4 independent experiments.

2.5.2 IL-21 & IL-22

Initially it was suggested that IL-21 and IL-22 were also expressed by Th17 cells (292). Recently a number of new T cell phenotypes have been described including IL-22^{+} IL-17^{-} cells, which have been termed Th22 cells (207, 214). Th22 cells are thought to be important in the pathogenesis of the autoimmune disease psoriasis (211). THi and TLo cells were investigated for their expression of IL-21 and IL-22 in conjunction with IL-17 by ICS and flow cytometry following PMA/Ionomycin stimulation at day 6 of culture. THi stimulation resulted in almost no detectable IL-21 or IL-22 expression as demonstrated in Figure 19. TLo cells had increased expression of both IL-21 and IL-22 when compared to THi cells. However, very little IL-21 production was observed in TLo stimulated cell cultures. This is a potentially surprising finding as Th17 cell secretion of IL-21 is a mechanism reported to result in Th17 cell phenotype maintenance. Autocrine IL-21 signalling initiates a positive feedback signal that maintains RORγt expression, via the activation of the STAT3 signalling pathway (426). The pro-Th17 cytokine cocktail added to THi/TLo stimulated cell cultures does not contain IL-6 which is required to initiate IL-21 expression. For human Th17 cell induction a cytokine cocktail containing IL-1β is used rather than IL-6 (332). Moreover IL-23 can activate the STAT3 signalling

pathway which may also negate the requirement for IL-21 (367). A large proportion of the cells were IL-22^{+}/IL-17^{+}, a finding similar to previous investigations (427). In addition, a small proportion of the cells were IL-22^{+}/IL-17^{-}, which may represent the more recently described Th22 cell subset. It has been proposed that the Aryl hydrocabon receptor (AhR) is the Th22 cell-specific transcription factor, although it is also associated with *in vivo* Th17 cells (208, 216). As TLo cells are cultured in IMDM medium which is known to contain AhR-activating ligands, IL-22^{+} cells may be induced or expanded through the AhR signalling and transcription pathway (217). All IL-17^{+} cells are not however IL-22^{+}, this may be due to the cytokine *milieu* employed. IL-23 in conjunction with IL-1β has been described to inhibit IL-22 expression and can regulate the Th17/Th22 cell balance in favour of Th17 cells (428). TLo induced Th17 cells express both IL-17 and the signature transcription factor RORc, however expression of IL-17 in conjunction with IL-21 or IL-22 is not always observed.

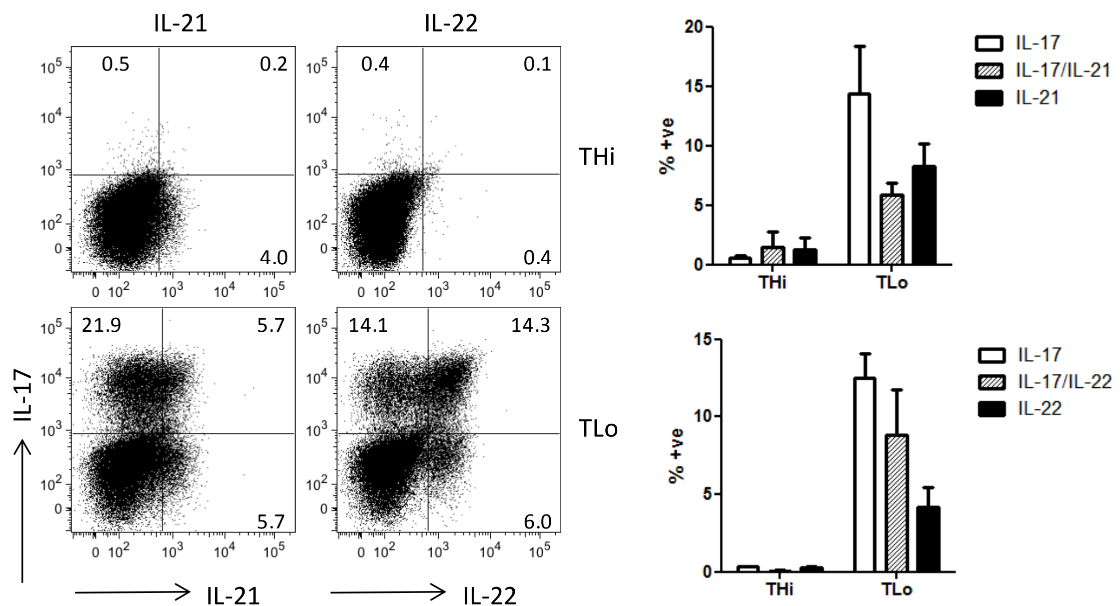


Figure 19. Expression of IL-21 and IL-22 by THi and TLo cells. CD4^{+} T-cells were stimulated under THi or TLo conditions. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- , IL-21- and IL-22-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Plots are representative of 3 independent experiments and the adjacent histograms demonstrate the percentage of IL-17 and/or IL-21 or IL-22 expressing cells from the 3 independent experiments. Error bars represent SEM

2.5.3 Proliferation & Viability

A consideration when assessing the culture of $CD4^+$ T cells activated with different stimulation strengths is that T-cell proliferation and viability will differ. The difference in the number of cell divisions can also affect the T-cell effector phenotype (429, 430).

2.5.4 Proliferation

Carboxyfluoroscein succinimidyl ester (CFSE) methodology was performed to assess the proliferative capability of THi and TLo stimulated cells. CFSE is a cell permeable fluorescent dye that upon cell proliferation is equally divided between daughter cells, therefore, when visualised by flow cytometry the T-cells that have divided have reduced fluorescent intensity. THi and TLo stimulated cells were stained on day 0 with CFSE and cultured until day 6 under pro-Th17 cell cytokine conditions. The dilution of CFSE and also the expression of IL-17 and IFNy were then assessed by ICS and flow cytometry. A control population of non-TCR-stimulated cells were also CFSE-labeled; these cells will contain undiluted CFSE as they have not been activated and therefore allow for gating of non-proliferative cells. The majority of THi and TLo stimulated cells were $CFSE^{dim}$ indicating that these cells had proliferated. However, a higher proportion of cells in the TLo cultures had not undergone division when compared to the THi stimulated cells (33% ± 3.7% for TLo vs 14% ± 3.4% for THi n=3) (Figure 20). The $IL\text{-}17^+$ cells (and $IFNy^+$ cells) largely resided within the $CFSE^{dim}$ fraction of the population, indicating that effector cytokine production is associated with cell division and is not induced from the non-proliferating cells by PMA/Ionomycin stimulation (Figure 20 A+B). Further computational analysis of CFSE dilution revealed four rounds of cell division (G1-G4) in THi and TLo cell cultures. Slightly greater proportions of THi stimulated cells were present within divisions G1-2 as compared to the G1-2 divisions of TLo stimulated cells (Figure 20C, D). However, both THi and TLo stimulated cells have similar proportions of T-cells in the later G3 and G4 phase of cell divisions. The greater proportion of THi stimulated cells present in the G1/2 phase of divisions may indicate that the proliferation of some THi stimulated cells has been halted at an earlier stage than in TLo stimulated cells. A further possibility is that these cells may have been activated at a later time point within the culture and have not reached the later G3-G4 division stages. The highest proportions of $IL\text{-}17^+$ cells were present within

the G4 stage of division. Despite comparable proportions of T-cells in THi and TLo stimulated cell cultures having reached this stage, only TLo cells have IL-17 expression, suggesting that low IL-17 expression in THi is not because they have not proliferated to the G3/G4 stage.

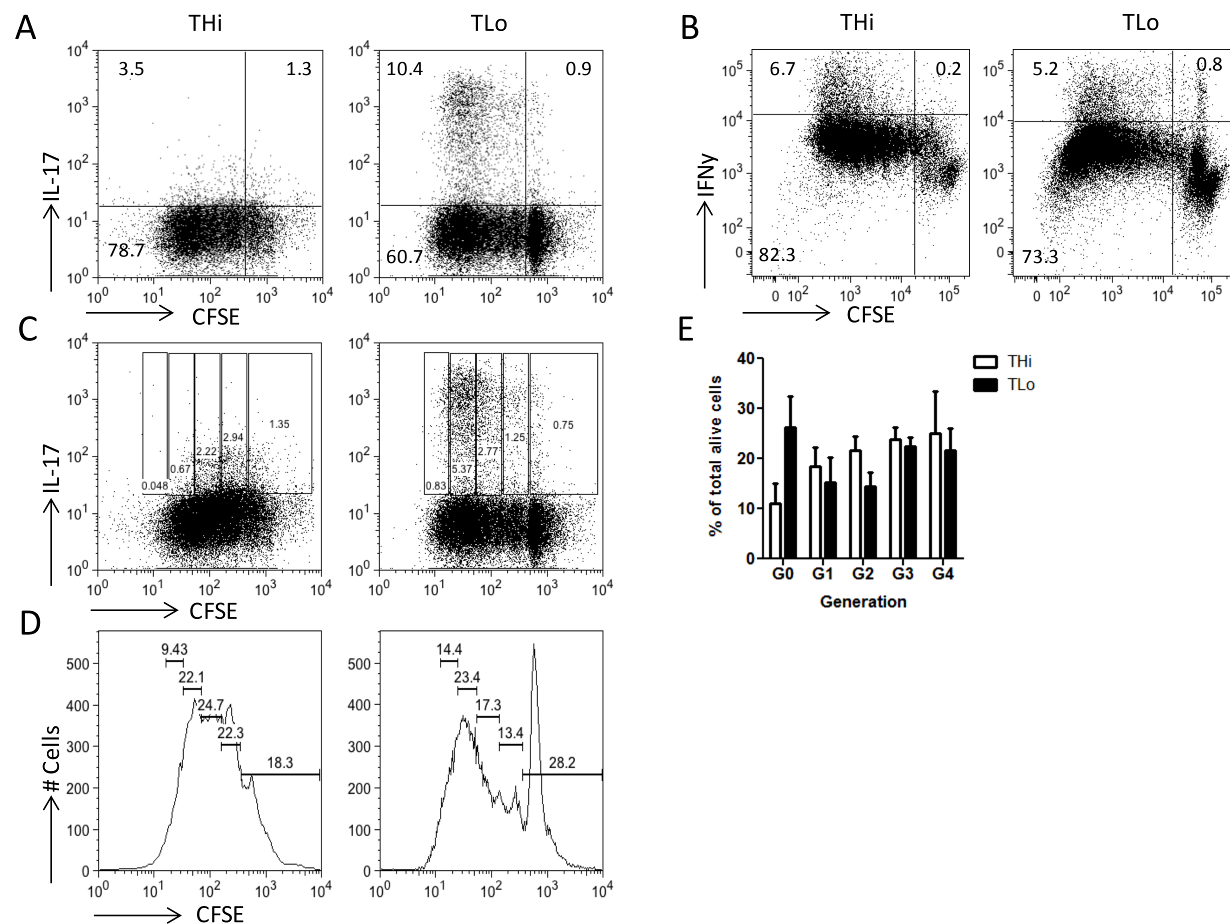


Figure 20. The proliferative response of T-cells stimulated under THi or TLo conditions. Human CD4+ T-cells were labelled with CFSE and cultured under THi or TLo conditions. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 (A) or IFNγ (B) in proliferating cells was assessed by flow cytometry. Plots are representative of 3 independent experiments (C) Cell generation gates were determined by FlowJo Proliferation Platform, and are representative of 3 independent experiments. (D) Percentages of cells in each cell generation, gates determined as in A and C; data representative of 3 independent experiments. (E) The percentage of cells present in each generation gate from the total cell population; N=3 independent experiments.

2.5.5 Absolute numbers of Th17 cells

I next explored the possibility that as a greater proportion of THi cells had proliferated compared to TLo cells, enrichment of Th17-cells within the TLo population may be proportional rather than absolute. To investigate this, on day 6 of THi/TLo stimulated culture, the total numbers of living cells were counted and used to calculate the absolute number of Th17 cells resulting from the THi and TLo cultures. Figure 21 reveals that TLo cultures resulted in significantly greater absolute numbers of IL-17^{+} cells in comparison to THi cultures. Thus, TLo stimulation results in both proportionally and absolutely more Th17 cells than THi stimulation.

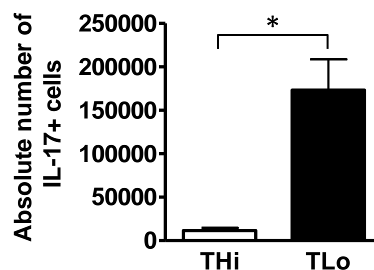


Figure 21. The increase in IL-17^{+} cells in TLo stimulated cultures is absolute. Human CD4^{+} T-cells were cultured for 6 days under THi or TLo stimulation conditions. The number of living T-cells at day 6 was determined by trypan blue viability staining. The absolute number of IL-17^{+} T-cells was calculated using the proportion of IL-17^{+} cells determined by flow cytometry; N=3. Error bars represent SEM; p-values calculated using a T-test *p<0.05.

2.5.6 Viability

Activation induced cell death (AICD) is an essential mechanism in the maintenance of T-cell homeostasis that activates the programmed cell death pathway (431). AICD is usually mediated by expression and binding of death ligand FasL to Fas receptor present on T-cells. Cell death may occur in a cell-autonomous manner termed 'suicide'

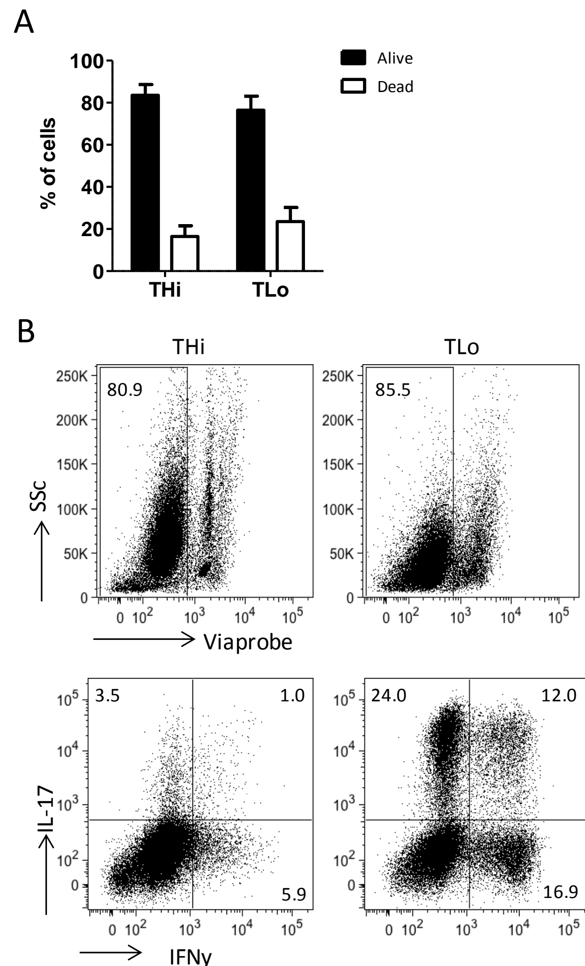


Figure 22. THi and TLo stimulated T-cells have comparable viability. Human CD4$^+$ T-cells were cultured under THi or TLo conditions for 6 days. (A) 1 well of THi- or TLo-stimulated T-cells was harvested and the percentage of dead and alive cells was determined by trypan blue staining. N=3, error bars represent SEM. (B) At day 6 following 5 hours restimulation with PMA/Ionomycin, cells were stained with Viaprobe and expression of intracellular IL-17 and IFNy was determined by intracellular cytokine staining followed by flow cytometry. The top row represents the viability of ungated cells acquired. The bottom row depict representative plots of IL-17 and IFNy expression from Viaprobe-negative cells. Plots are representative of 3 independent experiments.

or as the result of cell-cell contract termed 'fratricide' (432). AICD can be induced *in vitro* by activating T-cells via TCR in the presence of IL-2, followed by activating the cells via TCR ligation alone (433, 434). The resulting effect is the expansion of the T-cell population and then a decrease in T-cell number. To assess if differences in cell death between THi and TLo cultures was a possible cause for phenotypic differences, cell viability was assessed. Viability of cells in THi and TLo cultures was primarily assessed by viability staining of cells with trypan blue on day 6, and counting alive and dead cell populations. Comparable percentages of viable and non-viable cells between THi and TLo cultures were found (Figure 22A). Further phenotypic assessment was carried out

using a flow cytometric viability stain, Viaprobe. Within both the THi and TLo cell cultures assessed at day 6, ~15-20% of cells were dead (Viaprobe^{+}), similarly to results observed by trypan blue staining (Figure 22A, B (top row)). Furthermore, when viable cells were gated (Viaprobe^{-}), co-staining with IL-17 revealed that TLo stimulation still resulted in a greater proportion of IL-17^{+} cells than THi stimulation (Figure 22 B bottom row). The low proportion of IL-17^{+} cells in THi cultures is therefore unlikely to be a result of a dilution effect caused by the greater numbers of dead cells.

2.5.7 Exhaustion

I next hypothesised that THi stimulation may result in an exhausted cell state, preventing THi cell responsiveness to PMA/Ionomycin. T-cell exhaustion results in a dysregulated effector function, and is commonly associated with chronic infection and cancer. Unlike T-cell anergy, exhaustion arises in the presence of co-stimulation, and onset occurs gradually over time (435). To assess for exhaustion THi and TLo cells were assessed for their IL-17/IFNγ response following PMA/Ionomycin stimulation at day 6 or after resting T cells for an additional 4 or 8 days (i.e. day 10 or 14 of culture). Resting the cells until day 10 further increased proportions of both IL-17^{+} and IFNγ^{+} cells in TLo cell cultures (Figure 23). Conversely, within THi stimulated cell cultures a marginal increase in the proportion of IFNγ^{+} cells occured following resting, but no such increase was associated within the IL-17^{+} fraction. Resting of THi cells, if they are in an exhausted state, did not enhance IL-17 responses; however, TLo stimulated Th17 cell responses were further benefitted by a resting period of 4 days.

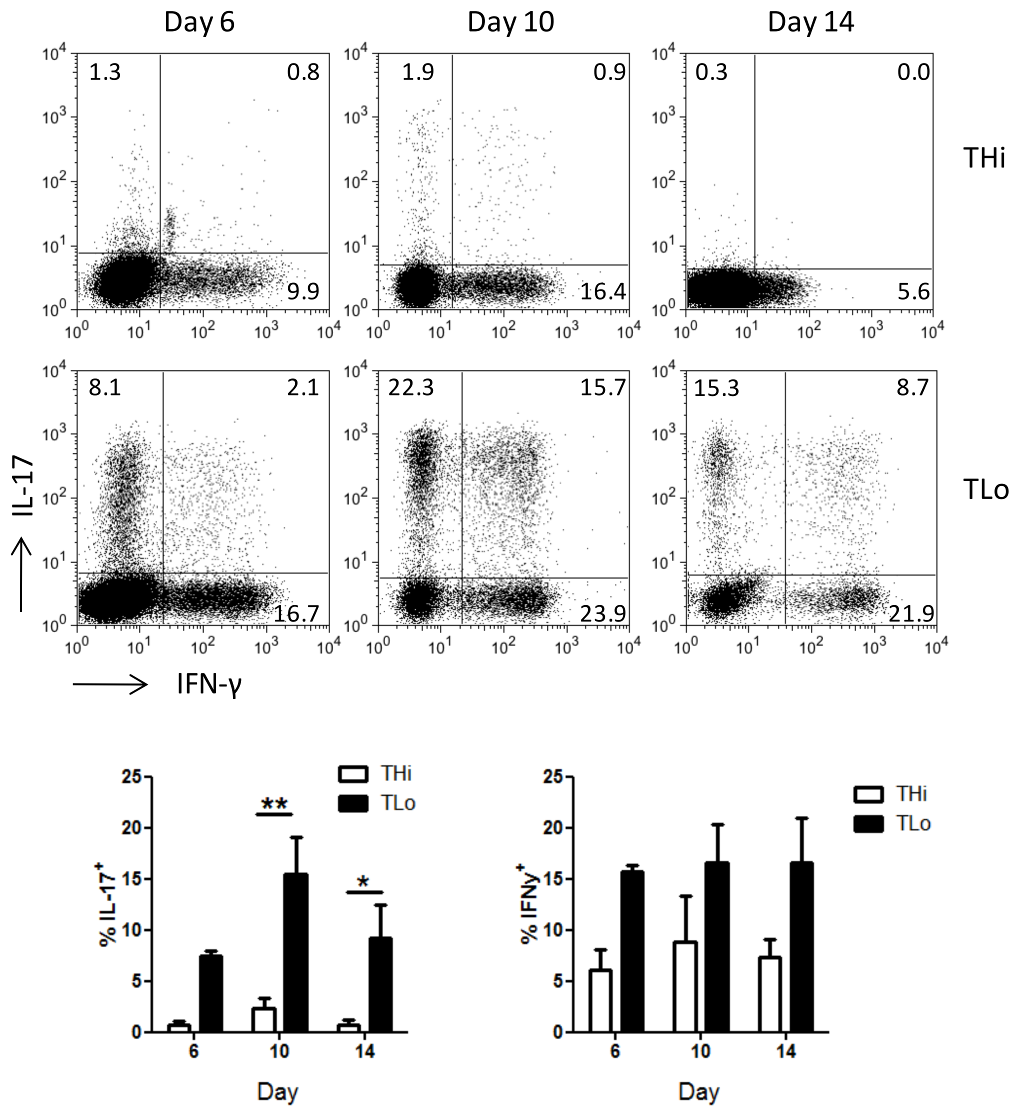


Figure 23. Resting of THi cells does not increase the proportion of IL-17$^+$ cells. Human CD4$^+$ T-cells were cultured under THi or TLo conditions. At day 6 the T-cells were either restimulated for flow cytometry analysis (left hand column) or anti-CD3/CD28 beads were removed and the CD4$^+$ T-cells were replated in the presence of IL-2 and IL-23 until day 10 (middle column) or until day 14 (right column). At each time point the T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A, and assessed for intracellular IL-17 and IFNγ by flow cytometry. Plots are representative of 3 independent experiments and the histograms below demonstrate the percentage of cells expressing IL-17 (left) or IFNγ (right) from the 3 independent experiments. Error bars represent SEM; ; p-values calculated using a 2 way ANOVA *p<0.05 **p<0.01.

2.5.8 Th17 responses of naïve versus memory CD4$^+$ T-cells

Within the periphery CD4$^+$ T-cells exist in different states of maturation, depending on antigen experience. Antigen-inexperienced naive T-cells were originally thought to differentiate into terminally differentiated effector T-cell lineages. However, recent investigations clearly demonstrate that T helper cells retain the capability to switch

phenotype following antigen encounter. Furthermore, induction of a Th17 cell phenotype from naive human CD4$^+$ T-cells has still not been convincingly achieved, potentially due in part to employing the incorrect cytokine *milieu* or stimulation strength. The response of naive and memory T-cells to THi and TLo stimulation was next compared (Figure 24). As with total CD4$^+$ T-cells both naive and memory Th17 cell responses were favoured by TLo stimulation. However, memory CD4$^+$ T-cells cultured with TLo stimulation resulted in a far greater IL-17 response than naive CD4+ T-cell cultures. As with total CD4$^+$ T-cells, TLo stimulation of memory CD4$^+$ T-cells compared to THi stimulation, resulted in a less marked increase in the proportion of IFNγ$^+$ cells than IL-17$^+$ T-cells. Conversely, in the naive culture the proportion of IFNγ$^+$ expression was reduced following TLo stimulation compared to THi stimulation, indicating that TLo culture does not favour naive IFNγ responses. However, the effect of pro-Th1 cytokines in concert with TLo stimulation remains to be determined. Due to imperfections in magnetic separation techniques, it cannot be excluded that the IL-17$^+$ cells induced from TLo stimulated naive T-cells were a result of contaminating memory T-cells rather than true Th17 cell differentiation, as proportions of IL-17$^+$ cells are low.

2.5.9 Duration of TCR stimulation affects Th17 cell responses

Prior investigations have demonstrated that TCR signal strength can be modulated by the duration of TCR stimulation. I therefore assessed if Th17 cell responses could be modulated by the duration of TCR stimulation. Using a THi stimulation (1 bead: 1 T-cell), memory CD4$^+$ T-cells were activated for 24, 48 or 72 hours with anti-CD3/CD28 beads under pro-Th17 cell cytokine conditions. At each time point the anti-CD3/anti-CD28 beads were removed and T-cells washed and re-cultured under pro-Th17 cell conditions until day 6 (control conditions underwent the same procedure except activating beads remained in culture). Data in Figure 25 reveal that if TCR activating beads were removed prior to 72 hours, Th17 cell responses increased when compared to the time point control. However by 72 hours of activation, if the stimulus is removed no increase in IL-17 response is observed in comparison to the continued presence of the beads for the 6 days. These data indicate that high strength stimulation for greater than 48 h is inhibitory to Th17 cell responses. TCR stimulation for 48 hours appeared to be optimal for Th17 cell responses though as short as 24

hours also yielded enhanced Th17 cell responses. These data may indicate how Th17 cell responses correspond with the signalling duration requirements of Th1 and Th2 cells (409). Iezzi *et al* found that optimal murine Th2 cell responses (>60% IL-4^{+}) were induced by a 96 hour stimulation with anti-CD3/anti-CD28 96 hour stimulation. Whereas, murine Th1 cell responses (>70% IFNy^{+}) were induced by a 24 hour stimulation, therefore the duration of CD3/CD28 stimulation required by Th17 cells may fall in between that required by Th1 and Th2 cells. Although, the duration of

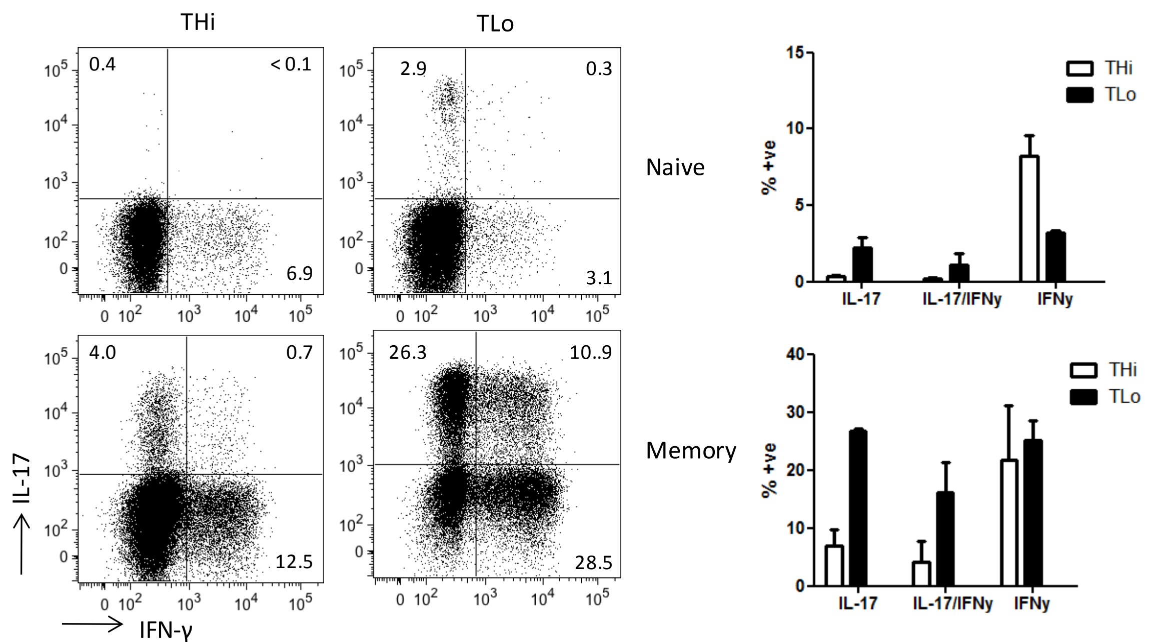


Figure 24. Naive and memory T-cell responses to THi and TLo stimulation . Human CD45RA+CD4+ naive or CD45RO+CD4+ memory T-cells were cultured under THi or TLo conditions. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-?-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Plots are representative of 2 independent experiments and the histograms adjacent (Top- Naive) (Bottom- Memory) demonstrate the percentage of cells expressing IL-17 and/or IFNy from the 2 independent experiments. Error bars represent SEM.

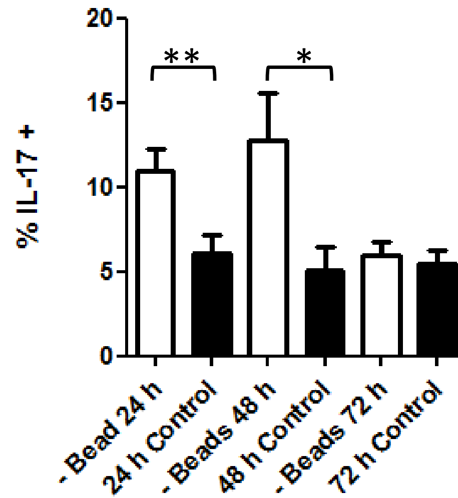


Figure 25. A shorter duration of TCR stimulation promotes Th17 cell responses. Human $CD45RO^{+}CD4^{+}$ memory T-cells were cultured under THi conditions. After either 24, 48 or 72 hours of stimulation, anti-CD3/anti-CD28 beads were removed and the T-cells were washed, replated and cultured until day 6. Control populations at each time point were washed and cultured but remained in the presence of anti-CD3/CD28 beads. At day 6, T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A and the expression of intracellular IL-17 was assessed by flow cytometry. Data are of 3 independent experiments. Error bars represent SEM. P values were calculated using a Paired T-test; *< 0.05, **<0.01.

stimulation required by naive and memory T-cells also differs, naive T-cells require prolonged TCR activation compared to memory (392).

2.5.10 Modulation of APC derived stimulation strength

Anti-CD3/anti-CD28 beads, are a useful tool for in vitro T-cell activation, but provide a less physiologically relevant stimulus than APC. Antibody engagement of TCR occurs at higher orders of magnitude than those of pMHC (436). To assess the effect of stimulation strength on Th17 cell responses by modulation of APC derived stimulation, total CD4+ T-cells were cultured with different numbers of allogeneic DC that had been matured with peptidoglycan. Peptidoglycan is a TLR2 agonist that activates signalling pathways which lead to the secretion of pro-Th17 cytokines including IL-1β and IL-23 (331, 437). A high strength stimulus of 1 DC:1 T-cell (DCHi) was compared to a low strength stimulus of 1 DC:10 T-cells (DCLo). IL-17 and IFNy responses were compared at day 6 of culture. Interestingly, low strength DC stimulation increased the proportion of IL-17+ cells in comparison with high strength DC stimulation (Figure 26).

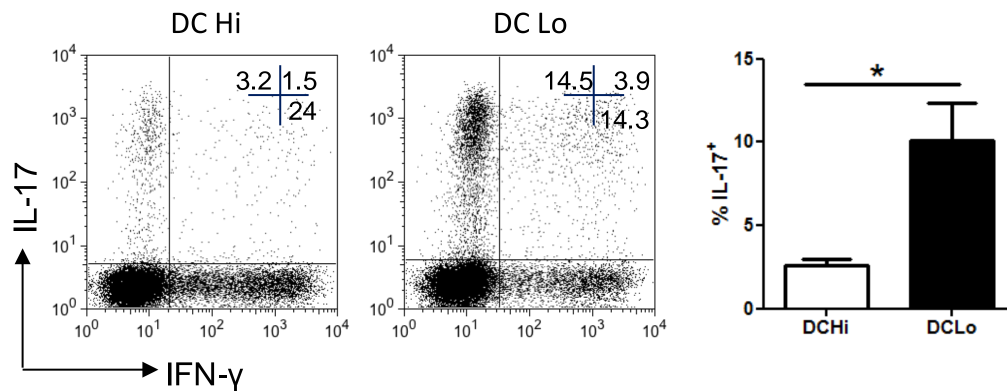


Figure 26. Modulating the strength of T-cell stimulation with moDC. Human CD4+ T-cells were cultured for 6 days with allogeneic PGN-activated moDC at either a 1:1 moDC:CD4$^+$ T-cell ratio (DC Hi) or a 1:10 moDC: CD4$^+$ T-cell ratio (DC Lo). Day 6 cells were restimulated for 5 hours with PMA/Ionomycin and expression of IL-17 and IFN-γ were determined by intracellular flow cytometry. Plots are representative of 3 independent experiments and the histograms adjacent demonstrate the percentage of cells expressing IL-17 from the DCHi (white bars) and DCLo (black bars) of the 3 independent experiments. Error bars represent SEM. P values calculated using an unpaired T-test; *p=<0.05.

However, whilst indicating that low strength stimulation favours Th17 cell induction, modulating the number of allogenic DC also affects a number of parameters other than TCR/co-timulation including: the concentration of DC released cytokines, surface molecule interactions, and the overall cell culture density. To minimise these differences and to specifically assess the differences in TCR activation a different model using APC and super antigen was employed. Superantigens are toxins released by bacteria such as *Staphylococcus aureus* which are capable of non-antigen-specific T-cell activation, by cross-linking the Vβ region of the TCR to MHC molecules expressed on APC (438). Co-culture of immature DC with autologous CD4$^+$ T-cells at a 1:10 ratio, in the presence of superantigen (staphylococcus enterotoxin B (SEB)), revealed that decreased concentrations of SEB and thus TCR stimulation favours IL-17 responses (Figure 27). In addition, high concentrations of SEB appeared inhibitory to IL-17 responses, with proportions of IL-17$^+$ cells reduced in comparison to background IL-17$^+$. The highest proportion of IFNy$^+$ cells were induced from an SEB concentration 10-fold higher than the concentration required for optimal proportions of IL-17$^+$ cells. In addition when compared to the IL-17 response, the IFNy response did not appear to be as sensitive to inhibition by high strength stimulation.

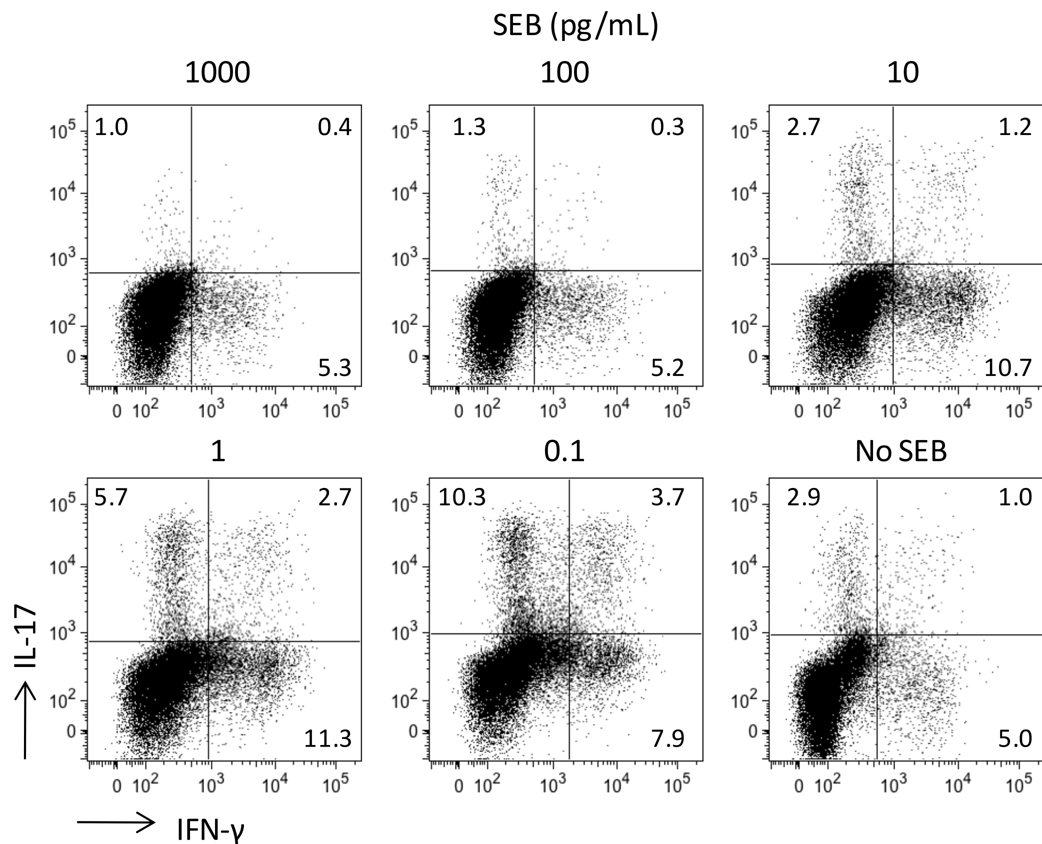


Figure 27. Low strength activation by autologous moDC and SEB favours Th17 generation. Human CD4+ T-cell were cultured for 6 days with autologous moDC at a 1moDC:10CD4+ T-cell ratio with decreasing concentrations of SEB (1000-0 pg/ml). Day 6 cells were restimulated for 5 hours with PMA/Ionomycin and expression of IL-17 and IFN-γ were determined by intracellular flow cytometry. Results are representative of 3 independent experiments.

2.5.11 Modulating stimulation strength via either anti-CD3 or anti-CD28

Anti-CD3/anti-CD28 bead stimulation did not reveal if low strength signals via the TCR or CD28 determined Th17 cell responses. Previous data in murine models indicates CD28 co-stimulation inhibits Th17 cell responses (421). My SEB titration data, however, indicated that low strength stimulation via the TCR may promote IL-17 expression. I therefore next addressed if lowering the stimulation strength via TCR/CD3 alone was sufficient for Th17 cell responses. To this aim, biotinylated beads were coated with different concentrations of anti-CD3 in the presence or absence of anti-CD28. T-cells were cultured at a constant ratio of 1 bead: 2 $CD4^+$ T-cells (manufacturer's recommend ratio) whilst providing different signal strengths via the different anti-CD3/CD28 concentrations and combinations. Figure 28 indicates that only a marginal change to the proportion of $IFNy^+$ cells was observed and these were not correlated to the

change in CD3 or CD28 stimulation; this may be due to the presence of pro-Th17 cell cytokines rather than pro-Th1 cell cytokines. However IL-17 responses were similar to those observed during SEB titration (Figure 27) whereby lower concentrations of anti-CD3 mAb and thus TCR stimulation favoured Th17 cell responses. Importantly, these effects were only observed in the presence of CD28 co-stimulation; the absence of anti-CD28 abrogated a Th17 cell response but not Th1 cell response (Figure 28). Together these data provide novel insights into the regulation of human Th17 cell responses demonstrating that, alongside pro-Th17 cytokines, low strength TCR stimulation in the presence of CD28 co-stimulation promotes human Th17 cells.

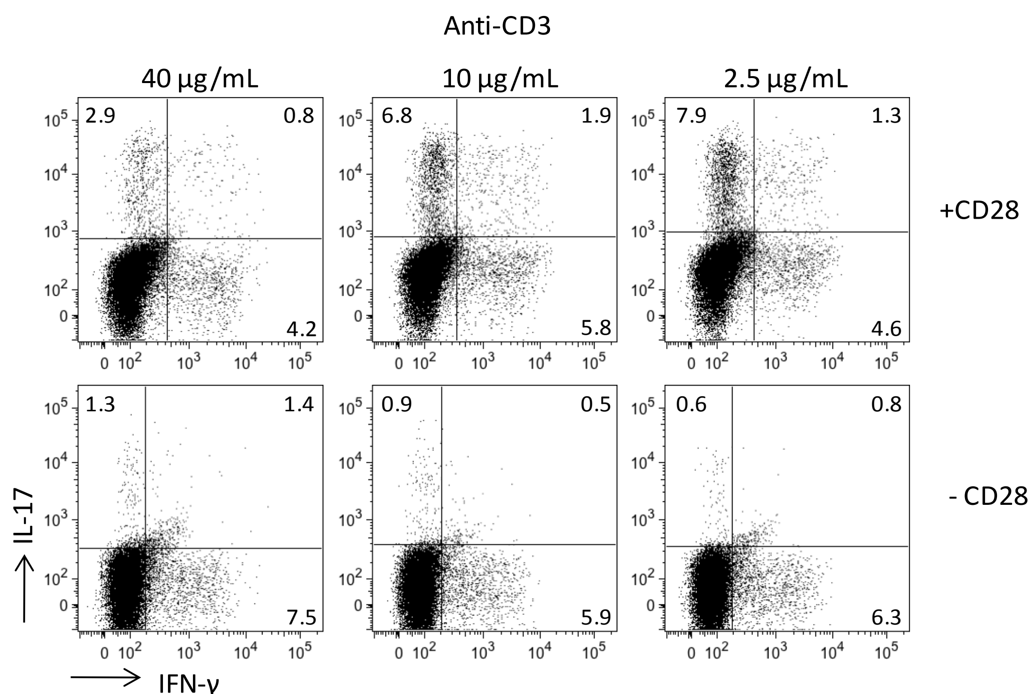


Figure 28. Low strength CD3/TCR stimulation promotes Th17 cell responses and is dependent on CD28 stimulation. Human CD4+ T-cells were cultured for 6 days at a 1:10 bead:T-cell ratio. Beads were loaded with decreasing concentrations of (40-2.5µg/ml) of anti-CD3 +/- 10µg/ml CD28. At day 6 T-cells were restimulated for 5 hours with PMA/Ionomycin and expression of IL-17 and IFN-γ was determined by intracellular flow cytometry. Results are representative of 3 independent experiments.

2.6 Discussion

Th17 cells are required for protection against certain pathogens but also play a role in the pathogenesis of autoimmunity (290). Since the discovery of Th17 cells investigations have focussed on the factors which control their induction. Although a clear role has been demonstrated for cytokine signalling in regulating Th17 cell induction, my data have revealed a novel role for TCR stimulation strength in regulating human Th17 cell responses. Low strength stimulation via anti-CD3/anti-CD28 beads or APC in a pro-Th17 cell cytokine *milieu* promotes Th17 cell responses *in vitro,* whereas high strength stimulation poorly supported IL-17 responses. Furthermore, CD28 stimulation was found to be required for optimal human Th17 cell responses. It was previously suggested that the strength of TCR stimulation was unlikely to be a determinant of T-cell phenotype as polarising cytokines are capable of overruling the effects of stimulation strength (67). Contrary to this, my data have revealed that reduced Th17 cell responses occur as a result of high strength stimulation but are not reversed by the presence of pro-Th17 cell cytokines. Although it remains a possibility that a different cytokine *milieu* may allow THi stimulated cells to make a Th17 cell response; my data suggest that the cytokine *milieu* in conjunction with the appropriate strength of TCR signalling promote Th17 cell responses.

2.6.1 Stimulation strength and Th17 cells

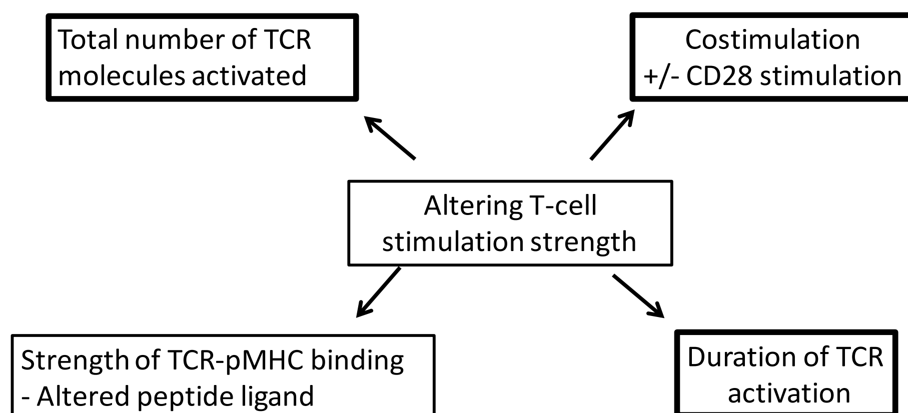


Figure 29. Methods of altering T-cell stimulation strength. Boxes with bold outline indicate methods of altering T-cell stimulation strength performed within this study.

The overall strength of T-cell stimulation may be determined by a number of factors including the rate and duration of TCR triggering, the affinity of TCR for pMHC, the number of TCR activated, and the signal threshold which can be modulated via CD28 signalling (Figure 29) (388). Our investigations vary the strength of stimulation by modulating the number of expander beads/DC, by varying the amount of anti-CD3 or superantigen present per bead or DC, respectively, or by modulating the duration of T cell stimulation (**Bold** out-lined boxes in Figure 29). Data of each model consistently demonstrated that Th17 cell responses were promoted by low strength T cell activation; the anti-CD3/superantigen titration data suggest that this effect is mediated through varying the signalling strength through the TCR/CD3 complex. Furthermore, my data demonstrated a requirement for CD28 co-stimulation for Th17 cell responses. More investigations are required to assess if different anti-CD28 concentrations in the presence or absence of anti-CD3 have more discrete effects on modulating Th17 cell responses. Some investigations into the effect of TCR stimulation strength on murine Th17 cell responses have already been conducted. Bouguermouh *et al* found that high concentrations of anti-CD3 alone induced optimal Th17 cell responses in mouse T-cells but that CD28 co-stimulation in the presence of anti-CD3 had an inhibitory effect. Furthermore, using LPS-matured DC, Bouguermouh *et al* also found that Th17 cell responses were increased at higher DC:T cell ratios and were further increased when stimulated with immature DC. This further increase in the presence of immature DC was thought to be due to the inhibitory effect of increased CD80/86 expression on mature DC, leading to activation of the CD28 signalling pathway. These investigations contradict my data; however, Bouguermouh *et al* also demonstrated differences between the naive and memory $CD4^+$ T-cell response towards CD28 co-stimulation. I believe that the majority of the Th17 cell responses resulting from TLo culture are derived from memory T-cells because Th17 cell proportions are diminished in naive cultures, but heightened in memory cultures (Figure 24). Bouguermouh *et al* found that memory T-cells were not affected by the presence of anti-CD28 stimulation, which due to the importance of memory T-cells to TLo Th17 responses, most likely contributes to the differences between our results. Like Bouguermouh *et al,* Gomez Rodriguez *et al* also demonstrated that IL-17A expression required a high dose of anti-CD3 stimulation. Differences in anti-CD3 dosing

ranges most likely explain this discrepancy; our low dose (2.5 ug/mL) is more comparable with their high dose (5 ug/mL). However, the strength of TCR signal induced by soluble vs plate bound anti-CD3 can differ and so may not be directly comparable (439). Plate bound anti-CD3 slowly induced a high level of Erk signalling which was sustained, whereas soluble anti-CD3 rapidly induced Erk signalling but signalling declined after 30 minutes. My data also reveal that using low concentrations of SEB (0.01 pg/mL) (Figure 27) resulted in increased Th17 cell responses, whereas comparisons to more physiological stimuli have not been drawn by Gomez-Rodriguez *et al*. Furthermore, data are conflicting within murine investigations regarding the role of CD28 co-stimulation on Th17 cell responses. Gomez-Rodriguez *et al* have demonstrated optimal Th17 cell responses in the presence of CD28 and higher concentrations of CD3, where as Bouguermouh *et al* found CD28 co-stimulation inhibitory to Th17 cell development (421, 422). More recently, raftlin, a protein present within the lipid raft capable of TCR signal modulation, was demonstrated to affect Th17 cell responses (422, 440). Raftlin modulates the amount of Lck in resting T-cells. *Raftlin*$^{-/-}$ T-cells have reduced resting Lck and a reduced intensity of TCR signalling. *Raftlin*$^{-/-}$ mice produced less IL-17, and data in wild-type mice also suggested that high strength anti-CD3 stimulation (10 ug/mL) promoted IL-17 responses (440). These differences may be attributed to the type and concentration of CD3/CD28 antibody and/or differences in the cytokine *milieu*. Our data, like that of another recent human Th17 cell study by Evans *et al*, demonstrate a clear requirement for CD28 co-stimulation for Th17 cell responses (99).

My experiments have assessed the avidity of TCR signalling rather than the affinity of TCR: pMHC interactions. Assessing the effect of TCR binding affinity on Th17 cell responses would be an interesting future research avenue and could be explored using altered peptide ligands (APL). *Ex vivo* human T-cells express a vast array of TCR with different pMHC specificities and so APL experiments could not be performed with *ex vivo* polyclonal human T-cells. The effect of TCR:pMHC affinity on Th17 cell responses could be explored in TCR transgenic mice specific for a certain peptide, by comparing the wild-type peptide to an APL with either enhanced or decreased affinity for the TCR.

It would also be beneficial to assess a wider array of effector phenotypes when assessing the stimulation strength requirements of certain subsets. Th1 and Th2 cells have already been demonstrated to be regulated by stimulation strength (400). More recent data demonstrate that other $CD4^+$ T-cell phenotypes are regulated by stimulation strength. Low doses of a strong agonist APL were found to promote $Foxp3^+$ Treg (425). Follicular helper T-cells may also be regulated by strength of TCR binding; high TCR signal strength induces IL-21 expression, promoting follicular helper T-cells (441). A high strength TCR signal requirement for IL-21 expression may be an alternate cause of reduced IL-21 expression from TLo stimulated cells; however, THi stimulation also did not result in IL-21 expression (Figure 19). As with prior investigations into the effects of stimulation strength on Th1 and Th2 cell responses, a lack of consistency in the dosing ranges and the type of peptide used can lead to different conclusions. Therefore if more phenotypes were assessed using the same dosing ranges our understanding of how TCR signalling regulates effector $CD4^+$ T-cell phenotypes would be substantially improved.

Different Th17 cell markers were assessed to characterise THi and TLo stimulation induced Th17 cells, the most important marker being IL-17A (IL-17). IL-17A allows Th17 cells to recruit neutrophils and drives the inflammatory responses to clear certain infections. As a result of TLo stimulation, IL-17A responses were increased both at the level of number of IL-17-producing cells and the total level of secreted IL-17 protein (Figure 16). Gomez-Rodriguez *et al* report that IL-17A and IL-17F are differentially regulated by TCR stimulation: IL-17A is more sensitive to Ca^{2+} signalling than IL-17F (422). This is because the *IL-17A* promoter contains a cross-species-specific binding site for the Ca^{2+} regulated transcription factor NFATc1, important for *IL-17A* expression. Within my investigation only IL-17A was assessed. However IL-17A has increased potency compared to IL-17F and as both types are capable of binding to the IL-17 receptor, the high levels of IL-17A secretion by TLo-induced Th17 cells would indicate that these cells are capable of mediating Th17 cell responses (276). However, it may be interesting to assess the expression of IL-17F in THi and TLo stimulated cells in future studies. IL-17A can suppress the expression of IL-17F expression (442). $IL\text{-}17A^{-/-}$ mice

over-expressed IL-17F, which resulted in increased GM-CSF secretion and neutrophil numbers, indicating that IL-17F is capable of functionally compensating for the absent IL-17A. As IL-17F expression is not regulated by TCR stimulation and THi stimulated cells have low IL-17A expression, IL-17F may still be expressed and allow THi stimulated cells to function as Th17 cells.

2.6.2 RORc in THi and TLo

RORγt/RORc is a transcription factor critical to IL-17 responses (371). Both THi and TLo cell populations contained a similar proportion of RORc$^+$ cells, though a large proportion of RORc$^+$/IL-17$^-$ cells existed within both populations, particularly within the THi cell population (Figure 18). *IL-17* transcription is regulated by a complex range of factors and RORc expression alone is most likely not sufficient for IL-17 expression. Possibly the presence of the RORc$^+$/IL-17$^-$ population is explained by the absence of further transcription factors, such as STAT3, which together with RORc mediate *IL-17* transcription (443). Conversely, inhibitory transcription factors, such as Foxp3 may be present preventing the RORc expressed from transcribing *IL-17* (374). It is known that in human T-cells TCR activation results in Foxp3 expression (444), and the involvement of Foxp3 in the THi/TLo stimulation model is further investigated in Chapter 3. The IL-17$^+$ cell population is RORc$^+$ confirming its importance in *IL-17* expression.

2.6.3 Differentiation vs Expansion

A key issue, due to using a CD4$^+$ population containing both naive and memory T-cells, is whether the Th17 cell population observed arises due to *de novo* Th17 cell differentiation or preferential expansion of a pre-existing IL-17$^+$ population. The *ex-vivo* CD4$^+$ T cell population following PMA/Ionomycin stimulation was <0.5% IL-17$^+$. Evans *et al* argue that it is unlikely that the resulting IL-17 population (up to 28%) resulting from 3 day culture is due to expansion of a pre-existing IL-17$^+$ cells alone, however THi/TLo stimulated cells are phenotyped at day 6, increasing the chances of pre-existing Th17 cell expansion (98). The doubling time of CD4$^+$ T-cells during the first 7 days of activation is ~11 hours (445). Therefore as T-cell expansion is exponential it is possible that selective expansion of pre-exisiting Th17 cells could account for the proportion of IL-17$^+$ cells observed rather than Th17 cell differentiation. Determining

whether low strength TCR signalling favours expansion of existing Th17 cells or *de novo* Th17 cell induction would require *ex vivo* sorting of Th17 cells. As production of IL-17 is the most important hallmark of Th17 cells, cells would have to be stimulated with, for instance, PMA/Ionomycin or anti-CD3/anti-CD28 beads, in order to perform a cytokine secretion assay for the isolation of Th17 cells. These cells could then be labelled with CFSE and their ability to proliferate and produce IL-17 could be followed in response to THi and TLo stimulation over time. However, such an approach would require a high initial stimulus on day 0, most likely counteracting the effects of subsequent TLo stimulation, and may therefore not be appropriate for this purpose. Ideally, a specific surface marker for Th17 cells would be used to track differentiation or expansion of Th17 cells. However, our current knowledge indicates that although Th17 cells are associated with the surface expression of molecules CCR6 or CD161, these markers may also be expressed, to a lower extent, by Th1 cells (362). Therefore in this model determining if the increase in Th17 cells observed following TLo stimulation is due to differentiation of Th17 cells or expansion of pre-existing Th17 cells is difficult to assess.

2.6.4 Naive T-cells

Naive T-cells may offer a means of assessing Th17 cell differentiation vs expansion as naive T-cells have not been polarised and so pre-existing Th1 or Th17 cells are not present. However *in vitro* human naive T-cell Th17 differentiation has not been convincingly achieved (327, 328). Figure 24 demonstrated that culture of naive T-cells under TLo conditions resulted in reduced IL-17 responses compared to memory T-cell TLo stimulation. The small proportion of IL-17^{+} cells observed in naïve TLo cell populations could be due to the expansion of contaminating memory T-cells from the magnetic cell sort. Sorting naive T-cells to >99%$^{+}$ by flow cytometry would reduce the chance of contaminating memory T-cells and therefore a pre-existing Th17 cell population. In comparison to *in vitro* murine naive T-cell differentiation where a combination of TGF-β and IL-6 can induce up to 60% IL-17^{+} cells, human naive T-cell culture towards a Th17 cell phenotype induces very low Th17 cell numbers (322). Figure 17 demonstrated that the cytokine *milieu* is important in combination with the correct stimulation conditions. The potential remains that naive TLo responses were reduced due to an incorrect Th17 cell cytokine *milieu* for human naive T-cells. It would

therefore be interesting to assess the effect of any new cytokine factors found to promote Th17 cells in conjunction with THi/TLo stimulation in the future. In addition, due to T-cell plasticity, investigating the factors that regulate memory T-cell Th17 cell induction is also relevant. The development of new techniques may allow human Th17 cell induction or expansion to be assessed.

2.6.5 Potential contribution of CD45 to stimulation strength

By comparison to human naive Th17 cell differentiation, generation of Th17 cells from human memory CD45RO^{+} T-cells is far easier to achieve. These differences may be due simply to naive T-cells requiring a factor as yet unidentified to become Th17 cells; though it should be pointed out that murine naive and memory T-cells have similar cytokine requirements. Differences in the signalling pathways induced by TCR signalling in naive and memory T-cells may modulate the activation of transcription factors that regulate Th17 cell induction/expansion (393, 446). For instance, activation of the Erk signalling pathway was found to be increased following TCR triggering in human naive T-cells compared to memory T-cells (393). Furthermore, Erk signalling can act to decrease Ca^{2+} signalling which would modulate NFAT activation and transcriptional effects. A further unexplored possibility is the role of CD45. CD45 exists in multiple forms due to alternative splicing of 3 exons: A,B, and C, CD45R^{O} have all 3 exons spliced out (447). CD45 isoforms R^{A} and R^{O} are used to select human naive and memory T-cells, respectively, but are not used in the selection of murine naive and memory T-cells (448, 449). CD45 is abundantly expressed on the cell surface, but the ligand for CD45 remains unknown (450). CD45 affects TCR signalling by regulating the phosphorylation of Lck; sustaining a basal pool of active Lck. Modulating CD45 expression *in vivo* can alter both thymocyte differentiation and peripheral T-cell activation (451). Cell line studies have revealed that isoforms of CD45 can alter T-cell activation via the TCR, by activating different signalling pathways resulting in different expression of cytokines (452, 453). More specifically, CD45R^{ABC} isoforms are capable of significantly increasing Ca^{2+} mobilisation in response to TCR stimulation, whereas CD45R0 does not have the same capacity to modulate Ca^{2+} mobilisation (391). My data indicates an important role for stimulation strength in modulating Th17 cell responses. However differences in the naive and memory T-cell Th17 cell response to THi and TLo

stimulation are still observed. One possibility is that differences in CD45 isoform expression, used to select naive and memory T-cells, may contribute to the overall threshold of T-cell signal, and may determine their capability to generate a Th17 cell response (447, 454, 455). However, the role of discrete CD45 isoforms in TCR signalling has not been clarified. It is also thought that the anti-CD45 antibodies used within these investigations can affect the early TCR signalling events. Within my investigations negative selection is employed to separate naive and memory-cells and therefore is unlikely to affect early TCR signalling. However it may be interesting to explore if differences in Th17 responses from naive and memory T-cells in humans compared to mice is due to the enrichment of different CD45 isoforms (450), whose ligand remains unknown.

2.6.6 Cell death

T-cells in response to high strength stimulation can be killed via AICD through expression of FAS-FASL, whereas expression of anti-apoptotic factor FLIP can prevent AICD. Th17 cells have recently been reported to express higher levels of FLIP and have an increased capability to survive AICD (456). THi and TLo cultures contained similar proportions of dead cells at day 6; however one would expect that if THi stimulation did result in greater cell death that this should increase the proportion of Th17 cells rather than diminish it. The possibility that THi stimulated cells were exhausted was also explored. Day 6 T-cells were rested by removing the anti-CD3/CD28 stimulation with the aim to reverse the effects of exhaustion. However, although resting of TLo cultures resulted in a further increase in IL-17^{+} cells, resting of THi stimulated cells did not increase the proportion of IL-17^{+} cells. It may be that removing the anti-CD3/anti-CD28 stimulus is not sufficient to rest the cells. Methods to reverse the effects of exhaustion are not fully understood, at least not for CD4^{+} T-cells. In CD8^{+} T-cells, blocking PD-1 signalling can reverse exhaustion, providing evidence that exhaustion is not terminal (435). Furthermore, PD-1 blockade has been demonstrated to increase the secretion of Th17 and Th1 cell cytokines, both of which are low following THi stimulation (457). Therefore, it may be interesting to assess the effect of PD-1 blockade in conjunction with anti-CD3/anti-CD28 bead removal on the Th17 (and Th1) cell responses induced from THi stimulated cells. Furthermore, when assessing the

duration of T-cell stimulation, it was observed that a shorter duration of stimulation favoured Th17 cell responses despite the T-cell receiving a THi strength stimulus (Figure 25). It may therefore also be interesting to assess if there are differences in PD-1 expression following bead removal at early vs late time points, which may inhibit Th17 cell development. Previous investigations have also demonstrated that a persistent high dose of antigen beyond day 10 can impair memory T-cell differentiation (433). THi and TLo culture occurred over 6 days, 4 days less than found to impair memory T-cell responses, which may indicate that the duration of THi/TLo stimulation is not long enough to induce exhaustion. However the binding affinity of anti-CD3 is most likely orders of magnitude higher than of antigen, which may increase T-cell signalling leading to exhaustion of THi cells (436).

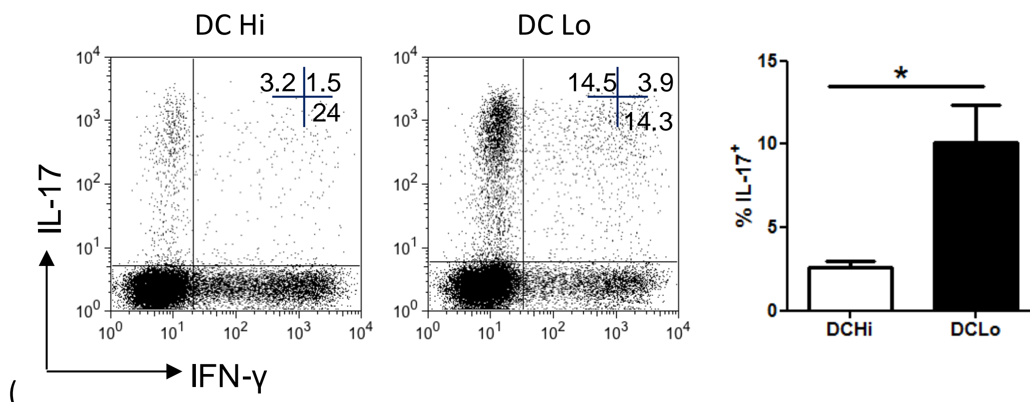
2.6.7 T-cell anergy

Our data indicate that anergy induced by THi stimulation is also not likely to contribute to the limited Th17 cell responses. THi stimulation (with anti-CD28) results in a high proportion of proliferative cells. Anergy is defined as being rapidly initiated following initial antigen encounter in the absence of co-stimulation preventing a proliferative response (434). There are also limited data indicating a role for cell division in regulating effector T-cell phenotype (409, 458). Th2 responses required 7-8 cell divisons whereas Th1 cell responses were observed following 2-3 divisions (409). Gett and Hodgkin also found that IL-4 expression increased at divisons 7-8 although Th1 cell responses in the presence of IL-12 were not examined (458). However, a difference in the number of cell divisions does not appear to be a cause of the difference in THi and TLo Th17 cell response. Both THi and TLo cultures have a comparable number of cells within the later cell division stages as observed by CFSE labelling. These later division stages are where the majority of IL-17 expression is observed in TLo but not THi stimulated cells (Figure 20), indicating that a failure to reach the same number of cellular divisions is not inhibiting Th17 cell responses under THi conditions.

2.6.8 Stimulation strength *in vivo*

Investigations continue to indicate an effect of stimulation strength upon T-cell effector phenotypes. It is therefore interesting to consider what role this may have *in*

vivo. Kapsenburg argues that if stimulation strength alone can dictate the T-cell phenotype then the protective T-cell response during infection would be induced by bacterial load rather than by the type of pathogen (67). It is widely accepted that the type of immune response is dictated by the type of pathogen rather than by pathogen load. However, successful clearance of certain pathogens is dictated by antigen load; *Leishmania major* infection at low doses induces a protective Th1 cell response, whereas at high doses it suppresses Th1 responses, and induces a non-protective Th2 response (459). More recently, antigen dose has been demonstrated to affect the Akt/mTOR pathway, which is a determinant of Treg vs effector T-cell induction (460). Low doses of peptide induced Foxp3 expression, whereas high doses induced effector cytokine production such as IL-17 and IL-6, but also higher IL-10. However, antigen dose is not the only way by which stimulation strength can be modulated. The number of APC and the distances between APC and T-cells can modulate T-cell responses *in vitro* (461). This may represent different *in vivo* situations such as T-cell-APC interactions within the lymph nodes compared to the periphery. Two photon microscopy technology is beginning to increase our understanding of APC-T-cell interactions within lymph nodes, indicating a role for the duration of stable contacts in determining effector function (462). Interestingly, APL with reduced MHC affinities also have a reduction in the half-life of MHC binding and this results in a more than 10-fold reduction in the number of DC presenting the low affinity antigen within lymph nodes (39). Modulating the strength of stimulation by changing the number of activating monocyte derived dendritic cells (moDC) was observed to affect Th17 cell responses



(Figure 26). Furthermore the affinity of antigen for the MHC complex can also determine which effector T-cell phenotype is induced (402, 408). Naturally occurring

APLs also exist: four naturally occurring APLs have been identified within the flagellar FliC protein homologues of both Salmonella serovars and other Gram-negative bacteria. IFNy responses were reduced in FliC APL stimulated T-cells compared to wild type peptide (463). Therefore, *in vivo* stimulation strength in the lymph node may be altered via the amount of peptide capable of being expressed by DC, which may affect the type of T cell phenotype induced.

2.6.9 Stimulation strength and autoimmunity

My data may also provide an explanation as to why a variety of autoimmune diseases are characterised by Th17 cells. Autoreactive T cells that escape negative selection have been shown to express low affinity TCR and may therefore, by nature of their TCR, preferentially develop a Th17 cell responses favoured by low strength TCR activation (464, 465). Furthermore, many genes associated with autoimmune diseases are those relating to TCR signalling including MHC class II, Protein Tyrosine Phosphatase-N22 (PTPN22 – the focus of Chapter 4) and CTLA-4 (466), indicating that regulation of TCR signalling plays an important role in maintaining tolerance. The effect of stimulation strength in regulating T-cell responses has also been harnessed as a therapeutic tool. Antigen-specific immunotherapies have been used to treat autoimmune diseases such as MS where some of the T-cell antigen specificity is known (467). High dose antigen specific therapy induces IL-10 secretion however also induces a harmful burst of cytokine release following T-cell activation. This problem was not observed following administration of low antigen doses. More recently dose escalation studies have revealed that when doses of a peptide analogue are slowly increased, a tolerogenic population of IL-10-producing T-cells are induced (468, 469). Potentially, if administration of the low dose therapy were continued in mice, rather than escalated, the outcome may be a harmful Th17 cell response, rather than a tolerogenic IL-10 response. Prior investigation have sought to harness APL to modulate the immune response in MS (470). Compared to wild-type peptide, administration of an APL with reduced binding can switch the immune response away from an inflammatory Th1 cell response towards a Th2 cell response. Th17 cell responses are also important in the pathogenesis of MS. Investigating how Th17 cells are affected by differences in TCR-

affinity rather than avidity may reveal if Th17 cell responses can be ameliorated in response to APL therapy.

2.6.10 Conclusion

The data presented within this Chapter indicate a novel role for low strength TCR stimulation in promoting human Th17 cell responses. Investigating how the strength of TCR signalling pathway promotes Th17 cell responses will further contribute to our understanding in this field. Therefore the focus of the next Chapter will be to determine by what mechanism TLo stimulation promotes Th17 cell responses and, conversely, how THi stimulation prevents Th17 cell responses.

3 Chapter 3: How does low strength stimulation favour Th17 responses?

3.1 Introduction

In Chapter 2, I demonstrated that low strength T-cell stimulation, administered by either anti-CD3/anti-CD28 coated beads or APC, promoted an increase in the proportion and absolute numbers of IL-17-producing T-cells. Th17 responses were only promoted by low strength stimulation when in the presence of pro-Th17 cell cytokines. I next aimed to establish the mechanism by which low strength stimulation (TLo) promotes Th17 responses or, conversely, by which high strength stimulation (THi) prevents Th17 responses. The factors below were explored for their capability to regulate the generation of Th17 cells in the context of THi and TLo stimulation.

3.1.1 IL-10

The strength of stimulation can affect the expression of many transcription factors and cytokines, some of which can have an inhibitory effect on the generation of Th17 cell responses. Treg-associated cytokines such as IL-10 and TGF-β, and also STAT1 activating cytokines IFNγ and IL-27 are capable of suppressing Th17 cell responses (471, 472). IL-10 is a cytokine typically associated with a Treg phenotype, although is also expressed, to a lesser degree, by most effector T-cells in order to restrain the inflammatory response (473). High affinity peptides have been shown to promote the generation of IL-10- secreting Treg (474) and the IL-10R is found highly expressed on Th17 cells, making Th17 cells sensitive to IL-10 (475). In addition, IL-10, secreted by macrophages, has been demonstrated to inhibit Th17 cell generation (476). Therefore, THi stimulation may promote the expression of IL-10 to inhibit Th17 responses.

3.1.2 TGF-β

TGF-β is also produced by Treg and is required for the induction of both Treg and Th17 cell phenotypes (326). Investigations have indicated that in the absence of pro-inflammatory cytokines, high concentrations of TGF-β can promote Treg phenotypes, particularly the $Foxp3^{+}$ Treg (383). Conversely, TGF-β in combination with pro-

inflammatory cytokines such as IL-1β promoted human Th17 responses (326). However, the requirement for TGF-β in Th17 responses has been greatly debated (477). High concentrations of TGF-β were found to exert an inhibitory effect on Th17 cell differentiation by preventing IL-23R expression and inducing Foxp3 expression in naïve T-cells (383). Furthermore, TGF-β is capable of increasing the proportion of Foxp3$^+$/RORγt$^+$ T-cells, and Foxp3 is able to directly bind RORγt inhibiting RORγt directed *IL-17* expression, altering the transcriptional balance in favour of Foxp3 and a regulatory phenotype. Investigations have also demonstrated that TGF-β promotes Th17 cell differentiation indirectly by inhibiting Th1 and Th2 responses (329, 477). In addition, T-cell responsiveness to TGF-β can be modulated by TCR stimulation (478, 479). Therefore, assessing the role of TGF-β within THi and TLo stimulated cell cultures may reveal how Th17 cell responses are regulated by T-cell stimulation strength.

3.1.3 Foxp3

The transcription factor Foxp3 is a defining marker of natural Treg and is critical to their function (480). As mentioned above, Th17 and Treg cells have reciprocal developmental pathways: the expression of both Foxp3 and RORγt can be induced by TGF-β (481). Foxp3 is a master regulatory gene, and is thought to be capable of inhibiting RORγt-mediated *Il-17* transcription by directly binding to RORγt (373). Evidence links TCR signal strength to the induction of Foxp3 expression in CD4$^+$ T-cells (425). Low doses of strong agonist peptides increased the number of Foxp3 expressing T-cells; however the presence of TGF-β raised the maximum dose of TCR agonist required to induce *in vitro* Foxp3. In addition, it has also been demonstrated that repeated exposure to superantigen increased the proportion of Foxp3$^+$ Treg (482). Low strength TCR signals have also been demonstrated to promote the induction of peripheral Treg (149, 425). The addition of TGF-β and retinoic acid was capable of overcoming the inhibition of Foxp3 expression during high strength stimulation (483). Furthermore, in human CD4$^+$ T-cells, following *in vitro* TCR activation, Foxp3 is transiently expressed an event termed activation-induced Foxp3 (444). Activation-induced Foxp3 does not, however, confer suppressive activity (484). Differential Foxp3 and IL-17 regulation has also been linked to the TCR signalling pathway (485). IL-1 receptor associated kinase 1 (IRAK-1) is capable of inactivating TCR activated

transcription factor NFATc2. NFATc2 inactivation by IRAK-1 prevented NFAT:Smad3 interactions and resulted in the inhibition of Foxp3 expression, but promoted Th17 responses by activating STAT3. The regulation of Foxp3 expression in THi and TLo stimulated cells may therefore contribute to the difference in Th17 response observed.

3.1.4 Nuclear-Factor of activated T-cells (NFAT)

The NFAT transcription factor family are regulated by a Ca^{2+} dependent signalling pathway induced by TCR activation (446). NFATs allow differences in TCR signal strength to be translated into the expression of different genetic profiles and therefore T-cell phenotype. TCR activation results in Ca^{2+} release from both intracellular stores and the cytosol, which then binds to calmodulin and in turn activates the phosphatase calcineurin (486). Active calcineurin is capable of dephosphorylating NFATs leading to NFAT activation and nuclear translocation, where they can regulate gene transcription, depicted in Figure 30 (446). The NFAT family is comprised of five distinct family members: NFAT1 (also known as NFATc2 and NFATp) NFAT2 (also known as NFATc1 and NFATc), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATc3 and NFATx) and NFAT5 (also known as TonEBP and OREBP) (487). NFAT1, 2 & 4 are all Ca^{2+} regulated and present in immune cells. NFATs have weak DNA binding capabilities, meaning they generally implement their effect in concert with other transcriptional partners, including cytokine induced transcription factors: AP-1, Runx and Smads (446, 488). Evidence indicates that the activation of particular NFAT family members can depend on the strength of TCR signalling/Ca^{2+} signalling, which, can in turn, induce different T- cell phenotypes (489). The transcription of Th1 and Th2 cell cytokines is in part regulated by different NFAT family members whose activation/translocation can be dictated by the amount of Ca^{2+} signalling induced as a result of the strength of T-cell stimulation. More recently, subsets of T helper cells have been observed to express molecules that allow the level of Ca^{2+} signalling induced to be determined (490). Th2 cells have higher Trpm4 expression than Th1 cells, which acts to increase Ca^{2+} flux allowing NFATc1 nuclear localisation in Th2 cells. Foxp3 is capable of forming a transcriptional complex with NFATs, and it is proposed that depending on the choice of NFAT transcriptional partner an inflammatory transcriptional program can be converted to a regulatory programme (491). More recently, NFATc1 and c2 have been

demonstrated to be crucial to TCR-mediated *IL-17* expression in human T-cells (492). Therefore THi and TLo stimulation may alter the regulation of NFAT activity to either promote or inhibit the Th17 cell response.

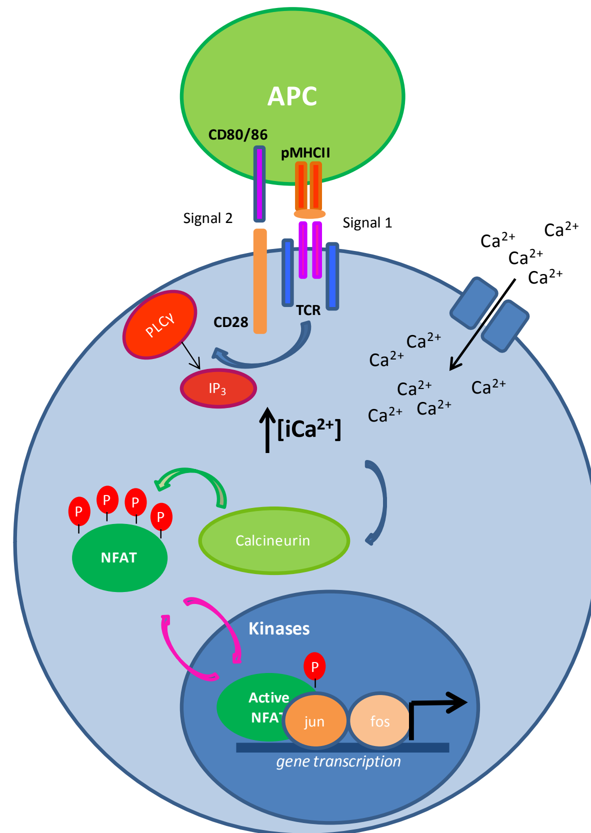


Figure 30. The calcium, calcineurin, NFAT signalling pathway in T-cells. TCR ligation and subsequent activation of PCLγ allows IP3 to bind to the IP3 receptor to initiate intracellular Ca^{2+} store release. Store depletion allows CRAC channels in the plasma membrane to open allowing an influx of Ca^{2+}. Ultimately this allows the activation of the serine phosphatase, calcineurin, and the dephosphorylation of NFAT. Dephosphorylated NFAT can then translocate into the nucleus and bind with other transcription factors to regulate gene expression. NFATs are then returned to the cytoplasm following phosphorylation by kinases (493).

3.2 Aims

A number of factors regulated by the strength of T-cell stimulation may affect Th17 cell responses. The aims of this Chapter were to establish the mechanism by which THi stimulated cells may be prevented from generating an IL-17 response, or the mechanism promoting TLo stimulated IL-17 responses.

- Assess if inhibitory secreted factors cause the difference in THi/TLo Th17 responses
- Establish if activation-induced Foxp3 modulates the capability of THi/TLo stimulated cells to generate Th17 cell responses.
- Investigate the differences in the regulation of the NFATc1 pathway in THi and TLo cells

3.3 Experimental Approach

To assess the mechanism underpinning the difference in Th17 cell response conferred by high strength or low strength stimulation, I used the THi and TLo model of T-cell stimulation outlined in Chapter 2. Based upon literature searches I generated hypotheses that may explain why high strength stimulation may result in reduced Th17 cell responses in comparison to low strength. Using the THi/TLo model the possibility that the factors: IL-10, TGF-β, Foxp3, or Ca^{2+}/NFAT signalling were the cause of the differential IL-17 response was explored.

3.4 Results

3.4.1 The role of IL-10 in determining Th17 responses in high and low strength stimulated T-cell cultures

High strength TCR signalling administered by an APL can drive an IL-10 regulatory T cell phenotype in murine models (474). I firstly investigated if THi stimulation results in increased endogenous IL-10 production compared to TLo stimulation, which may be inhibitory to Th17 responses. IL-10 secretion was assessed by ELISA in day 3 supernatants of THi and TLo cultures. Figure 31 shows that, although not significant that the average concentration of IL-10 secreted by THi cells is raised compared to TLo cells.

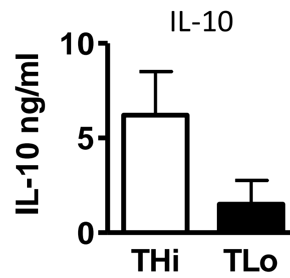


Figure 31. THi cells secrete higher levels of the regulatory cytokine IL-10 than TLo cells. Human CD4$^+$ T cells were stimulated under THi or TLo conditions. After 3 days cell-free supernatants were harvested and levels of IL-10 measured by ELISA; N=3; Error bars represent SEM.

I next assessed if the level of IL-10 produced by THi stimulated cells was inhibitory to their capability of generating a Th17 cell response. THi and TLo cells were cultured in the presence of an anti-IL-10 receptor antibody to block IL-10 signalling. The addition of αIL-10R antibody to THi cultures however, did not affect the proportion of IL-17$^+$ cells in comparison to the isotype matched control. In addition, comparable proportions of intracellular IL-17$^+$ and IFNy$^+$ cells were observed within the control and isotype control populations of THi cultures (Figure 32), indicating minimal non-specific binding or activity of the isotype control. IL-10R blockade was also performed on TLo cultures. TLo cells secreted lower concentrations of IL-10 than THi cells. Therefore, the Th17 response was not strikingly affected by IL-10R blockade in TLo cultures. The possibility remained that αIL-10R was inactive and therefore could not affect the Th17 cell response in THi cultures. To exclude this possibility, recombinant IL-10 was added

to TLo stimulated cells to assess if high IL-10 concentrations prevented Th17 cell responses. The maximum concentration detected within THi cultures (10 ng/mL) was added to TLo cultures on day 0. The addition of rIL-10 to TLo conditions, however, had no inhibitory effect on the proportion of IL-17$^+$ or IFNγ$^+$ T-cells present within the culture when compared to the control populations (Figure 33). Together these data suggest that IL-10 was not responsible for the low Th17 cell response in THi cultures.

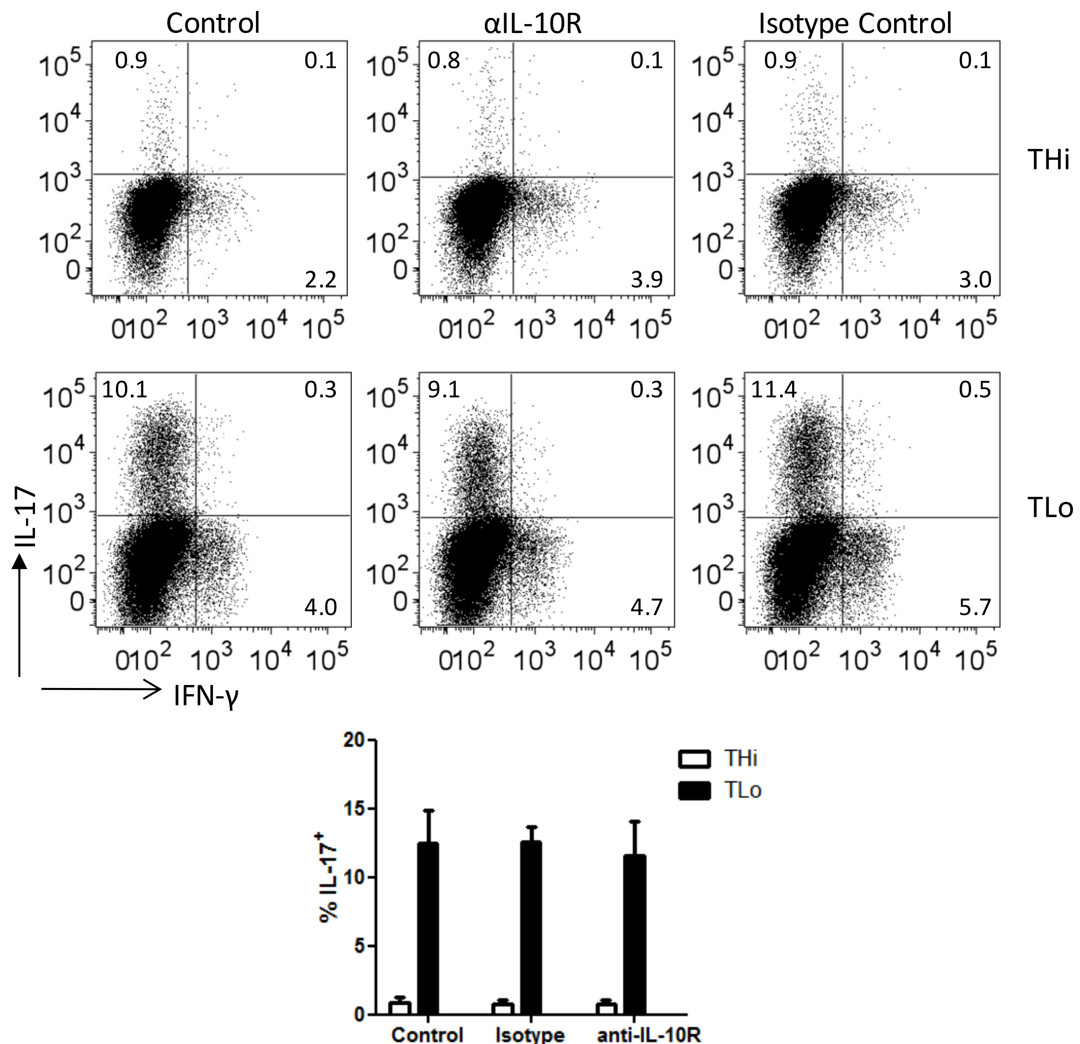


Figure 32. Blockade of IL-10 signalling does not increase THi Th17 responses. Human CD4$^+$ T cells were stimulated under THi or TLo conditions in the presence or absence of anti-IL-10R or isotype control. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Plots are representative of 3 independent experiments and the histogram below demonstrates the percentage of cells expressing IL-17 from the 3 independent experiments. Error bars represent SEM.

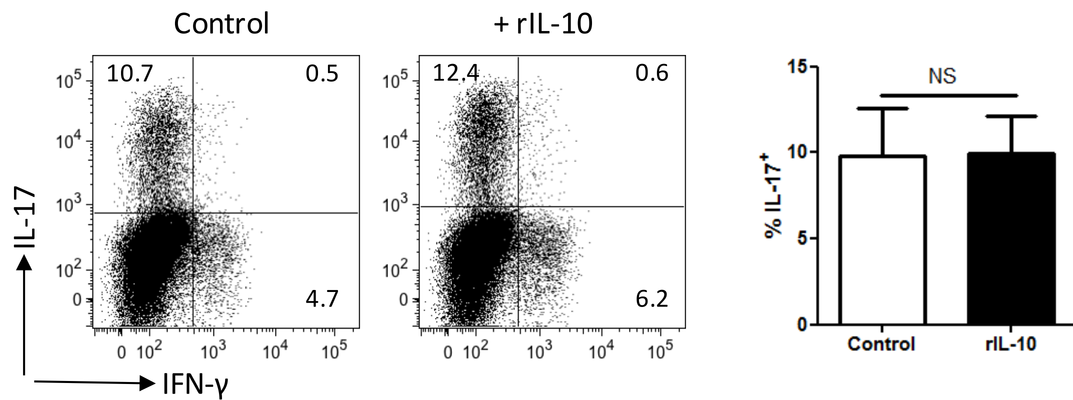


Figure 33. Addition of rIL-10 to TLo does not inhibit Th17 responses. Human CD4+ T cells were stimulated under THi or TLo conditions in the presence or absence of 10ng/ml rIL-10. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Plots are representative of 3 independent experiments and the histogram adjacent demonstrates the percentage of cells expressing IL-17 from the 3 independent experiments. Error bars represent SEM.

3.4.2 The role of TGF-β in determining Th17 cell responses in high and low strength stimulated T-cell cultures

TGF-β is a highly pleotropic cytokine, and TGF-β signalling can have opposing effects depending on the cell type and surrounding environment (494). As described above TGF-β is required for Th17 cell responses, however, at high concentrations it can be inhibitory to Th17 cell development, instead inducing a regulatory T-cell phenotype. I proposed that THi stimulation may induce endogenous TGF-β expression and that in concert with exogenous TGF-β, added as part of the pro-Th17 cell cytokine cocktail, may contribute to the inhibition of Th17 cell responses.

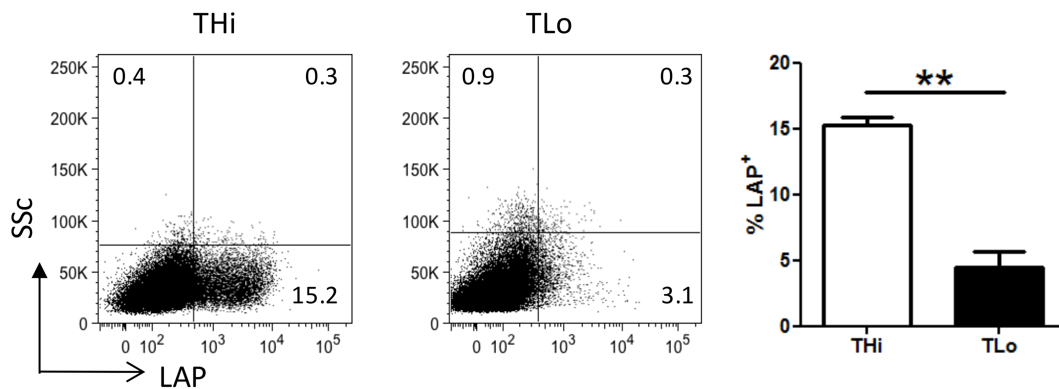


Figure 34. THi cells express increased LAP/TGF-β compared to TLo cells. Human $CD4^+$ T cells were stimulated under THi or TLo conditions. After 6 days expression of LAP was determined by flow cytometry. Plots are representative of 3 independent experiments and the histogram adjacent demonstrates the percentage of LAP expressing cells from the 3 independent experiments. Error bars represent SEM. P values calculated using a paired T-test; **p=<0.01.

I firstly assessed if THi had greater expression of membrane-bound TGF-β compared to TLo. Following THi or TLo culture for 6 days, T-cells were surface stained for the expression of latency associated peptide (LAP)-TGF-β. THi cells were observed to have increased cell surface expression of LAP-TGF-β compared to TLo cells (Figure 34). It was next assessed if the increased expression of TGF-β-LAP was inhibitory to Th17 cell responses under THi conditions. A small molecular inhibitor (SB505124) of the activin receptor-like kinase (Alk) 4, 5, and 7 mediated TGF-β signalling pathway was used to block TGF-β signalling (495). In Figure 35 different concentrations of SB505124 were used in the presence or absence of exogenous TGF-β to assess if an optimal level of TGF-β could be achieved that would promote Th17 cell responses in THi cultures. TGF-β blockade in the presence or absence of exogenous TGF-β increased the proportion of $IFNy^+$ cells in THi and TLo conditions. However, TGF-β blockade in the presence/absence of exogenous TGF-β did not enhance the proportion of $IL\text{-}17^+$ cells in THi cultures above that of the +TGF-β control condition. Whereas, TGF-β was found to be important for TLo IL-17 responses; in the absence of exogenous TGF-β or in the presence of SB505124, the proportion of $IL\text{-}17^+$ cells was decreased. The activity of SB505124 was confirmed by the dose dependent increase in the proportion of $IFNy^+$ cells and the decreased proportion of $IL\text{-}17^+$ cells (Figure 35 bottom left). Together these data indicate that although TGF-β is important for TLo IL-17 responses, that increased expression of LAP-TGF-β on THi is not inhibitory to Th17 cell responses.

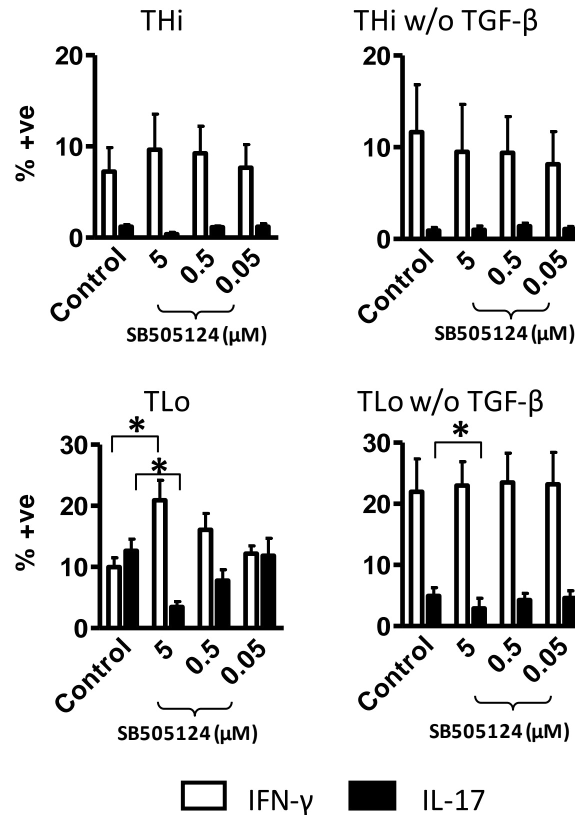


Figure 35. TGF-β is required for high IL-17 production by TLo stimulated cells, and high LAP/TGF-β expression by THi cells does not inhibit Th17 responses. Human CD4$^+$ T cells were stimulated under THi or TLo conditions in the presence or absence of TGF-β and in the presence of absence of SB505124 (5-0.05uM). After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry, N=3. Error bars represent SEM; p-values calculated using a T-test *p<0.05.

The addition of as little as 25 pg/ml of TGF-β to LPS activated DC co-cultured with Treg depleted naive T-cells has been found to deviate T-cells from a Th1 to a Th17 cell response (322). TGF-β added to THi/TLo cultures as part of the pro-Th17 cytokine *milieu* is 400-times in excess of this and therefore may be too high and potentially limit TLo Th17 responses. The concentration of TGF-β within TLo cultures was therefore titrated and the proportions of IL-17- and IFNy-producing cells were assessed by ICS and flow cytometry. Figure 36 shows that as the TGF-β concentration decreased from 10 ng/mL, to 0.1 ng/mL the proportion of IL-17$^+$/IFNy$^-$ cells marginally decreased. Interestingly, the total proportion of IL-17$^+$ cells (single and double positive) remained constant. Reduced TGF-β appeared to increase the number of dual IL-17$^+$/IFNy$^+$ cells along with the proportion of IFNy$^+$ cells. These data indicated that reducing the concentration of TGF-β below 10 ng/mL did not enhance the proportion of IL-17$^+$ cells,

and in addition suggested that TGF-β may support Th17 cell responses in part through inhibition of IFNy expression. TGF-β may encourage phenotype switching towards IL-17^{+} rather than IFNy^{+}/IL-17^{+}, thereby promoting Th17 cell induction or expansion in the culture (479).

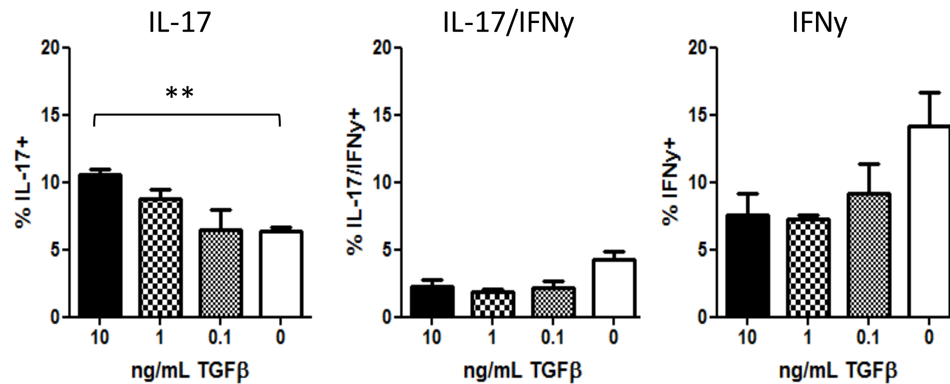


Figure 36. Higher concentrations of TGF-β are required for optimal TLo-induced Th17 generation. Human CD4^{+} T cells were stimulated under TLo conditions in the presence or absence of TGF-β (10-0.1 ng/mL). After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17, IL-17/IFNy-γ, and IFN-γ producing cells were determined by intracellular cytokine staining followed by flow cytometry; n=3; error bars represent SEM p-value calculated using a paired T-test **p<0.01.

I also assessed if inhibition of TGF-β and IL-10 signalling in concert would promote Th17 responses in THi cultures. IL-10 is capable of enhancing TGF-βRII expression on active T-cells, making them more responsive to TGF-β (478). Another investigation has demonstrated that TGF-β is important for Th17 cell expression of IL-10 in mouse T-cells (473). Therefore heightened IL-10 secretion may make THi stimulated cells more responsive to the increased LAP-TGF-β available on the cell surface and/or increased LAP-TGF-β expression may stimulate further IL-10, which may inhibit THi TH17 cell responses. I therefore assessed if combined inhibition of the TGF-β and IL-10 signalling pathways would promote Th17 cell responses in THi stimulated cells. Initial data (Figure 37) however, did not indicate any effect of combined IL-10R and TGF-β signalling pathway inhibition on THi IL-17 responses. I therefore ceased to pursue this line of investigation any further in order to investigate new hypotheses.

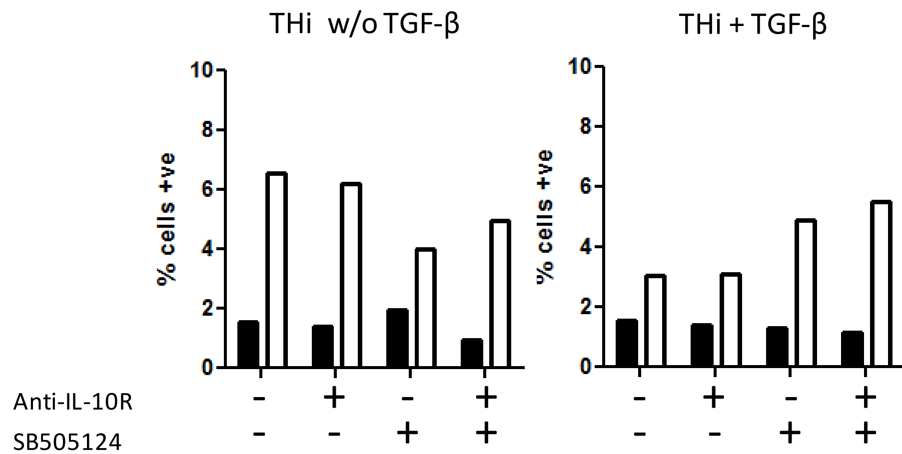


Figure 37. Combined inhibition of IL-10 and TGF-β signalling does not promote Th17 responses from THi stimulated cells. Human CD4$^+$ T cells were stimulated under THi conditions in the presence or absence of TGF-β (10ng/ml), SB505124 (0.05µM), and/or αIL-10R. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry; black bars =IL-17 and white bars =IFNy; N=1.

3.4.3 Do THi secrete Th17 inhibitory factors?

To rule out the possibility that a secreted factor was responsible for THi being unable to generate Th17 cells, conditioned media experiments were performed. Day 3 conditioned media from THi or TLo cells were added to TLo cells. If inhibitory factors were secreted from THi stimulated cells into the media, reduced TLo Th17 cell responses would be expected. Addition of either THi or TLo conditioned media to day 0 TLo culture did not affect the proportion of IL-17$^+$ or IFNy$^+$ cells (Figure 38).

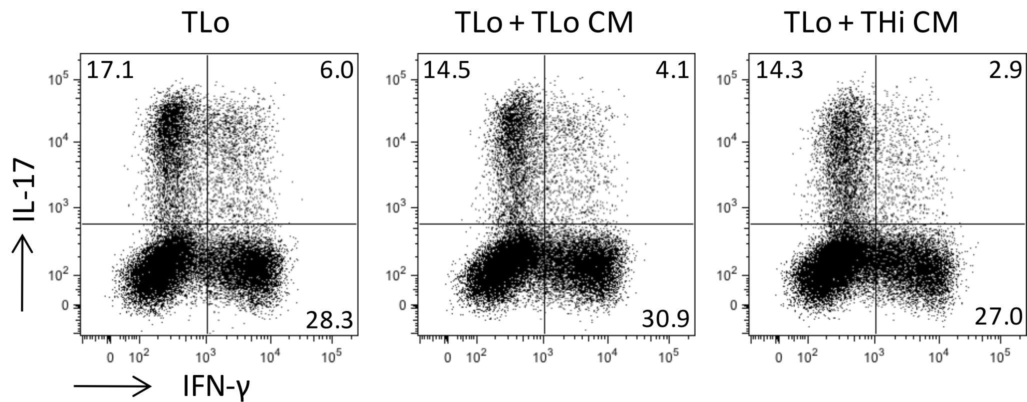


Figure 38. THi conditioned medium does not contain a factor inhibitory to Th17 generation. Human CD4+ T-cells were stimulated under THi or TLo conditions. After 72 hours cell-free supernatant was harvested. Human CD4+ T-cells stimulated under TLo conditions for 6 days in the presence of absence of 50% THi or TLo 72h conditioned media. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Data are representative of 3 independent experiments.

The small decreases observed are most likely reflected by decreased quality of media following 3 days culture and a freeze thaw cycle rather than by specific Th17 inhibitors. Potentially a better control would have been to take culture media from unstimulated day 3 cells, and freeze thawing in the same way as above; however unstimulated cells would not have the same metabolic activity and so would also not be directly comparable either. The possibility remains that the 72 hour time point may be too late to observe the inhibitory effects of rapidly secreted and stimulation strength dependent factors such as IL-2 upon Th17 cell responses. However conditioned media experiments performed after this thesis indicated that THi 24 hour or 48 hour conditioned media did not have an inhibitory effect upon TLo conditions. Furthermore certain soluble factors e.g. indolamine 2-3-dioxygenase (IDO) are short-lived and so won't be picked up in conditioned media experiments. Furthermore, addition of recombinant IFNy to TLo cultures did not reduce the proportion of IL-17+ cells compared to control conditions (Figure 39). Together with the IL-10 and TGF-β neutralisation data, these data indicate that a T-cell secreted factor is most likely not the cause of the inability to generate a Th17 cell response under THi conditions.

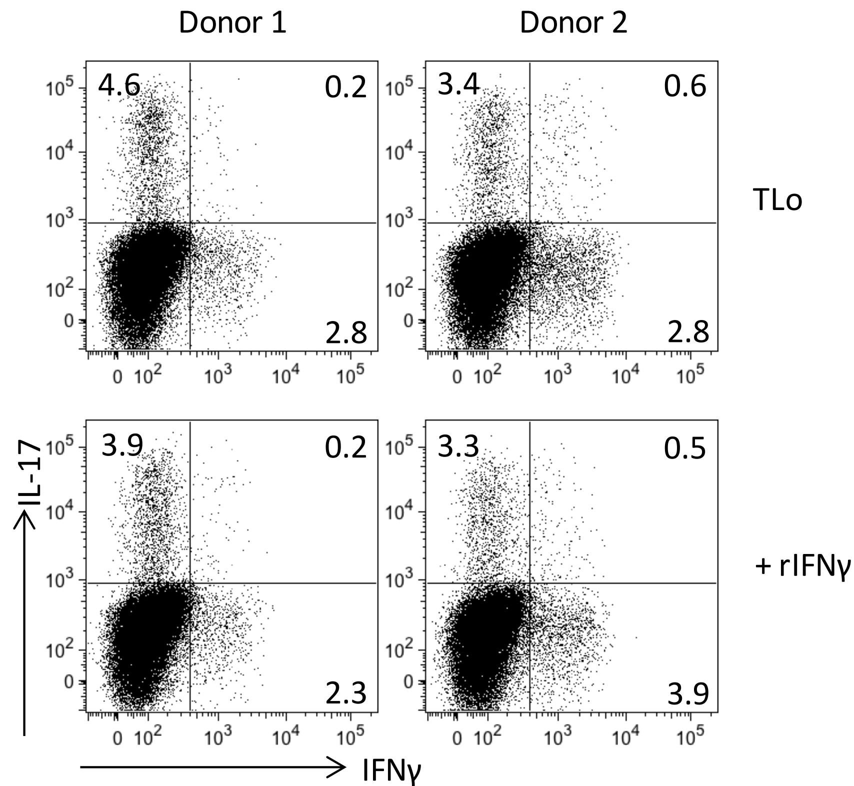


Figure 39. Addition of rIFNγ does not inhibit TLo Th17 responses. Human CD4$^+$ T cells were stimulated under TLo conditions in the presence or absence of rIFNγ (10ng/ml). After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry. Plots are of two independent experiments.

3.4.4 THi stimulated cells express master regulatory transcription factor Foxp3

I next hypothesised that a T-cell intrinsic factor could inhibit the capability of THi cells to become IL-17$^+$ cells. A strong candidate for this is the master regulatory transcription factor Foxp3. It was hypothesised that a high strength T-cell stimulus may induce a higher proportion of T-cells to express activation-induced Foxp3, which may inhibit *IL-17* transcription. To assess if an increased number of THi cells express Foxp3 than TLo cells, intracellular Foxp3 was assessed on day 6 of culture. I observed that a higher number of THi stimulated cells expressed Foxp3 at day 6 than TLo cells (Figure 40).

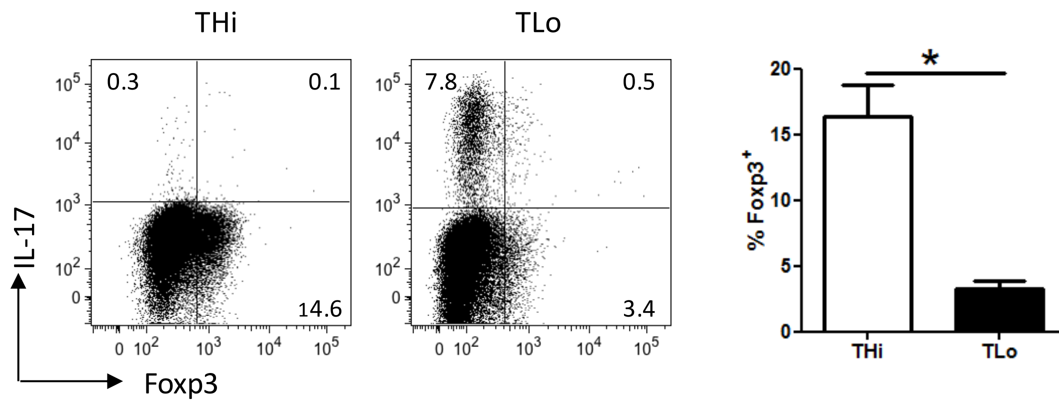


Figure 40. THi cells express increased Foxp3 compared to TLo. Human CD4^{+} T-cells were stimulated under THi or TLo conditions. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and Foxp3 expressing cells were determined by intracellular staining followed by flow cytometry. Plots are representative of 4 independent experiments and the histogram adjacent demonstrates the percentage of Foxp3 expressing cells from the 4 independent experiments. Error bars represent SEM. P values calculated using a paired T-test; *p=<0.05.

THi stimulation resulted in ~14% Foxp3^{+} T-cells at day 6 compared to ~3% in TLo cultures, and the IL-17^{+} cells were largely Foxp3^{-}. I next assessed the kinetics of Foxp3 expression in THi and TLo cultures. A time course was performed assessing intracellular Foxp3 and IL-17 expression at days 3, 6 and 10 in THi and TLo cultures. Interestingly, in both THi and TLo cultures the proportion of Foxp3 expressing cells was high at day 3 of culture, indicating the induction of activation-induced Foxp3 under both conditions (Figure 41). However, at day 3 almost double the proportion of THi stimulated cells express Foxp3 compared to TLo cells. Furthermore, in TLo cultures the proportion of Foxp3 expressing cells declined sharply from day 3 to 6 and was inversely proportional to the number of cells expressing IL-17. In contrast, the decline in the proportion of Foxp3 expressing cells in THi cultures from day 3 to 6 was less striking than that observed in TLo cultures, and a distinct Foxp3^{+} population was still observed in THi cultures at day 6. Despite the reduction in the proportion of Foxp3 expressing cells by day 10 in THi cultures, the proportion of cells expressing IL-17 did not increase to the proportion observed in TLo cultures. Unlike TLo cultures, the proportion of Foxp3 expressing cells in THi cells was not inversely proportional to the number of IL-17 expressing cells. The higher proportion of Foxp3^{+} cells at day 3 of THi culture and the prolonged kinetics of Foxp3 expression may prevent Th17 cell responses in THi cultures.

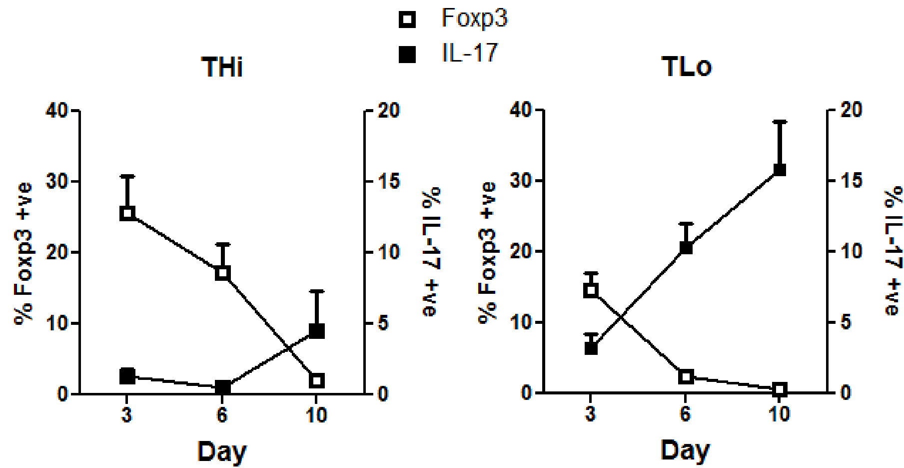


Figure 41. An inverse relationship between Foxp3 and IL-17 expression in TLo but not THi conditions. Human CD4$^+$ T-cells were stimulated under THi or TLo conditions for 3, 6, or 10 days. To generate day 10 cells, on day 6 anti-CD3/anti-CD28 beads were removed and the CD4$^+$ T-cells were replated in the presence of IL-2 and IL-23 until day 10. At each time point the T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A, and assessed for intracellular IL-17 and Foxp3 by flow cytometry. Data are representative of 3 independent experiments; n=3; error bars represents SEM; IL-17 data shown in Figure 23 (Chapter 2) is plotted in this graph.

An observation arguing against a role for FoxP3 in inhibiting Th17 responses under THi condition was made during the experiments using the TGF-β signalling inhibitor SB505124. I measured the expression of intracellular FoxP3 in one of these experiments and found that SB505124 reduced Foxp3 expression in THi stimulated cells (Figure 42). Despite the reduction in Foxp3$^+$ cells the proportion of IL-17$^+$ cells remained low, however as TGF-β is also important for pro-Th17 pathways this is not the optimal experiment to address if activation-induced Foxp3 inhibits THi IL-17 responses. One way to achieve this would be to knock-down expression of the *Foxp3* gene. Knockdown systems do not result in 100% inhibition of gene expression and are difficult to achieve in human primary T-cells. Moreover, gene knock-downs are often only transient and do not carry over inhibition into daughter cells. THi and TLo stimulated

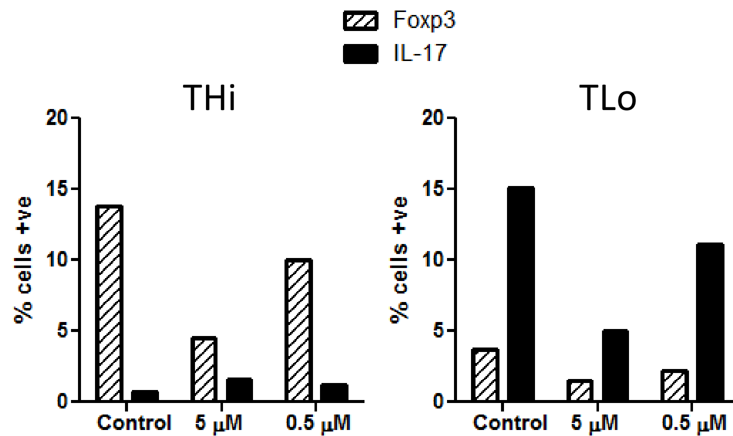


Figure 42. TGF-β inhibition inhibits Foxp3 expression in THi and TLo cells. Human CD4$^+$ T-cells were stimulated under THi or TLo conditions in the presence or absence of SB505124 (5 or 0.5 µM). After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and Foxp3-expressing cells were determined by intracellular staining followed by flow cytometry; N=1.

cultures over a 6 day period undergo multiple rounds of cell division, and would most likely not maintain the gene knockdown. Therefore, *in vitro* genetic knockout was deemed not be an appropriate system to address this question. Another way to achieve this is to use a natural human knockout system. Immunodysregulation polyendocrinopathy and enteropathy X-linked (IPEX) syndrome, is a rare disease caused by mutations within the *Foxp3* gene which causes either a complete absence of Foxp3 or a loss of Foxp3 function in the patient: providing a human knockout system for functional Foxp3. IPEX patients are very rare and symptoms become apparent in infancy (496). I obtained a Peripheral blood mononuclear cell (PBMC) sample from an infant with IPEX syndrome. The mutation was present in exon 10 of the *Foxp3* gene, resulting in non-functional rather than absent Foxp3. It was hypothesised that if high Foxp3 induced by THi stimulated culture was the cause of reduced IL-17 responses then this would not be observed within the IPEX cells when cultured under THi and TLo conditions.

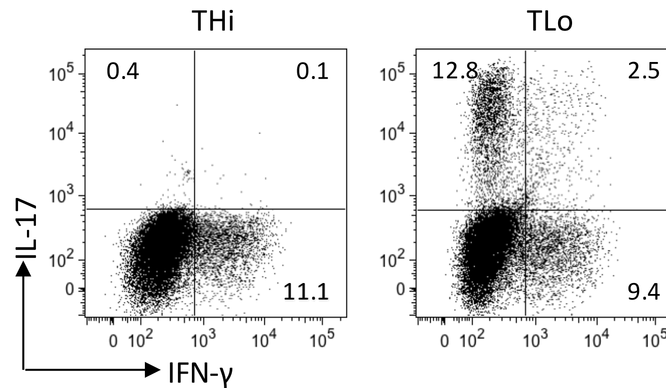


Figure 43. Presence of non-functional Foxp3 does not reverse THi inhibition of Th17 generation. IPEX patient PBMC were stimulated under THi or TLo conditions. T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry; N=1.

Data in Figure 43 demonstrates that in IPEX PBMC THi culture still resulted in diminished Th17 cell responses compared to TLo. Despite the absence of functional Foxp3, TLo stimulation still resulted in greater proportions of IL-17^{+} cells than THi stimulation. There was little difference in the proportion of IFNy^{+} cells between THi and TLo cultures. These data suggest that the high proportions of Foxp3^{+} cells induced by THi stimulation did not prevent Th17 cell responses. Cumulatively, although the expression of these factors is raised in THi stimulated T-cells, investigations indicate that these factors do not directly contribute to the reduced Th17 cell response observed in THi cells. I therefore next investigated how the strength of stimulation may directly affect Th17 cell responses.

3.4.5 TCR stimulation strength, Ca^{2+} signalling and NFATs

TCR activation leads to induction of a calcium-dependent signalling pathway which leads to expression of NFAT transcription factors (446). NFATs are important partners in mediating transcription of Th1- and Th2-cell associated cytokines IFNy and IL-4 (489, 490). Recently, an NFATc1 binding site has been described within the proximal *IL-17* promoter contributing to *IL-17* transcription (492). NFATc1 expression is controlled by the level of Ca^{2+} signalling in T-cells. I hypothesised that high strength TCR stimulation would increase Ca^{2+} signalling and that high Ca^{2+} signalling might be inhibitory to Th17 cell responses, via NFATc1. To assess this, a Ca^{2+} ionophore, ionomycin, was added to

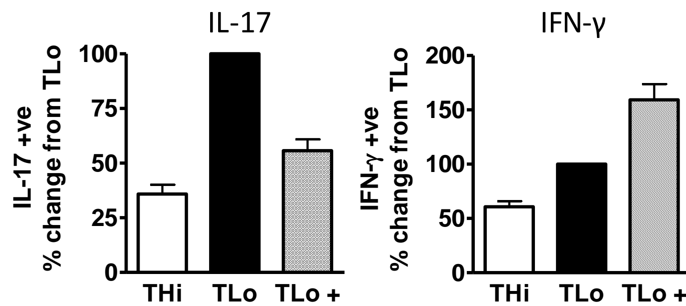


Figure 44. Ionomycin inhibits TLo induction of Th17. Human $CD4^+$ T-cells were stimulated under THi or TLo conditions. or TLo conditions in the presence or absence of 500nM Ionomycin (TLo+) added daily for the first 4 days of culture. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17- and IFN-γ-producing cells were determined by intracellular cytokine staining followed by flow cytometry; n=3; error bars represent SEM.

TLo cultures to investigate if increased Ca^{2+} signalling would have an inhibitory effect on Th17 cell responses. The addition of Ionomycin to TLo cultures was observed to decrease the proportion of IL-17^+ cells by nearly 50%, almost to the level of THi stimulated cells (Figure 44). Interestingly, in comparison to IL-17, the proportion of $IFNy^+$ cells increases by nearly 50%. These data indicate that higher Ca^{2+} signalling is inhibitory to Th17 responses. I therefore next hypothesised that TCR stimulation strength may modulate the cellular location and/or capability of NFATc1 to bind to the *IL-17* promoter and therefore affect *IL-17* expression.

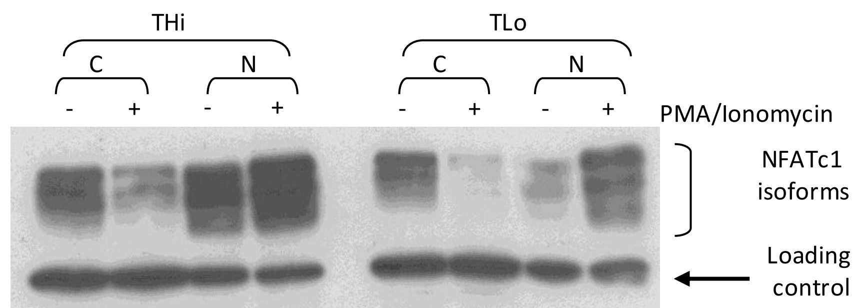


Figure 45. NFATc1 translocates from the cytoplasm into the nucleus in both THi and TLo stimulated cells. Human $CD4^+$ T-cells were stimulated under THi or TLo conditions. After 6 days T-cells were either restimulated for 2 hours with PMA/Ionomycin (+) or left untreated (-). Cytoplasmic and nuclear lysates were prepared and expression of NFATc1 determined by Western blotting. Blots are representative of 3 independent experiments.

I investigated if T-cell stimulation strength affected the induction, translocation or binding of NFACTc1 to the *IL-17* promoter in THi or TLo stimulated cells. Firstly, the presence and location of NFATc1 was assessed in day 6 THi and TLo stimulated cells by

western blot. Both THi and TLo stimulated cells were found to express NFACTc1 in the cytoplasm of day 6 cells (Figure 45). In both THi and TLo stimulated cells, when restimulated with PMA and ionomycin, NFATc1 expression in the cytoplasm was reduced. Nuclear expression of NFATc1 in THi cells was higher at day 6 than TLo. Following restimulation with PMA/Ionomycin, expression of NFATc1 was increased in the nucleus of TLo cells and was also further increased in THi stimulated cells. Together these data demonstrated that both THi and TLo stimulated cells express NFATc1 and both were capable of NFATc1 translocation to the nucleus following restimulation.

Having established that NFATc1 translocation is not impaired in THi or TLo cells, I next assessed if the nuclear NFATc1 was capable of binding to the *IL-17* promoter in THi and TLo stimulated conditions. Using chromatin immunoprecipitation (ChIP), Figure 46 reveals that, compared to the isotype control, there is no increase in NFATc1 binding to the *IL-17* promoter in either day 6 THi cells or those restimulated with PMA ionomycin. Conversely, in day 6 TLo cells there was an ~20 fold increase in NFATc1/*IL-17* promoter binding after PMA/ionomycin stimulation. These data indicate that differences in the binding capabilities of a TCR regulated transcription factor may be the cause of reduced Th17 cell responses under THi conditions.

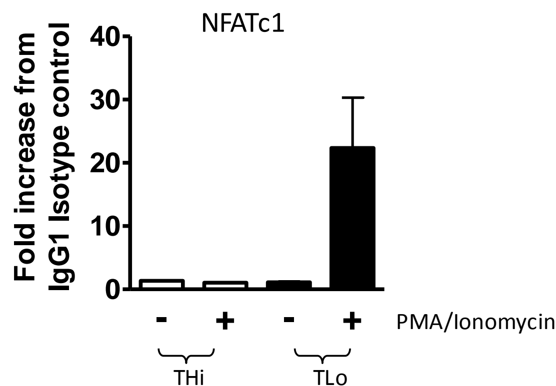


Figure 46. NFATc1 binds to the *IL-17* promoter in TLo but not THi stimulated cells. Human CD4$^+$ T-cells were stimulated under THi or TLo conditions. After 6 days T-cells were either restimulated for 2 hours with PMA/Ionomycin (+) or left untreated (-). NFATc1 binding to the IL-17 promoter was assessed by ChIP. N=2; error bars represent SEM. ChIP data were geneated by Dr Jelena Mann from cells cultured and restimulated by H. Purvis.

3.5 Discussion

The data described in this Chapter investigated the mechanism by which strength of stimulation is capable of determining the Th17 cell response, as observed in Chapter 2. Using the THi/TLo anti-CD3/anti-CD28 bead model in the presence of pro-Th17 cell cytokines, TLo stimulation was found to promote Th17 cell responses (Chapter 2). A number of potential mechanisms to explain the regulation of Th17 cells by stimulation strength were excluded. However, investigations within this Chapter revealed that the most likely cause of increased Th17 cell responses, resulting from TLo compared to THi stimulation, were due to differences in the regulation of the Ca^{2+}/NFATc1 signalling pathway. Reduced NFATc1 binding to the *IL-17* promoter was observed in THi cells compared to TLo cells, which has been demonstrated in previous studies to regulate *IL-17* expression (492). In addition Ca^{2+} signalling can be modulated depending on the strength of TCR stimulation and is the signalling molecule which determines NFATc1 activation (486, 497). Increased Ca^{2+} signalling via the addition of ionomycin to TLo cultures resulted in decreased IL-17 responses (Figure 44). High strength Ca^{2+} signalling, induced by ionomycin or high strength anti-CD3/anti-CD28 stimulation, appears to be inhibitory to Th17 cell responses. Together these data indicate that the strength of stimulation regulates Th17 cell responses, in part, by modulating the capacity of NFATc1 to bind to the *IL-17* promoter and that lL-17 expression is sensitive to the strength of Ca^{2+} signalling. Therefore low strength TCR stimulation mostly likely promotes Th17 cell responses via a Ca^{2+}/NFATc1 dependent mechanism.

3.5.1 Regulation of NFAT activation and localisation

Prior investigations have revealed that Th2 cell responses are favoured by low strength TCR stimulation, whereas Th1 cells are favoured by high strength TCR stimulation (403, 489). One mechanism for this was proposed to be due to differential NFAT activation (489). The activation of different NFAT family members (i.e. NFATc1 or NFATc2) has been suggested to be regulated by the level of Ca^{2+} flux induced in T-cells (489). Reduced Ca^{2+} signalling preferentially activates NFATc1 in $CD8^{+}$ T-cells (498). Furthermore low potency TCR signalling has been demonstrated to result in greater nuclear localisation of NFATc1 initiating Th2 cell responses, whereas NFATc2 remained located within the cytoplasm (489). The balance between NFATc1 and NFATc2 has not

been addressed within this study. However, in THi or TLo cultures no difference was observed in the capability of NFATc1 to localise to the nucleus. Despite the nuclear location, NFATc1 was only observed to bind to the *IL-17* promoter in TLo conditions. In addition, these data contradict that of Gomez Rodriguez *et al* who found that high strength TCR stimulation was required for NFAT-mediated *IL-17* expression (422). Although, as described in Chapter 2 these differences may be attributed to the use of mouse vs human T-cells, and in the definition of high vs low strength stimulation. Whilst data in Figure 45 demonstrated that THi and TLo stimulated cells are capable of increasing NFATc1 nuclear localisation following PMA/Ionomycin stimulation, a higher basal amount of NFATc1 was also observed within the nuclear fraction of THi. Recent investigations assessing NFAT localisation of NFATs in Treg have revealed that NFATc1 and NFATc2 are constitutively localised within the nucleus (499, 500). In addition, ChIP analysis revealed that NFATs are constitutively bound to known Foxp3 target genes including the *Il-2* promoter within Treg (500). In non-Treg, NFATs only bound to DNA following stimulation. These data may provide insights into the regulation of THi/TLo-induced Th17 responses. High constitutive NFATc1 in the nucleus of THi cells may indicate the induction of a Treg phenotype, or nuclear NFATc1 may be bound to target genes that repress Th17 target gene expression. Furthermore Li *et al* demonstrate that constitutive nuclear localisation of NFATs within Treg is independent of calcineurin activity (500), raising the question of how NFATs can become constitutively localised in the nucleus. In resting T-cells, NFAT proteins are retained within the cytoplasm by being highly phosphorylated at their nuclear localisation sequence by kinases such as casein kinase 1 and glycogen synthase kinase 3 (GSK-3). Ca^{2+} signalling activates the calmodulin-dependent phosphatase calcineurin, which dephosphorylates NFATs allowing nuclear localisation. The balance between calcineurin and NFAT kinases may be important to the retention of NFAT within the nucleus. Differences in the regulation of NFAT phosphatases and kinases may also affect the regulation of NFATc1 in THi and TLo cells. Investigations have revealed differences in the balance of GSK-3 in Treg compared to effector T-cells. Treg were observed to have increased GSK-3 following TCR stimulation, facilitating shuttling of NFATs out of the nucleus, whereas GSK-3 was decreased in non-Treg following TCR stimulation (499). Assessing the balance of NFAT

phosphatases vs kinases may reveal why NFATc1 has a different capacity to regulate *IL-17* expression in THi and TLo stimulated cells.

3.5.2 NFAT transcriptional partners

Both THi and TLo cells were capable of increased nuclear localisation of NFATc1 following PMA/Ionomycin restimulation, however, only in TLo cells was NFATc1 bound to the *IL-17* promoter. Evidence suggests that NFAT transcriptional activity is not only controlled by nuclear localisation, but that the transcriptional binding partners formed by NFATs are also important in determining the capability of NFATs to regulate gene expression (446). NFATs can bind to a number of transcriptional partners including: Foxp3, AP-1, Runx, and STATs (446). Potentially the difference observed, in NFATc1 binding to the *IL-17* promoter in THi/TLo stimulated cells, is due to the availability of other transcriptional partners. Data in Chapter 2 revealed that RORc availability is most likely not a constraint in either THi or TLo stimulated cells. However, Figure 40 in the current Chapter indicates that THi stimulated cells have higher expression of Foxp3 than TLo cells. NFATs are capable of inducing *de novo* Foxp3 expression (501) and can form transcriptional binding partners with Foxp3 (491). In addition, Foxp3 is capable of repressing the NFAT:AP-1 transcription activities (491), which may prevent *IL-17* expression. However, the reduction in Foxp3 expression, following T-cell resting or in IPEX PBMC did not 'rescue' IL-17 expression. AP-1 is a prominent NFAT binding partner and a key transcription factor induced by the MAPK pathway. Inhibition studies have indicated the importance of MAPK/AP-1 in *IL-17* transcription (492). Investigations have demonstrated that the MAPK pathway is regulated by stimulation strength; strong TCR stimulation increases MAPK signalling leading to IFNγ expression (497). Therefore the availability of AP-1 may also be modulated by stimulation strength and affect *IL-17* expression. Runx protein family members can also function as transcriptional co-factors for NFATs; Runx 3 is capable of inhibiting *Il-4* expression via interacting with NFATc1 (502). Runx protein expression was not assessed within this study but is known to be important to Th17 and Treg cell responses. Runx1 can bind to RORγt and activate *Il-17* transcription, however Runx1 is also capable of binding Foxp3 which is required for Foxp3 inhibition of RORγt-mediated *Il-17* transcription (374). Therefore, Runx family members may also be capable of binding to NFATs present in

the nucleus of THi cells and modulate their transcriptional capabilities. THi stimulation therefore may prevent Th17 cell responses by either inducing transcriptional co-factors that repress NFAT/*IL-17* promoter interactions or by preventing the expression of transcription factors that allow NFAT/*IL-17* promoter binding.

3.5.3 Epigenetic regulation

Epigenetic instability of both cytokine and transcription factor gene loci are thought to be a major factor mediating $CD4^+$ T-cell plasticity (503). DNA is wrapped around four core histones: post-translational modifications to histones controls transcription factor access to DNA (504). Epigenetic modifications to certain histones are associated with either gene activation or gene repression. Th1 cells have hyperacetylated histone H3 in the *Ifny* gene promoter, but not *Il-4*. In both Th1 and Th2 cells, *Il-17* and *Il-17F* promoter regions are hypoacetylated which corresponds to a lack of *Il-17* expression in these cells (504). In Th17 cells, hyper H3 actelylation of *Il-17* locus allows *Il-17* transcription (505). Cytokine stimulation can initiate these epigenetic modifications contributing the T-cell plasticity. IL-12 can cause *IL-17* gene silencing via epigenetic remodelling of the *RORc* locus. In Th17 cells the *TBX21* promoter encoding Tbet and the *IFNy* promoter are maintained in a 'poised' state, which is thought to contribute to the capacity of Th17 cells to readily switch to express *IFNy* (503, 506). TGF-β signalling may be important to preventing IFNy expression in Th17 cells (503). THi stimulation may result in repressive epigenetic modifications to the *IL-17* promoter potentially explaining why NFATc1 does not bind. It would therefore be interesting to assess the acetylation of H3 and/or further DNA modifications in THi vs TLo stimulated cells. Investigations into thymic T-cell development indicate that TCR signalling can control epigenetic modifications within T-cells (507). In addition, CD28 co-stimulation induced stable histone acetylation of the *Il-2* promoter/enhancer allowing IL-2 expression in peripheral T-cells (508). Investigating the contribution of TCR signal strength to the control of epigenetic modifications within T-cell may also reveal additional insights into how cytokine and TCR signals integrate to regulate T-cell function.

3.5.4 TGF-β function in THi and TLo cultures

TGF-β is important for the development of both Th17 cell and Treg cells. In the absence of inflammatory cytokines, Foxp3 expression and iTreg differentiation is induced, but in the presence of IL-1β or IL-6, RORγt expression and Th17 cell differentiation is induced. High concentrations of TGF-β are inhibitory to Th17 cell development, but favour Treg development (383). The T-cell culture model described in this thesis was set up with serum-free media containing a TGF-β-free serum-replacement. This prevents suppressive effects of endogenous TGF-β associated with the use of bovine sera (323). Addition of TGF-β at a concentration of 10 ng/mL was found to be optimal in supporting Th17 cell responses in TLo cultures. However, varying TGF-β concentrations had no effect on THi Th17 cell responses. Expression of LAP-TGF-β is higher on THi cells than TLo however the inhibition of TGF-β signalling did not result in increased Th17 cell responses in THi cells. In TLo cultures, TGF-β signalling appears to be critical to Th17 cell responses (Figure 35). Inhibition of TGF-β signalling reduced IL-17 expression and increased the number of IFNγ-producing T-cells. Reduced TGF-β signalling correlated with an increased IL-17/IFNγ dual positive response; this may be due to TGF-β capacity to control the *IFNγ* 'poised' transcriptional state (503). Expression of latent TGF-β on the cell surface is a proposed mechanism by which Treg deliver TGF-β to mediate their suppressive effects (509). However, a remaining possibility is that THi stimulated cells have a reduced capacity to activate LAP-TGF-β. For example, it has been shown that reduced expression of TGF-β-activating factors (e.g. thrombospondin) can lead to an accumulation of latent TGF-β on the cell surface (510). Thus, the observed high expression of latent TGF-β on THi cells does not necessarily result in enhanced TGF-β activity. Furthermore, T-cell responsiveness to TGF-β can also be modulated by TCR stimulation. Expression of TGF-βRII is reduced following TCR stimulation although IL-10 signalling is capable of enhancing TGF-βRII expression and restoring TGF-β responsiveness (478). T-cell responsiveness to TGF-β can also be reduced by CD28 co-stimulation in the presence of high avidity TCR stimulation (479). The expression of the TGF-β receptors by THi and TLo cells was not examined. Differences in receptor expression would contribute to TGF-β sensitivity and signalling of THi cells and therefore would also contribute to differences in the capacity to generate a Th17 cell response (511). However, the decrease in Foxp3 expression

that resulted from TGF-β blockade in both HiD and LoD cultures indicates that THi/TLo cells are responsive to TGF-β (Figure 42) and so this is most likely not the reason for low Th17 in THi, although further experiments would be required to confirm this finding.

3.5.5 Are THi regulatory T-cells?

An increased proportion of THi stimulated cells expressed Foxp3 at day 3 and 6 compared to TLo cells (Figure 41). Foxp3 expression was detected using the 206D antibody clone, which is not associated with non-specific binding following T-cell activation (512). The transiency of Foxp3 expression in THi and TLo cells suggests that the Foxp3 expressed is activation-induced in both cultures. A greater number of THi cells expressed Foxp3 than TLo, but as Foxp3 expression was transient, it does not indicate that THi stimulated cells are regulatory T-cells. However, a population of IL-10-secreting Foxp3$^-$ regulatory T-cells has been described in TCR transgenic mice. IL-10-secreting Foxp3$^-$ Treg were induced *in vitro* in the presence of immunosuppressive drugs vitamin D3 and Dexamethasone, or *in vivo* via high dose peptide administration (154). It would be interesting to assess if the THi stimulated cells that have not developed into either an IL-17- or IFNγ-producing T-cell are a type of iTreg. Collectively, these data indicate that the THi stimulated cells display a number of Treg characteristics: high surface TGF-β, high IL-10 secretion, higher basal nuclear NFAT, higher (although transient) Foxp3 at day 6. Adaptive T-reg have been observed to be induced by low strength stimulation (425). The inhibitory effect of high strength stimulation on Foxp3 expression is prevented by TGF-β and IL-2, both of which are present during THi/TLo cultures (483). Assessing the capacity of THi cells to inhibit proliferation may indicate their regulatory potential.

3.5.6 Inhibition of Foxp3 expression

The increased expression of Foxp3 with the THi population did not appear to be the cause of reduced IL-17 responses in THi stimulated cells. Reducing Foxp3 expression by either resting T-cells until day 10 or inhibiting TGF-β signalling did not increase the proportion of IL-17 producers. Furthermore, THi stimulation of IPEX patient cells which have a defective form of Foxp3 also did not increase Th17 cell responses. One caveat in

this experiment is that the Foxp3 mutation is present within exon 10. Foxp3 binds to RORc via peptides encoded within exon 2, therefore the mutated Foxp3, although non functional in the patient, has possibly retained the capability to bind to RORc and inhibit *IL-17* expression (373, 513). To eliminate the possibility that increased expression of Foxp3 in THi cultures inhibits Th17 cell generation, development of a strong human T-cell Foxp3 knockout system would be required, or, alternatively, a sample from an IPEX patient with a mutation present in the RORc binding domain or absence of the Foxp3 protein would be helpful in resolving this issue. However, overall, investigations conducted so far do not indicate that activation-induced Foxp3 prevents THi stimulated Th17 cell responses. Furthermore, these data are in agreement with findings that show activation-induced Foxp3 does not confer suppressive effects on secretion of IL-2 or IFNγ from T-cells (444).

3.5.7 Conclusion

Together the data presented within this chapter have indicated that TLo stimulation promotes Th17 cell responses via a Ca^{2+}/NFATc1 dependent pathway. High strength TCR stimulation most likely results in increased Ca^{2+} signalling, which is inhibitory to Th17 cells responses by either directly affecting the capacity of NFATc1 to bind to the *IL-17* promoter, or potentially by affecting the expression of NFATc1 transcriptional partners, or by modulating DNA accessibility. Further investigations are required to precisely investigate how stimulation strength regulates the transcriptional apparatus leading to *IL-17* transcription and therefore Th17 cell responses. However, my research interests turned towards investigating how an RA associated genetic polymorphism, which modulates TCR signalling strength may affect Th17 cell responses.

4 Chapter 4: The regulation of Th17 cell responses by PTPN22

4.1 Introduction

I established in Chapters 2 and 3 that the strength of T-cell stimulation influenced Th17 cell responses. A low strength stimulus, administered by either anti-CD3/anti-CD28 coated beads or APC, promoted Th17 cell responses (514). Increased Th17 responses are known to contribute to the pathogenesis of a number of autoimmune diseases including RA (515). Genome wide association studies (GWAS) have uncovered an increasing number of genetic variants associated with increased susceptibility to RA that encode molecules involved in T-cell receptor (TCR) signalling and activation, including *CD28*, *CTLA4, CD2-CD58, PTPRC, CD40, CD247*, *PTPN22*, *PTPN2, IL2RA*, *IL2RB, RBPJ, PIP4K2C*, and *REL* (516). Notable among these are the protein tyrosine phosphatases (PTP) *PTPN22*, *PTPRC* and *PTPN2,* which function as negative regulators of TCR signalling and set the thresholds of T cell activation that control T-cell differentiation and effector function (404, 489). Thus, inherited perturbations in T-cell activation may underpin the development of autoimmunity. One of the strongest genetic risk factors for RA is a missense single nucleotide polymorphism (SNP) at the *PTPN22* locus. *PTPN22* encodes Lyp (or PEP in mice) and the C1858T SNP in *PTPN22* results in an arginine-tryptophan substitution at codon 620 (R620W) (517).

4.1.1 Regulation of TCR signalling by Lyp

PTP Lyp/Pep is known to negatively regulate immune-receptor signalling cascades, notably the src family kinases (SFKs) Lck, Zap-70, Fyn and TCRζ tyrosine residues in T-cells and Syk and Fyn in B-cells (518). In addition, Lyp also regulates FcγR signalling in NK cells and has been implicated in regulating DC maturation pathways (519). Lyp/Pep is a 105 KDa protein characterised by a 300 amino acid N terminal protein tyrosine phosphatase (PTP) motif and a 200 amino acid C terminal containing four polyproline motifs (P1-4) (520). The P1 motif, closest to the N terminal, allows Lyp to interact with the SH3 domain of protein tyrosine kinase (PTK) Csk, allowing negative regulation of TCR signalling. In resting T-cells, tyrosine phosphorylated Lyp is detectable, but is strongly induced by TCR signalling (521). Lyp negatively regulates downstream TCR

signals in part by dephosphorylating the autophosphorylation sites on TCR signalling molecules, including Lck, Fyn and Zap-70 (522). Lck is a SFK that positively regulates TCR signalling by phosphorylating ITAMs on TCRζ, CD3 and ZAP-70 to increase TCR signalling, events which are prevented by active Lyp (518). Recent investigations have found that Lyp forms a constitutive complex with Lck (521). Dephosphorylation of Lck Y505 and autophosphorylation of Y394 activates Lck. Lyp inactivates Lck via the Y394 autophosphorylation site. Activated Lck is also capable of inactivating Lyp by phosphorylating Lyp Y536 (521). ZAP-70 is a further substrate for Lyp, but Lyp may also negatively target ZAP-70 signalling intermediates such as Grb2 and Vav, affecting CD28/CTLA4 signalling and regulation (523). Further research is required to fully understand the molecular interactions made by Lyp and the signalling effects that occur.

4.1.2 C1858T conferred changes to Lyp function

The R620W substitution occurs within the P1 motif, which is critical for Lyp binding to Csk. Lyp acts with Csk to inhibit TCR signalling; therefore the mutation may be expected to be a loss-of-function mutation. However, the majority of data suggest the opposite is true; the substitution disrupts constitutive Csk dependent Lyp-Lck interactions, which reduces Lyp inactivation and increases T-cell signalling inhibition (521). Moreover, a recent investigation has revealed that Lyp can inhibit T-cell activation when dissociated from Csk (524). Further observations suggesting that the C1858T SNP confers a gain-of-function mutation include: increased catalytic activity of Lyp phosphatase, and decreased downstream Ca^{2+} signalling (525). In addition, the risk T-allele has a dose dependent effect on reducing TCR signalling. In the majority of autoimmune disorders associated with the C1858T SNP, including RA, the odds ratio (OR) for developing disease is significantly greater for 1858T homozygotes than the OR for heterozygotes (526) This is proposed to be due to functional compensation of LypW by LypR, which is not possible in LypW homozygotes.

4.1.3 PTPN22 and effector T-cell responses

PTPN22 1858T SNP can alter the peripheral T-cell repertoire (527, 528). The strength of TCR signal is critical to thymic selection and increased Lyp function can increase the

TCR signalling thresholds required for negative selection which is proposed to allow the release of self reactive T-cells into the periphery (529). Furthermore PTPN22 can alter the development of nTreg (530). In comparison to wild-type, PTPN22 knock-out mice had increased Ca^{2+} flux and an enhanced proportion of thymic nTreg; implying that the PTPN22 gain-of-function mutation may affect Treg development. Two further studies have demonstrated in human C1858T genotyped T-cells that TCR signalling, measured by Ca^{2+} flux, is decreased in T-allele carrying donors, supporting a gain-of-function mutation (528, 531). In addition, the number of effector T-cells was also increased in human T-allele carriers (528); and IL-2 and IL-10 production were reduced following TCR stimulation. Although data obtained from human cells indicates that C1858T is a gain-of-function mutation, this has not been supported in all experimental models. One study suggested that PTPN22 is a loss-of-function mutation (532), which was also observed in a study using a knock-in mouse strain where R619W mutation (equivalent of R620W) has been introduced into the germline (519).

I previously observed that Th17 cell responses were promoted by low strength T-cell stimulation and that this occurred by a NFAT/Ca^{2+} dependent mechanism (514). The presence of the minor T allele has been observed in human cells to affect T-cell signal strength by decreasing Ca^{2+} mobilisation and reducing T-cell IL-10 secretion (528). I proposed that the decrease in TCR signalling previously found to occur in PTPN22 T-allele carriers would promote the Th17 cell response, when compared to major C-allele carrying homozygotes.

4.2 Aims

In order to investigate the above hypothesis this Chapter aimed to:

- Genotype healthy donors for the PTPN22 C1858T allele
- Investigate the proportion T-cell phenotype of donor PBMC and assess if associations between genotype and IL-17 and IFNy expression can be found
- Assess the proportion and absolute numbers of cells expressing IL-17$^+$ and IFNy$^+$ that arise from the culture of PTPN22 genotyped memory CD4$^+$ T-cells stimulated with either: THi, Tint, or TLo stimulation strengths
- Assess the cytokine secretion profile associated with PTPN22 genotyped memory CD4$^+$ T-cells stimulated with either THi/Tint/TLo.

4.2.1 Experimental approach

To assess what affect the PTPN22 C1858T polymorphism has on Th17 cell responses 'healthy human' donors were genotyped for the C1858T polymorphism by restriction fragment length polymorphism. Either PBMC or Memory CD4+ T-cells (purity >95%) were cultured on 24 well plates in IMDM media supplemented with 5% serum replacement in a volume of 1 mL containing 1 x10^6 cells. PBMC or Memory CD4$^+$ T-cells were activated with either 1:1 1:10 or 1:50 bead:T-cell ratios. Cultures were performed in the presence of pro-Th17 cell cytokines IL-1β, IL-23, and TGF-β each at a concentration of 10 ng/mL. T-cells were cultured as depicted in Figure 47 until day 6. To assess differences in the primary response to stimulation of the genotyped donor T-cells, cell free supernatants were obtained and assessed for the presence of IL-17, IFNy and IL-10 by ELISA. At day 6 T-cells were restimulated with PMA/Ionomycin for one hour and then cultured in the presence of Brefeldin A for a further 4 hours. Alive T-cell counts were also determined by trypan blue staining and used in combination with flow cytometry data in order to calculate the absolute number of cytokine expressing cells within the cultures. These data from each genotyped donor were then assessed for correlations between PTPN22 C1858T genotype and Th17 cell response.

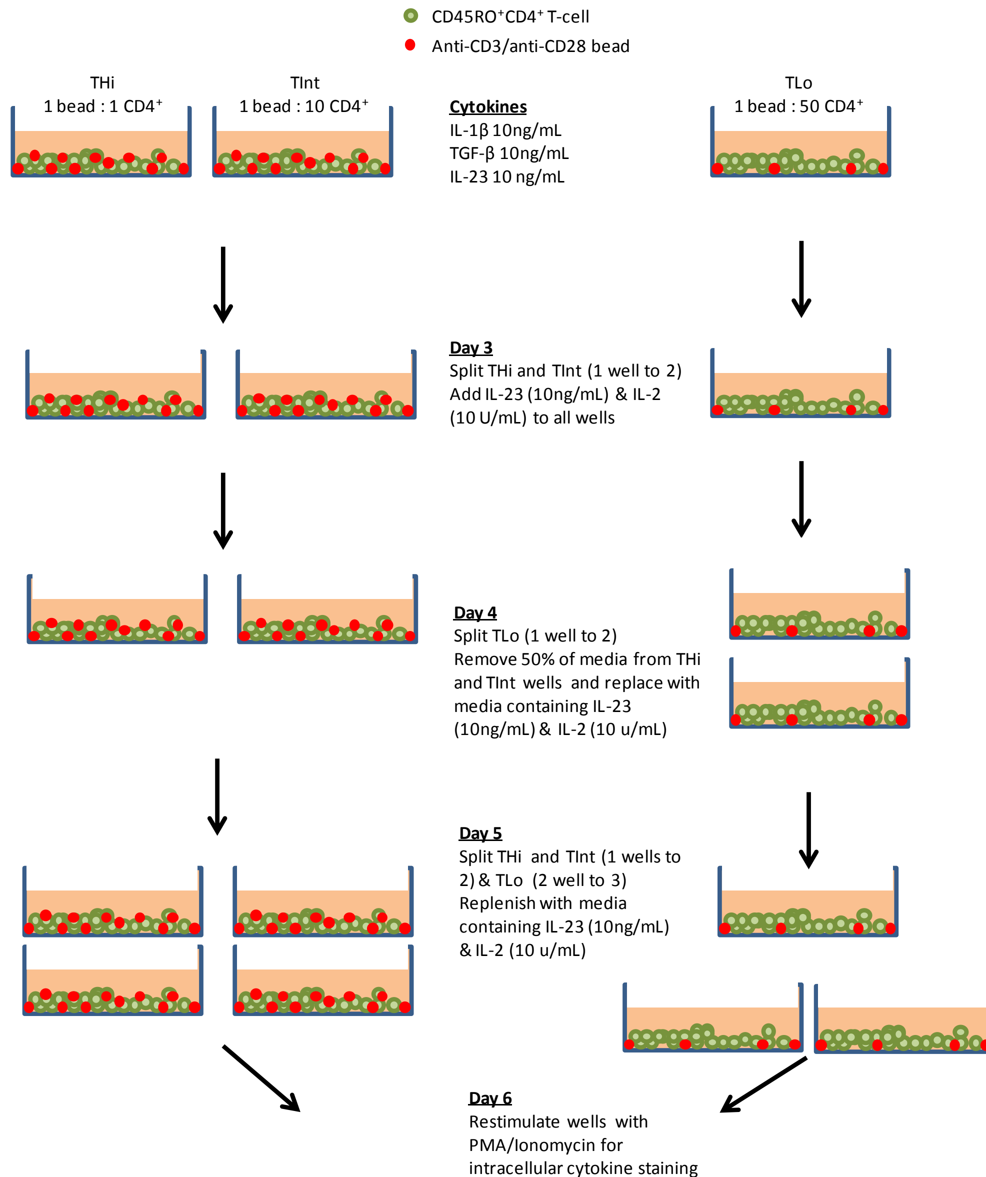


Figure 47. An experimental model to assess how the presence of the PTPN22 C1858T variant affects Th17 cell responses. 1 x 10^6 CD45RO$^+$CD4$^+$ T-cells were cultured in IMDM with 10% serum replacement in a 1mL volume on a 24 well plate. T-cells were cultured with either 1x10^6 (THi), 1x10^5 (Tint), or 0.02x10^6 (TLo anti-CD3/anti-CD28 beads in the presence of proTh17 cytokines IL-1β, IL-23 and TGF-β each at 10 ng/mL. T-cells were incubated at 37˚C 5% CO_2 for 6 days. During the 6 day culture cells were split as indicated above. Splitting of wells was based upon lightening of the media and a high cell confluency within the well. Day 4 and 5 media was replenished to a volume of 1 mL with IMDM + 10% serum replacement containing 10 ng/mL of IL-23 and 10 U/mL of IL-2. At day 6 T-cells were restimulated with PMA/Ionomycin as described in section 7.5.2 and results were analysed as indicated in Figure 11.

4.3 Results

4.3.1 Genotyping healthy donors

20 healthy Caucasian European blood donors were PTPN22 genotyped. Genotyping was performed using optimised primers for a C1858T natural restriction site by restriction fragment length polymorphism (RFLP). Table 1 shows that of the 20 individuals genotyped 16 were homozygous for the major C-allele (1858CC), 3 were heterozygous (1858CT), and 1 was homozygous for the minor T-allele (1858TT). My cohort had more than double the expected T-allele frequency of the UK (~10.5%), however only one donor was homozygous for the minor T-allele.

Number of donors	PTPN22 1858 genotype	Age range	Males	Females
16	CC	22-44	6	10
3	CT	23-56	1	2
1	TT	25	1	0

Table 1. PTPN22 genotypes of healthy donors. Whole blood from 20 healthy individuals was genotyped by restriction fragment length polymorphism for PTPN22 C1858T SNP.

4.3.2 Genotyped donor PBMC IL-17 responses

The T-allele is known to predominantly affect T-cells (particularly memory T-cells (528)) and B-cells and therefore variations in the proportion of T-cells within PBMC may affect the cytokine response observed following *in vitro* culture. I therefore firstly assessed the proportion of $CD3^+/CD4^+$ T-cells and memory $CD4^+$ T-cells present within the PBMC of genotyped donors. PBMC were isolated from genotyped donors and the proportion of *ex vivo* $CD3^+CD4^+$ T-cells and $CD3^+CD4^+CD45RO^+$ were determined by surface staining. Figure 48 A and B demonstrate that the proportion of $CD4^+$ T-cells and $CD4^+CD45RO^+$ T-cells between individual donor PBMC were highly variable, and did not appear to correlate with PTPN22 genotype. One explanation for variability in T-cell proportion may be due to differences in donor age; however, the variability observed, particularly in the proportion of memory T-cells, may mask any potentially subtle effects of PTPN22.

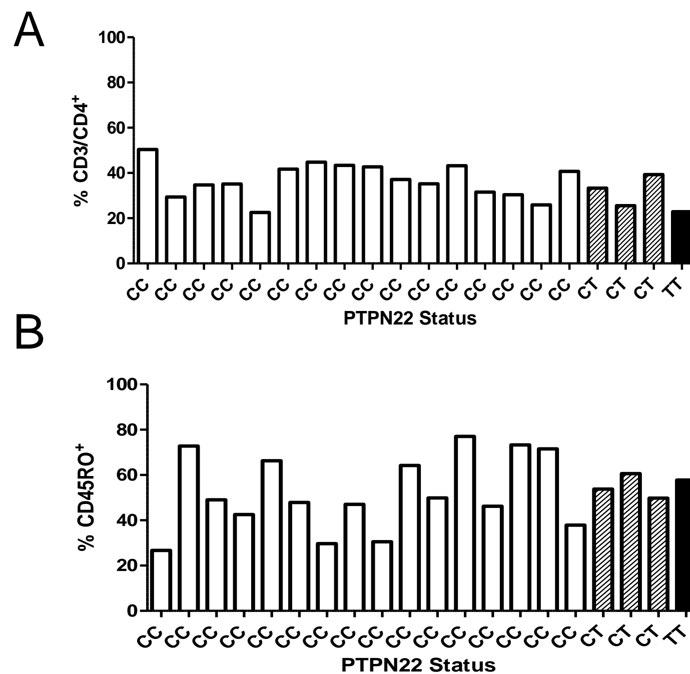


Figure 48. CD3/CD4 and CD45RO expression by PTPN22 genotyped donor PBMC populations. Human PBMC were isolated from genotyped donors. The proportions of CD3/CD4$^+$ and CD45RO$^+$ T-cells were determined by surface staining PBMC at day 0 for CD3, CD4 and CD45RO expression by flow cytometry. (A) The percentage of CD3/CD4$^+$ cells present within the total PMBC population acquired is depicted for each donor. (B) CD3/CD4$^+$ T-cells were gated and the proportion of CD45RO$^+$ T-cells present is depicted. Each bar represents one donor.

Nevertheless, the PBMC of the genotyped donors were cultured under pro-Th17 cytokine conditions at three different stimulation strengths THi (1 bead:1 T-cell), Tint (1 bead:10 T-cell) and TLo (1 bead:50 T-cell). As the PTPN22 SNP may alter the TCR signalling thresholds, different stimulation strengths were assessed to maximise the chance of observing if the PTPN22 risk-allele alters Th17 responses. As observed in Chapter 2, generally the highest IL-17 response is observed under TLo conditions (Figure 49). However the response is highly variable between donors and does not appear to correlate with PTPN22 genotype. Variations in the proportion of memory CD4$^+$ T-cells may contribute to the range of IL-17 responses and may alter or mask any potential effects of the PTPN22 allele variants. In addition, IL-17 can also be produced by CD8$^+$ T-cells (termed Tc17 cells) and γδT-cells within the PBMC population which may further any mask effect of PTPN22 variants (533, 534). Due to these variations, I decided to refine the experiment by selecting memory CD4$^+$ T-cells from PTPN22 C1858T genotyped donors.

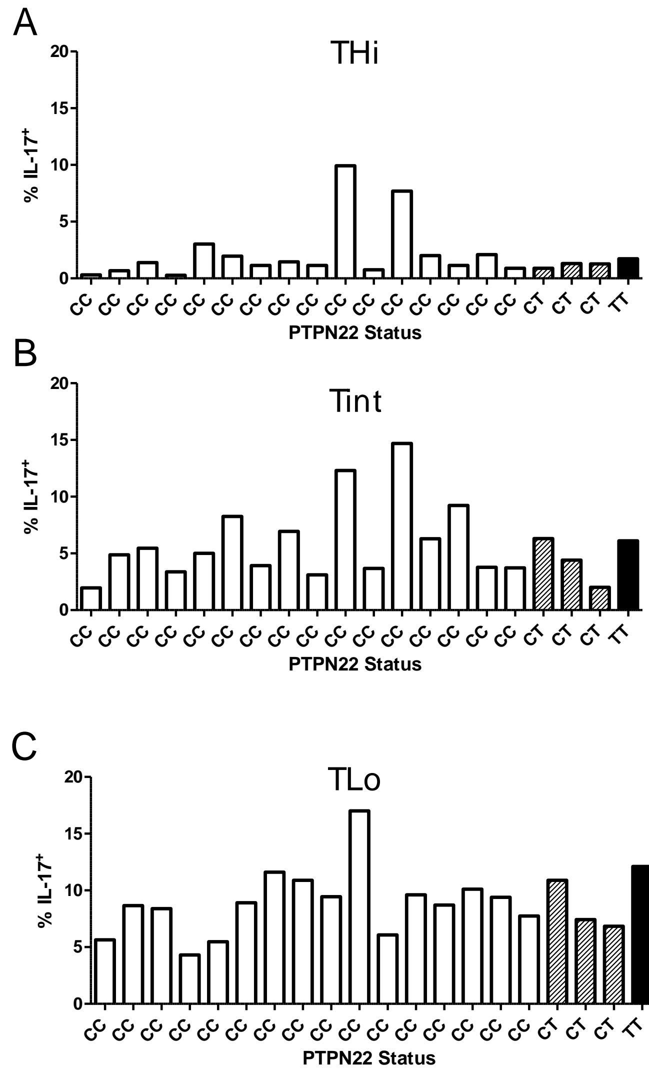


Figure 49. Proportion of IL-17 response is variable between genotyped donor PBMC populations. Human PBMC were cultured under THi (1 bead: 1T-cells), Tint (1 bead: 10 T-cells), or TLo (1 bead: 50 T-cells) conditions in the presence of pro-Th17 cytokines. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17-expressing cells were determined by intracellular staining followed by flow cytometry (A) THi, (B) Tint, (C) TLo.

4.3.3 Proportion of intracellular cytokine expression in memory CD4$^+$ T-cells

Memory T-cells were isolated from 8 donors including all 3 T-allele carrying donors. T-cells were stimulated under THi, Tint, and TLo conditions in the presence of pro-Th17 cell cytokines and the T-cell phenotype on day 6 was determined by intracellular cytokine staining. I firstly examined if the C1858T genotype correlated with differences in the proportion of IL-17$^+$, IL-17/IFNy$^+$, and IFNy$^+$ cells. As previously observed, TLo stimulation generally favoured IL-17 responses (Figure 50 TLo, right column), though in some donors Tint (Figure 50 middle column) also resulted in high proportions of IL-17$^+$

cells. The homozygote 1858T donor has a marked increase in IL-17 expression as stimulation strength decreased. Within some of the 1858CT and 1858CC donors the increase in the proportion of IL-17$^+$ cells was less marked, however this was variable.

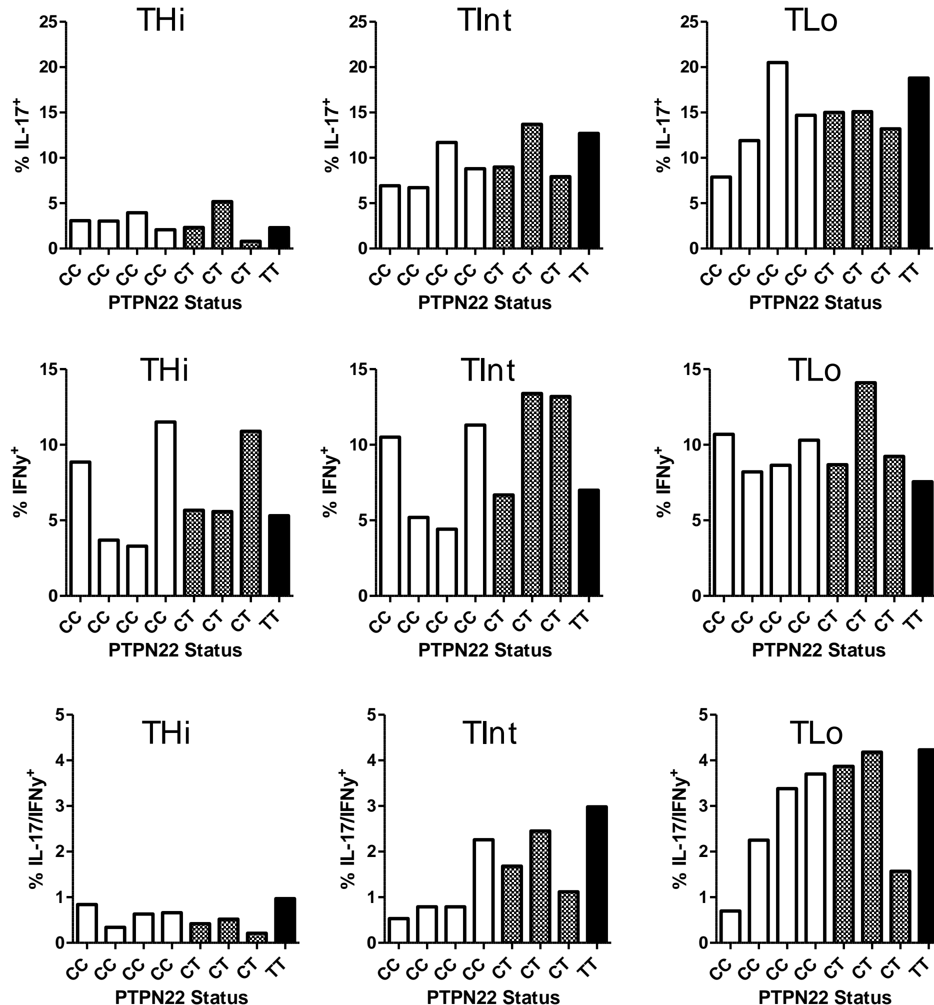


Figure 50. Percentage of individual donor cytokine responses. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence of pro-Th17 cytokines. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17 and IFNγ expressing cells were determined by intracellular staining followed by flow cytometry. Graphs depict the individual donor responses; the percentage of IL-17$^+$ cells (top row) IFNγ$^+$ (middle row) and IL-17/IFNγ$^+$ (bottom row).

As no striking pattern of cytokine expression was observed in Figure 50, I grouped the data based upon C1858T genotype. Interestingly, at a Tint stimulus the proportion of IL-17$^+$ cells appeared to increase as the T-allele dose increased, although this was not significant. However at a TLo stimulus, although the minor T allele homozygote had the highest proportion of IL-17$^+$ cells, a dose dependent effect was not observed. Compared to IL-17, the proportion of IFNγ producing cells were less affected by

stimulation strength and responses did not correlate to PTPN22 genotype. However, the proportion of IFNy$^+$ cells and the capability of IFNy responses to be generated may be limited by the presence of a pro-Th17 cytokine *milieu* and the variations observed may be due to differences in the proportion of *ex vivo* IFNy$^+$ cells. The most striking results were observed within the IL-17$^+$/IFNy$^+$ population under Tint and TLo conditions where a trend of increased IL-17$^+$/IFNy$^+$ cell proportions occurred as the dose of the T-allele increased. Although the proportion of IL-17$^+$/IFNy$^+$ expressing cells was low this phenotype has been described as highly pathogenic (330).

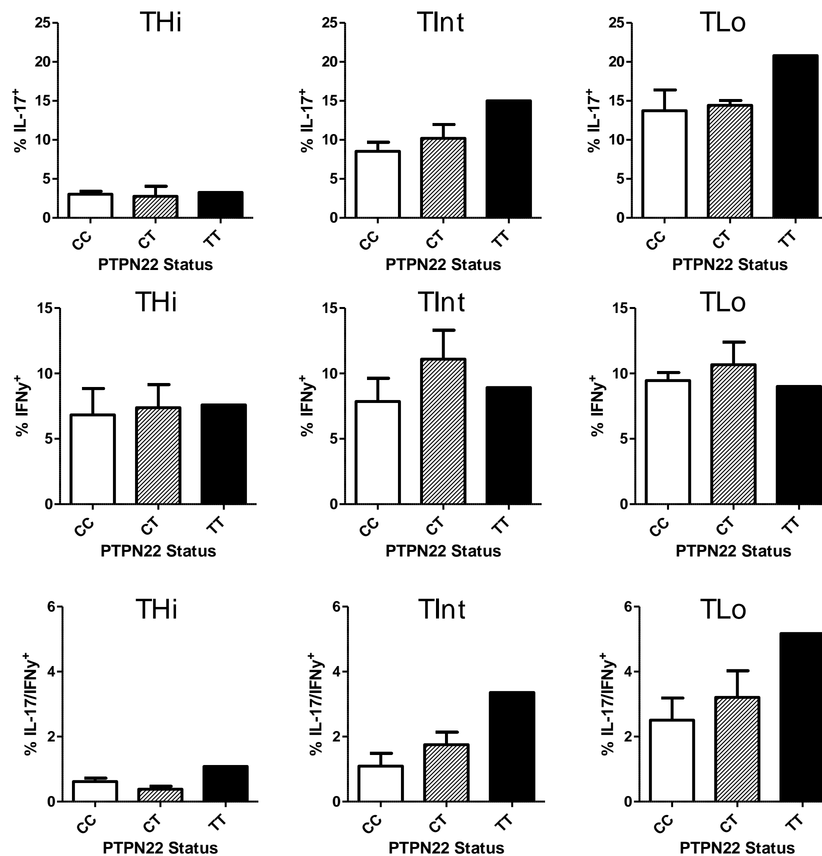


Figure 51. Percentage of cytokine response grouped by PTPN22 C1858T genotype. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence of pro-Th17 cytokines. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17 and IFNy expressing cells were determined by intracellular staining followed by flow cytometry. Graphs depict the proportion of cytokine response grouped by genotype. CC N=4, CT N=3, TT N=1. The IL-17+ cells (top row) IFNy+ (middle row) and IL-17/IFNy+ (bottom row).

As the cohort contains only one T-allele carrying donor I could not draw statistical significance from these results. I therefore assessed if the presence of the T-allele affects Th17 cell responses. I grouped the CT and TT donor results and compared to the C-allele homozygotes; however, despite increasing the sample size of each

population no statistical significance was observed. The data demonstrate a trend that at a Tint stimulus the presence of the T-allele marginally increased the proportion of IL-17$^+$ cells, but also the proportion of IFNy$^+$ cells. The analysis further demonstrates that at a TLo stimulus the proportion of IL-17$^+$ and IFNy$^+$ cells were similar between C-allele and T-allele groups. The most striking observation remains within the IL-17$^+$/IFNy$^+$ population at the Tint and TLo stimuli.

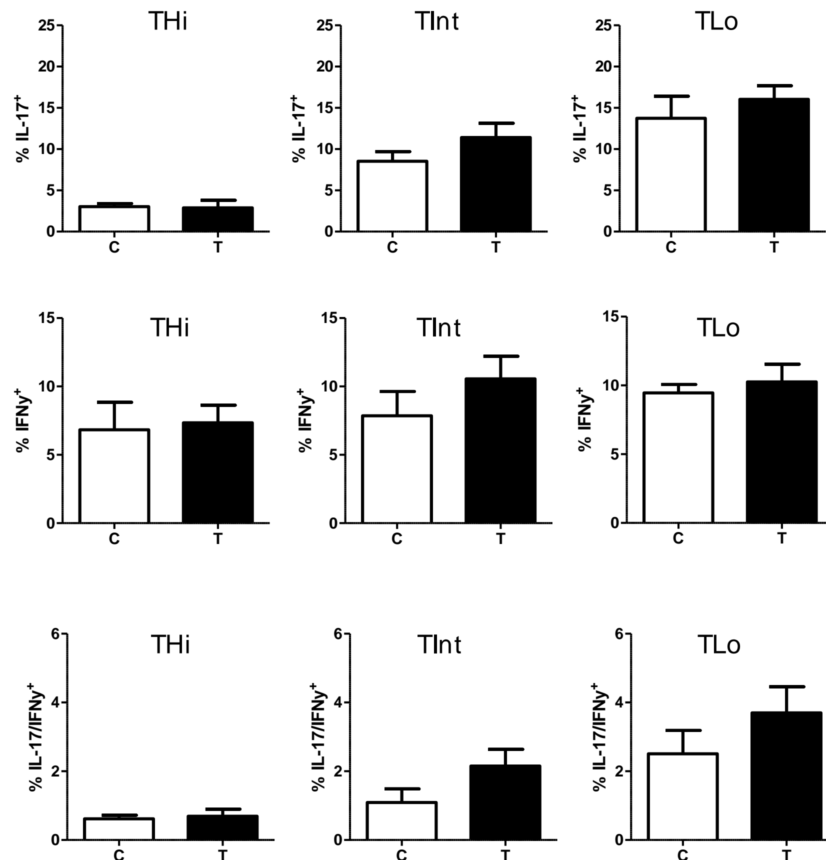


Figure 52. Percentage of cytokine response from T allele carriers vs non-carriers. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence of pro-Th17 cytokines. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17 and IFNy expressing cells were determined by intracellular staining followed by flow cytometry. Graphs depict the proportion of cytokine response grouped non-T-allele carriers vs T-allele carriers. C N=4, T N=4. The IL-17+ cells (top row) IFNy+ (middle row) and IL-17/IFNy+ (bottom row).

As I only had one T-allele donor for my experiments, I checked the reproducibility of the current data by repeating the T-allele donor data. I repeated the experiments with T-allele donor memory T-cells one month later. Figure 53 demonstrates that the proportions of cytokine producing T-cells are reproducible at a Tint stimulus with the same donor.

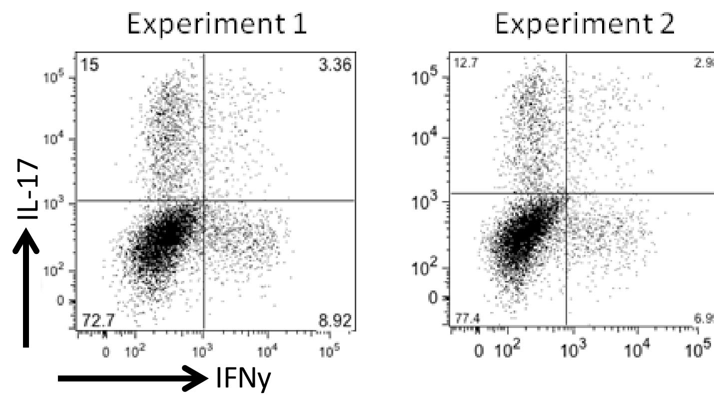


Figure 53. T-allele homozygote T-cell response is reproducible. Human memory T-cells from T-allele homozygote donor where isolated from peripheral blood one month apart. Memory T-cells were cultures with Tint stimulation in the presence of pro-Th17 cytokines. After 6 days T-cells were restimulated for 5 hours with PMA/Ionomycin, the last 4 hours in the presence of Brefeldin A. Proportions of IL-17 and IFNy expressing cells were determined by intracellular staining followed by flow cytometry. Each plot represents an independent experiment from the same donor.

Comparing C-allele homozygotes to T-allele carriers did not demonstrate any statistical significance. I therefore calculated the sample size required for the currently observed differences to be statistically significant and the power of the current data. Table 2 demonstrates that the power of the C vs T allele differences observed were generally low (<0.2) or very low (<0.1). However, the more striking results (Tint IL-17$^+$ and IL-17$^+$/IFNy$^+$) observed in Figure 52 had the highest power of >0.2. For the currently observed differences to be statistically significant sample sizes in excess of 15 would be required. Increasing the sample sizes does not guarantee the statistical significance of the resulting data, but these analyses provide an indication of the scale required for future experiments.

Condition	Sample size C vs T for each group	Difference between % means	Standard Deviation	Power	Target Power	Sample Size for each group
THi IL-17	4	0.15	1.29	0.05	0.9	1580
Tint IL-17	4	2.86	2.89	0.22	0.9	23
TLo IL-17	4	2.28	4.30	0.10	0.9	76
THi IFNy	4	0.51	3.29	0.06	0.9	743
Tint IFNy	4	2.69	3.43	0.16	0.9	35
TLo IFNy	4	0.79	1.9	0.08	0.9	123
THi IL-17/IFNy	4	0.08	0.31	0.06	0.9	309
TInt IL-17/IFNy	4	1.06	0.79	0.36	0.9	16
TLo IL-17/IFNy	4	1.19	1.44	0.17	0.9	32

Table 2. Power and sample size analysis of percentage of cytokine responses, comparing C-allele to T-allele carrier responses. The proportion of cytokine response resulting from THi, Tint or TLo were grouped as C-allele homozygotes vs T-allele carriers, N=4 for each grouping. The difference between the group mean cytokine percentages were calculated and the average standard deviation of the two groups was used as the assumed standard deviation. A 2-sample T-test was used to calculate the power of the observed results and to calculate the sample size required for a target power of 0.9 (90% confidence in rejecting the null hypothesis). Power and Sample Size analysis was performed using Mini-tab II.

4.3.4 Absolute number of intracellular cytokine expressing memory CD4$^+$T-cells

I next assessed for differences in the absolute numbers of IL-17$^+$, IL-17$^{+/}$IFNy$^+$ and IFNy$^+$ cells resulting from THi, Tint and TLo stimulation conditions between PTPN22 genotyped donors. The number of alive T-cells at day 6 of culture was determined and in combination with day 6 intracellular cytokine staining, used to calculate the absolute number of cytokine producing cells. Figure 54 demonstrates that as stimulation strength decreased, the number of IL-17$^+$ and IL-17$^+$/IFNy$^+$ cells increased within each donor population, whereas the number of IFNy$^+$ cells, although variable, did not change as substantially in response to stimulation strength.

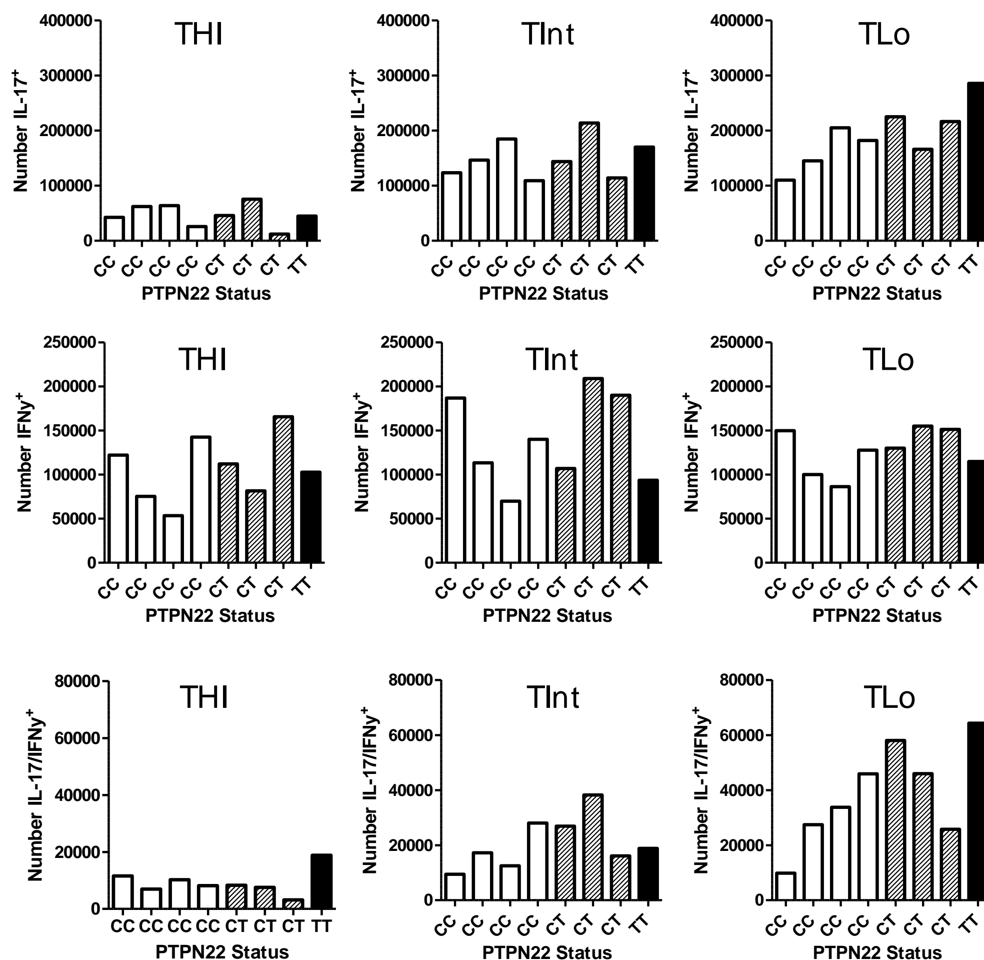


Figure 54. Absolute numbers of IL-17 and IFNy expressing cells. Human CD4$^+$ T-cells were cultured for 6 days under THi, TInt or TLo stimulation conditions. The number of living T-cells at day 6 was determined by trypan blue viability staining. The absolute number of IL-17$^+$ T-cells was calculated using the proportion of IL-17$^+$ cells determined by flow cytometry. Graphs depict the individual donor responses; the number of IL-17$^+$ cells (top row) IFNy$^+$ (middle row) and IL-17/IFNy$^+$ (bottom row).

As observed above, the variation between individual donors is high and an association with the number of cytokine-expressing cells is not readily observable. I therefore grouped the data based upon genotype. Figure 55 demonstrates that at a THi stimulus the number of cells producing IL-17, IFNy or IL-17/IFNy were similar between all three genotypes. In comparison to the proportion of cytokine producing cells only a minor increase in IL-17$^+$ cell numbers occurred in the presence of the T-allele at a Tint stimulus. Furthermore, the dose dependent increase in IL-17$^+$/IFNy$^+$ cell number was not observed. However, at a TLo stimulus the number of IL-17$^+$ and IL-17$^+$/IFNy$^+$ cells appeared to increase in a T-allele dose-dependent manner, however these observations are not significant and in a larger cohort these results may fall at the higher end of natural variation in this system.

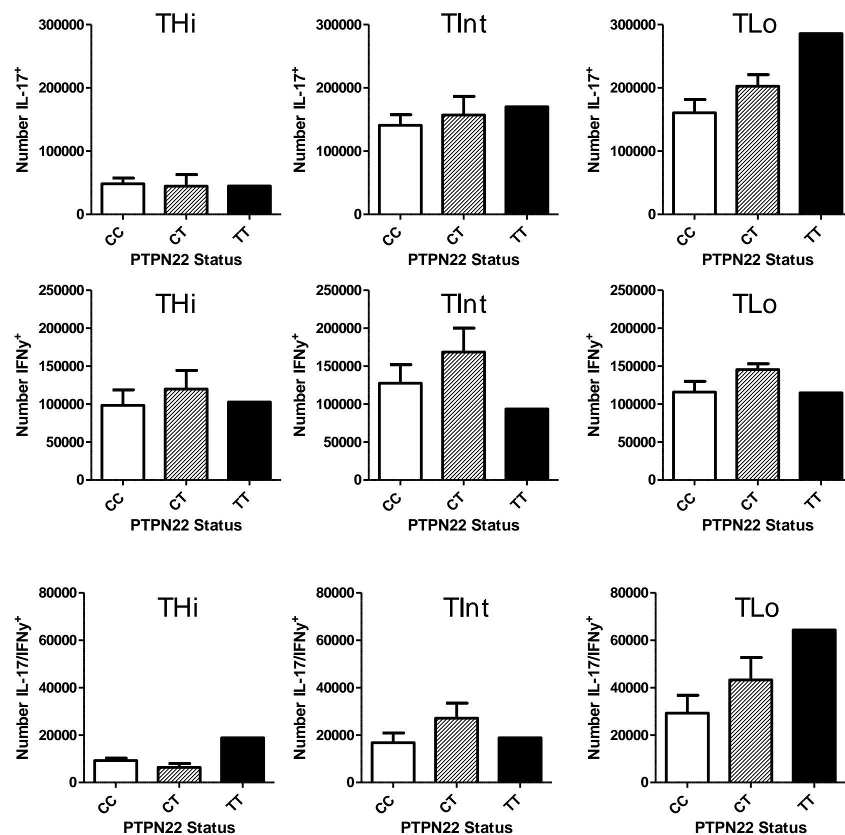


Figure 55. Absolute numbers of IL-17 and IFNy expressing cells grouped by PTPN22 C1858T genotype. Human CD4$^+$ T-cells were cultured for 6 days under THi, TInt or TLo stimulation conditions. The number of living T-cells at day 6 was determined by trypan blue viability staining. The absolute number of IL-17$^+$ T-cells was calculated using the proportion of IL-17$^+$ cells determined by flow cytometry. Graphs depict the number of cytokine producing cells grouped by genotype. CC N=4, CT N=3, TT N=1. The IL-17$^+$ cells (top row) IFNy$^+$ (middle row) and IL-17/IFNy$^+$ (bottom row).

4.3.5 Absolute T-cell response CC vs T-allele carriers

To assess if these results were statistically significant, I compared the C-allele homozygotes to the T-allele carriers, increasing the sample size of each group, however as observed above, no statistical significance was achieved (Figure 56).

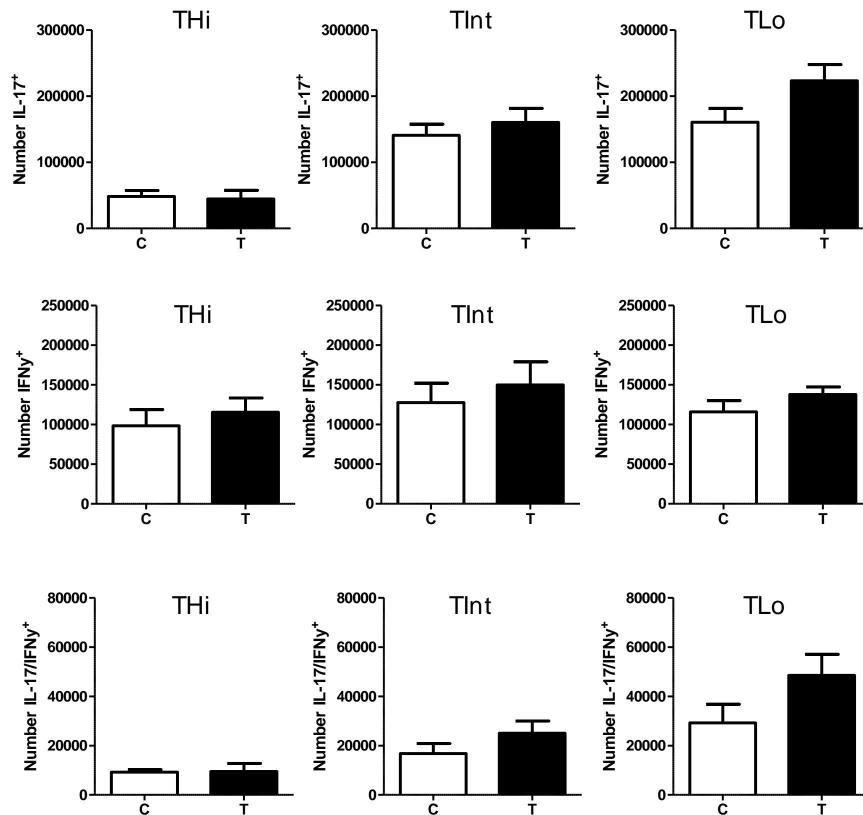


Figure 56. Absolute numbers of IL-17 and IFNy expressing cells grouped as T-allele carriers vs non-carriers. Human CD4$^+$ T-cells were cultured for 6 days under THi, TInt or TLo stimulation conditions. The number of living T-cells at day 6 was determined by trypan blue viability staining. The absolute number of IL-17$^+$ T-cells was calculated using the proportion of IL-17$^+$ cells determined by flow cytometry. Graphs depict the number of cytokine producing cells grouped non-T-allele carriers vs T-allele carriers. C N=4, T N=4. The IL-17$^+$ cells (top row) IFNy$^+$ (middle row) and IL-17/IFNy$^+$ (bottom row).

The analysis confirmed that the presence of the T-allele, in this model, had no effect on IFNy$^+$ cell numbers, but that under TLo stimulation the presence of the T-allele appeared to promote the number of IL-17$^+$ and IL-17$^+$/IFNy$^+$ cells, although this is not significant. To estimate the scale required for future experiments I performed power and sample size analysis (Table 3). The majority of results had low (<0.2) or very low (<0.1) power and correspondingly the sample size required for these differences to be significant were in excess of 30 donors/group. The difference in the number of IL-17

and IL-17/IFNy producing cells between C-allele homozygotes and T-allele carriers had the highest power of $\geq$0.3. The sample size required for the observed differences to be statistically significant was estimated to be 13-16 donors per group to reach a target power of 0.9. However, a weakness of these data is that the *ex vivo* proportion and absolute number of IL-17$^+$ and IFNy$^+$ cells were not assessed, which would have allowed the data to be normalised to the starting IL-17 and IFNy proportions.

Condition	Sample size C vs T for each group	Difference between means Cell number	Standard Deviation	Power	Target Power	Sample Size for each group
THi IL-17	4	3872	21957	0.05	0.9	13387
Tint IL-17	4	19454	37639	0.10	0.9	80
TLo IL-17	4	62640	45364	0.38	0.9	13
THi IFNy	4	17192	19963	0.18	0.9	30
Tint IFNy	4	23363	53564	0.08	0.9	112
TLo IFNy	4	21875	23583	0.2	0.9	26
THi IL-17/IFNy	4	250	4349	0.05	0.9	361
TInt IL-17/IFNy	4	8221	9033	0.19	0.9	27
TLo IL-17/IFNy	4	19286	16010	0.30	0.9	16

Table 3. Power and sample size analysis of absolute cell numbers, comparing C-allele to T-allele carrier responses. The proportion number of cytokine producing cells resulting from THi, Tint or TLo were grouped as C-allele homozygotes vs T-allele carriers, N=4 for each grouping. The difference between the group mean cytokine percentages were calculated and the average standard deviation of the two groups was used as the assumed standard deviation. A 2-sample T-test was used to calculate the power of the observed results and to calculate the sample size required for a target power of 0.9 (90% confidence in rejecting the null hypothesis). Power and Sample Size analysis was performed using Mini-tab II.

4.4 Secretion of IL-17, IFNγ and IL-10

I lastly assessed if the secretion of cytokines IL-17, IFNy and IL-10 would be affected by the presence of the 1858T allele. Prior investigations have revealed that IL-10 responses are decreased within T-allele carriers compared to C-allele carriers, whereas IFNy responses were not affected (528). Due to the low yield of memory T-cells from two donors, there were only sufficient T-cells from 6 donors to perform ELISA analysis on. T-cell cytokine secretion was assessed in 3 CC-homozygotes, 2 CT-heterozygotes and 1 TT-homozygote.

4.4.1 IL-17 secretion

To assess the primary cytokine secretion response, memory $CD4^+$ T-cells from genotyped donors were stimulated in the presence of pro-Th17 cell cytokines under THi, Tint, or TLo stimulation conditions for 72 hours. Supernatants were assessed for IL-17, IFNy, and IL-10 by ELISA. Figure 57 demonstrates IL-17 secretion from memory T-cells was highest within THi stimulated cultures. This was true for the secretion of all measured cytokines, which differs from the flow data, though is most likely due to the earlier time point assessed and the activation of *ex vivo* memory T-cells already capable of expressing IL-17, IFNy, or IL-10. At a THi stimulus all donors were observed to secrete a similar concentration of IL-17, except for one of the C-allele homozygote donors whose IL-17 response was ~3-fold higher than the other donors. To observe differences between genotypes, the data were grouped accordingly (Figure 58). The IL-17 response at THi stimulus appear greatest from the C-allele homozygotes, however, the outlying donor may have affected the result. IL-17 secretion from Tint and TLo stimulated cells, were very low compared to THi stimulation and differences between the genotypes were not observed. Similar observations were made when comparing C-allele homozygotes to T-allele carriers (Figure 59).

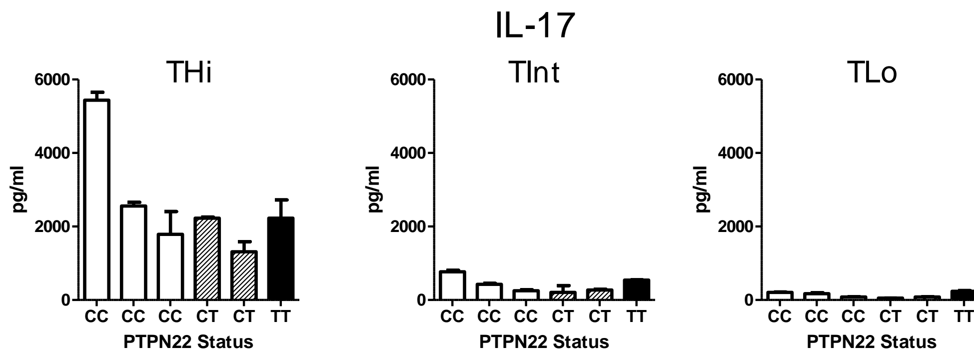


Figure 57. Individual donor secretion of IL-17. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. At day 3 supernatants were collected and IL-17 secretion was determined by ELISA. Graphs depict the individual donor responses performed in triplicate. Error bars represent SD.

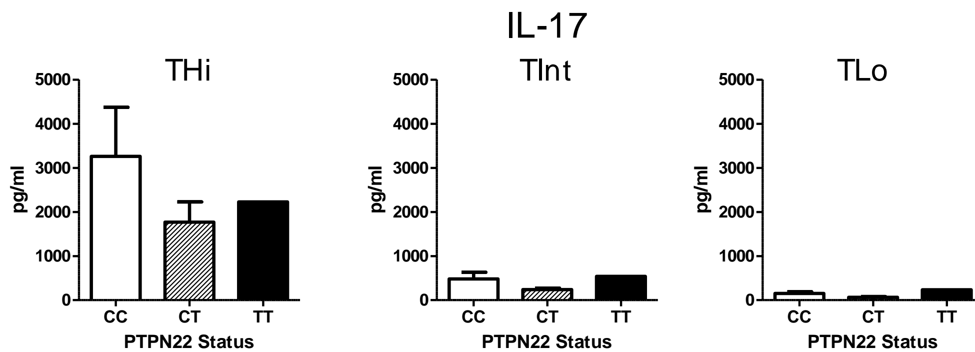


Figure 58. Secretion of IL-17 grouped by PTPN22 C1858T genotype. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. At day 3 supernatants were collected and IL-17 secretion was determined by ELISA. Graphs depict the mean genotype grouped donor responses. CC N=4, CT N=3, TT N=1. Error bars represent SD.

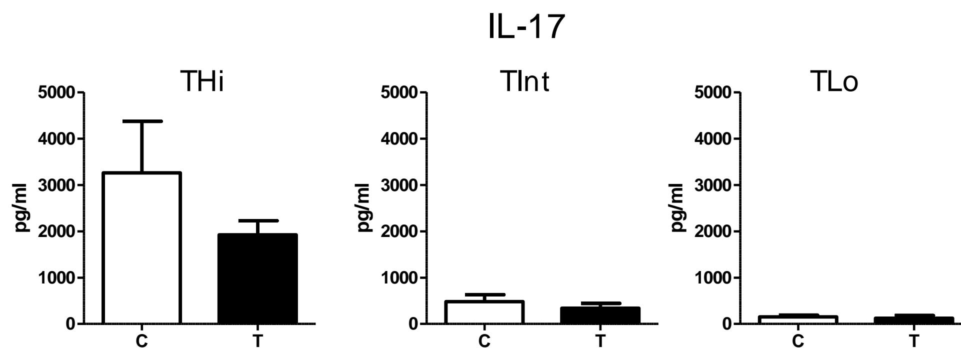


Figure 59. Secretion of IL-17 T-allele carriers vs non-T-allele carriers. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. At day 3 supernatants were collected and IL-17 secretion was determined by ELISA. Graphs depict the mean genotype grouped donor responses. Error bars represent SD. Graphs depict the mean genotype grouped donor responses. C N=4, T N=4. Error bars represent SD.

4.4.2 IFNγ secretion

I next examined the secretion of IFNγ after 72 hours THi, Tint, or TLo stimulation. The same donor that had 3-fold higher IL-17 also had 3-fold higher IFNγ secretion (Figure 60). The raised cytokine secretion from this donor is not likely to be due to a technical error as the error bars depict 3 technical repeats and minimal intra-assay variation is observed. Compared to the remaining donors the T-allele homozygote had the highest IFNγ secretion, though when data are compared based on genotype this response is similar to the C-allele homozygotes (Figure 61). The secretion of IFNγ appeared to be reduced in T-allele carriers compared to C-allele homozygotes (Figure 62), although the low sample size and variability in donor response means these data should be interpreted cautiously.

4.4.3 IL-10 secretion

I lastly assessed the secretion of IL-10 by PTPN22 C1858T genotyped donors. IL-10 secretion is highly variable between individuals at both THi and Tint stimulation strengths, though intra-assay variation is small (Figure 63). The variations observed in IL-10 secretion were not due to a single outlying donor. When the data were grouped according to donor genotype, the T-allele homozygote appears to have reduced IL-10 secretion compared to C-allele homozygotes at THi and Tint stimuli (Figure 64). However, a dose dependent effect with the heterozygote IL-10 response falling between C-allele and T-allele homozygotes was not observed. Most interestingly, at a Tint stimulus when T-allele carriers were compared to C-allele homozygotes (Figure 65), IL-10 secretion was lower in T-allele carrying donors, similar to that observed by Rieck *et al,* however this result was also not significant (528).

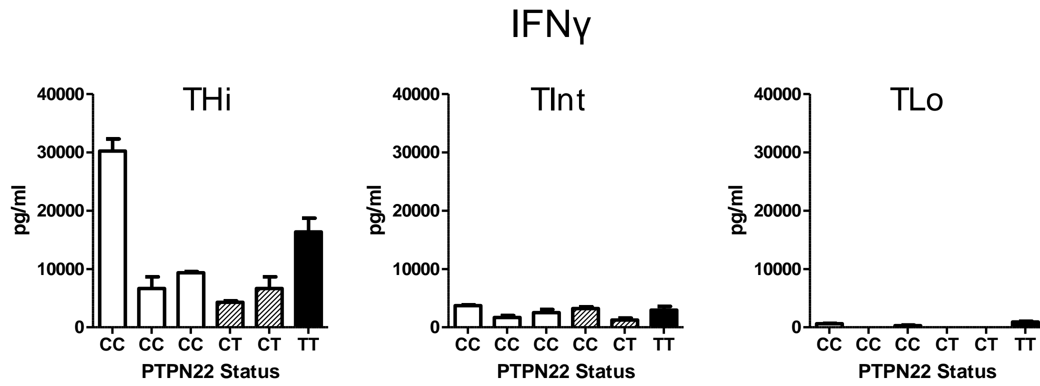


Figure 60. Individual donor secretion of IFNγ. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. A day 3 supernatants were collected and IFNγ secretion was determined by ELISA. Graphs depict the individual donor responses performed in triplicate. Error bars represent SD.

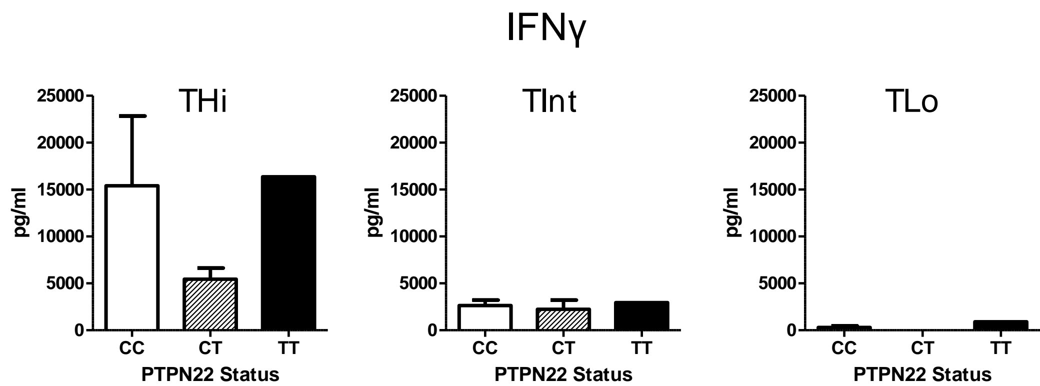


Figure 61. Secretion of IFNγ grouped by PTPN22 C1858T genotype. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. At day 3 supernatants were collected and IFNγ secretion was determined by ELISA. Graphs depict the mean genotype grouped donor responses. CC N=4, CT N=3, TT N=1. Error bars represent SD.

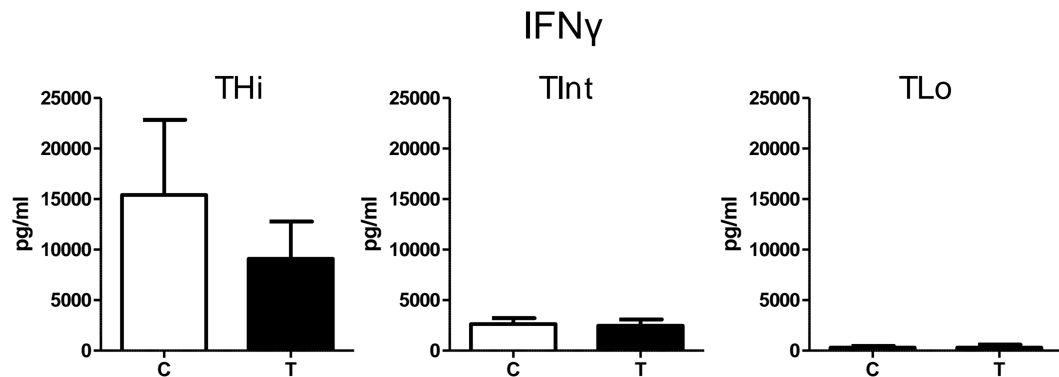


Figure 62. Secretion of IFNγ T-allele carriers vs non-T-allele carriers. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. At day 3 supernatants were collected and IFNγ secretion was determined by ELISA. Graphs depict the mean genotype grouped donor responses. Error bars represent SD. Graphs depict the mean genotype grouped donor responses. C N=4, T N=4. Error bars represent SD.

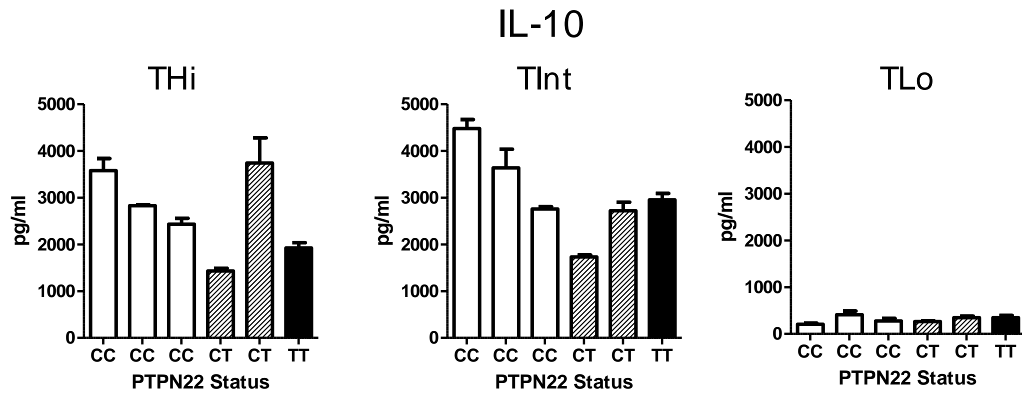


Figure 63. Individual donor secretion of IL-10. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. At day 3 supernatants were collected and IL-10 secretion was determined by ELISA. Graphs depict the individual donor responses performed in triplicate. Error bars represent SD.

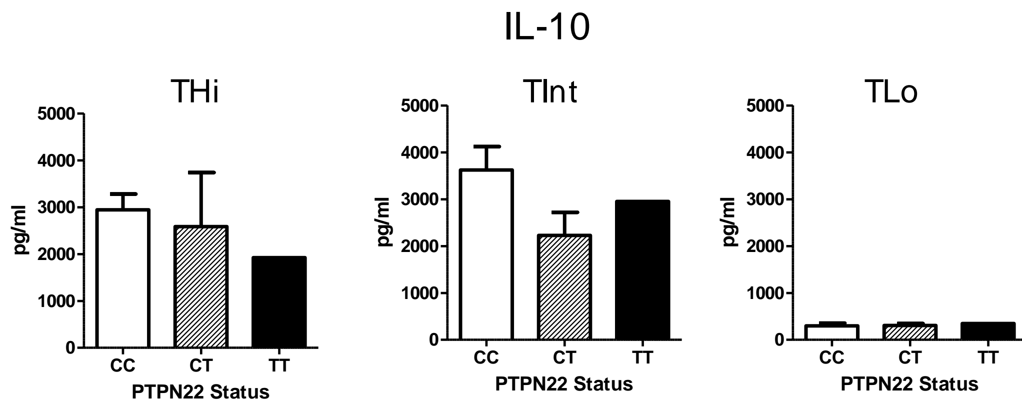


Figure 64. Secretion of IL-10 grouped by PTPN22 C1858T genotype. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. At day 3 supernatants were collected and IL-10 secretion was determined by ELISA. Graphs depict the mean genotype grouped donor responses. CC N=4, CT N=3, TT N=1. Error bars represent SD.

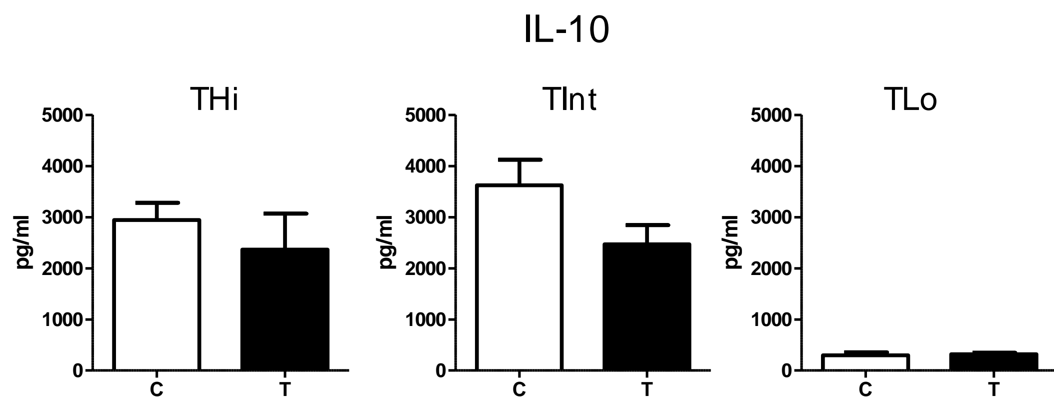


Figure 65. Secretion of IL-10 T-allele carriers vs non-T-allele carriers. Human memory T-cells were cultured under THi, Tint, or TLo conditions in the presence pro-Th17 cytokines for 3 days. At day 3 supernatants were collected and IL-10 secretion was determined by ELISA. Graphs depict the mean genotype grouped donor responses. Error bars represent SD. Graphs depict the mean genotype grouped donor responses. C N=4, T N=4. Error bars represent SD.

Condition	Sample size C vs T for each group	Difference between means pg/mL	Standard Deviation	Power	Target Power	Sample Size for each group
THi IL-17	3	1338	1226	0.26	0.9	19
Tint IL-17	3	142	217.9	0.12	0.9	51
TLo IL-17	3	31.4	82.4	0.07	0.9	146
THi IFNy	3	6314	9652	0.12	0.9	51
Tint IFNy	3	159	1039	0.05	0.9	899
TLo IFNy	3	1.7	412	0.05	0.9	1237905
THi IL-10	3	580	899	0.12	0.9	51
TInt IL-10	3	1156	805	0.4	0.9	12
TLo IL-10	3	21.5	75	0.06	0.9	260

Table 4. Power and sample size analysis of ELISA data, comparing C-allele to T-allele carrier responses. The cytokine secretion responses resulting from 3 days THi, Tint or TLo stimulation in the presence of pro-Th17 cytokines were grouped as C-allele homozygotes vs T-allele carriers, N=3 for each grouping. The difference between the group mean cytokine percentages were calculated and the average standard deviation of the two groups was used as the assumed standard deviation. A 2-sample T-test was used to calculate the power of the observed results and to calculate the sample size required for a target power of 0.9 (90% confidence in rejecting the null hypothesis). Power and Sample Size analysis was performed using Mini-tab II.

4.4.4 ELISA power and sample size analysis

Lastly I performed power and sample size analysis on the ELISA data for the C-allele homozygotes vs T-allele carrier data within Figure 59, 58, and 61. Generally lower powers were observed for these data than for the flow cytometry data, due in part to increased donor-to-donor variation and the very low sample size. The most interesting trend, and that also observed by Rieck *et al,* of reduced IL-10 secretion by T allele carriers had a power of 0.4. For this response to achieve a power of 0.9 a sample size of 12 would be required for future experiments. However, largely the slight differences observed were small and therefore far greater sample sizes are required. Differences in cytokine secretion will, at early time points, be affected by the proportion of cells capable of secreting certain cytokines. Therefore greater efforts in normalising for T cell effector phenotype may allow the true contribution of the PTPN22 C1858T SNP to be assessed. By obtaining larger sample sizes, true differences are more likely to be observed.

4.4.5 Conclusion

Together the data in this Chapter provide initial insights in to how variations in the function of a TCR signalling component contributed by a single nucleotide polymorphism, may affect T cell responses. A trend of marginally increased IL-17 responses was observed in donors with the risk T-allele, which is thought to confer reduced TCR signalling. Like a previous study (528) IL-10 secretion also appeared to be reduced in T-allele carriers compared to C allele homozygotes. However, groups contained very low sample sizes and to assess the significance of these initial observations, the study needs extending to include greater numbers of genotyped individuals. These data suggest that variations in the C1858T SNP might contribute to T-cell effector phenotype, though further investigations are required to confirm these data.

4.5 Discussion

Investigations within previous Chapters revealed that Th17 cell responses were promoted by low strength TCR stimulation via a Ca^{2+} dependent mechanism. I assessed how an autoimmune-associated genetic polymorphism in the *PTPN22* gene, that has been described to alter TCR signalling, might affect Th17 cell responses. Th17 cell responses are known to be important to the pathogenesis of a number of autoimmune diseases including RA. One of the genetic polymorphisms most highly associated with RA is the PTPN22 C1858T SNP. Presence of the risk T-allele results in the expression of Lyp peptide which has increased activity compared to the wild-type Lyp peptide (535). Lyp is thought to reduce TCR signalling in humans, and the SNP confers increased activity in an allele-dose dependent manner, therefore further reducing TCR signalling. I hypothesised that the presence of the risk T-allele would decrease TCR signalling and would promote Th17 cell responses. Some data presented within this Chapter may support this hypothesis. T-cell stimulation with Tint or TLo stimuli marginally enhanced the proportion/number of IL-17 and IL-17/IFNγ populations in (risk) T-allele carrying donors compared to C-allele homozygotes. Furthermore, regulatory IL-10 secretion by T-allele carriers appeared reduced compared to C-allele homozygotes. However, only four out of the 20 donors genotyped had the risk T-allele and the differences observed were not statistically significant. Sample size calculations have revealed that depending on the stimulus and the biological effect measured, that sample sizes in excess of 12 are required to assess if the currently observed differences in cytokine response by T-allele carriers compared to C-allele homozygotes are statistically significant.

4.5.1 Increasing the donor sample size

Due to the low frequency of T-allele carriers, particularly homozygotes, analysing the effect of the C1858T SNP was difficult. A recent meta-analysis of PTPN22 1858C/T-allele distribution found that 10.5% of healthy individuals are expected to carry the T-allele in UK, rising to 15.3% in RA patients (536). Tables 2, 3, and 4 demonstrate that increased sample sizes are required to assess the effect of the risk-allele on T-cell phenotype. A future project would need to increase the C and T-allele sample sizes to ~20 donors. Obtaining T-allele carrier T-cells is a limiting factor as they make up only

~10% of the population. A potential for increasing the frequency of T-allele carrying donors is to use RA patients whose T-allele frequency is increased to 15.3%. However, RA patients have an inflammatory disease which may affect the T-cell phenotype and capability to respond within this model. If RA patients were to be used the efforts would need to be made to reduce variations in: age, disease state, and management. Ideally sample sizes could be increased by gaining access to a large cohort such as the Twins-UK bioresource (>600 twins) allowing peripheral blood to be obtained from healthy genotyped donors. A further way to assess the effect of Lyp on T-cell responses would be to use a Lyp inhibitor (524); such experiments would indicate how Lyp contributes to effector T-cell responses but not reveal how the SNP modulates T-cell function. However, currently the main conclusion drawn from these data is that the study needs extending to include greater donor sample sizes, in order to confirm if the interesting observations so far are true.

4.5.2 1858T and effector T-cell function

Data within this Chapter sought to address the effect of the PTPN22 1858T allele on T-cell effector function by stimulating T-cells with different stimulation strengths in the presence of pro-Th17 cell cytokines. Previously Rieck *et al* demonstrated that $CD4^+$ T-cells from T-allele carriers, when activated with anti-CD3/CD28 beads for 24 hours, had a significant decrease in IL-10 secretion, and also observed decreased trends in IL-2 and IL-4 secretion, when compared to C-allele homozygotes (528). The number of memory T-cells in T-allele carriers was found to be reduced compared to C-allele homozygotes, as was the capability of memory T-cells to induce Ca^{2+} flux suggesting that T-cell activation was reduced. I also observed a small decrease in IL-10 secretion by T-allele carriers compared to C-allele, though this was not significant. Rieck *et al* had a sample size of 6 for each allele. My sample size calculations revealed that for the difference observed in my data to be significant, 12 donors would be required, double that of the previous study. However, the presence of pro-Th17 cytokines may have affected IL-10 secretion. Differences in IFNy and IL-17 secretion appeared distorted by a potential outlier, and although efforts were made to control for age and sex, differences in these may contribute to the variations observed. However, the proportion and number of IL-17^+ and IL-17/$IFNy^+$ cells were slightly enhanced in T-

allele carrying donors. With further investigation this may reveal that perturbations in T-cell signalling conferred by the C1858T SNP may favour inflammatory T-cell responses. To extend this study, the expression of IL-10 by Th17 cells (473, 537) could be explored by flow cytometry. As the proportion and number of IL-17$^+$ cells may be increased within T-allele carriers, but IL-10 secretion may be decreased this might imply differences in the capacity of T-allele carrier Th17 cells to express IL-10. Th17 cell expression of IL-10 is important to limiting Th17 cell inflammatory responses; it is therefore possible that a reduction of Th17 cell IL-10 expression may potentiate an inflammatory state (537). Therefore, it may be interesting to assess if the presence of the T allele effects Th17 cell secretion of IL-10, via flow cytometry. Th17 cells which are not capable of limiting their inflammatory response may contribute to the chronicity of autoimmune disease. Currently, data presented within this Chapter suggest that the risk T-allele may reduce regulatory responses (as previously observed) and enhance inflammatory IL-17 and IL-17/IFNγ responses, but further investigation is required.

4.5.3 Type of stimulus

T-cells were activated with anti-CD3/anti-CD28 beads to assess the effect of the PTPN22 C1858T SNP on T-cell effector function. The majority of differences in the proportion of IL-17 responses were observed at a Tint stimulus, which may be due to differences in Ca^{2+} mobilisation by C- vs T-allele carriers. It would be interesting to assess the capability of C-allele and T-allele carriers to mobilise Ca^{2+} in response to the different bead stimuli via flow cytometry, which would also confirm that Ca^{2+} mobilisation is affected by the T-allele SNP. Furthermore, decreased T-cell proliferation has been observed in 1858T allele carriers and so it may be interesting to assess effector T-cell responses in combination with CFSE labelling (531, 538). Stimulation using bead-bound antibodies is less physiologically relevant than the use of APC such as DC. However, using DC to stimulate T-cells may entail problems particularly in an autologous T-cell/DC system. Recent investigations have demonstrated that DC in 619W knock-in mice (equivalent of T-allele carriers) are hyperresponsive, indicating the Lyp may regulate DC maturation pathways (519). Lyp is expressed in DC, however if Lyp regulates SFK-dependent pathways important to DC maturation and function is currently unknown. SFKs expressed in DC can regulate TLR, chemokine and cytokine

signalling (539) and have been shown to regulate the balance of IL-12 and IL-23 secreted by DC (540). Therefore the T-cell response of T-allele carriers may be further affected within autologous DC-T-cell co-cultures by effect on DC function. To assess the effect of the T-allele using a physiological stimulus an MLR would be best comparing co-cultures of genotyped DC (CC, CT, TT) with T-cells (CC, CT, TT).

4.5.4 Does the minor T-allele increase or decrease TCR signalling

A recent investigation has revealed that the 1858T SNP may be a loss-of-function rather than a gain-of-function mutation (519). The investigation by Zhang *et al* used a knock-in mouse model to demonstrate that although the SNP causes a gain-of-function *per se*, the presence of the T-allele enhances Lyp/Pep protein degradation, which can reduce TCR signalling. The degradation was mediated by the calcium-dependent calpain protease calpain-1 which reduced the cellular expression of Pep/Lyp in risk allele carriers. A loss-of-function mutation was further suggested by progressive thymus and spleen enlargement in addition to increased T-cell numbers within the thymus, lymph nodes, spleen and peripheral memory T-cells of Pep619W (Lyp620W equivalent in mice) mice, suggestive of enhanced activation. These findings suggest that Lyp mutations increase rather than decrease TCR signalling strength (541). If Th17 cell responses are favoured by low strength TCR stimulation, a reduction in Th17 cell responses would be expected. Initial data within this Chapter indicated that IL-17 responses at an intermediate stimulus observed a minor increase in the presence of the T-allele. If TCR signalling is increased as a result of the 1858T allele this may counter our previous findings. Data in Chapter 3 demonstrated that Th17 responses are sensitive to high Ca^{2+}. Depending on the affinity/avidity of TCR binding, the level of Ca^{2+} signalling induced may differ. Calpain1 is a calcium-dependent protease and so the strength of TCR stimulation may affect the activity of calpain1, and in turn affect the capability of calpain-1 to mediate Lyp/Pep degradation (542). Zhang *et al* did not assess for differences in Ca^{2+} mobilisation between knock-in and wild-type mice. Previous investigations have demonstrated that Ca^{2+} mobilisation is impaired within 1858 T/T human individuals, which may affect the capability of calpain1 to degrade Pep/Lyp (528). Prior investigations have demonstrated that Ca^{2+} mobilisation is specifically decreased within memory $CD4^+$ T-cell and memory B cell populations (519,

528). Therefore, although Zhang *et al* demonstrates similar Lyp degradation in human T-allele carrying PBMC populations, the use of PBMC may 'mask' the effect of the T-allele on Ca^{2+} mobilisation within memory $CD4^{+}$ T-cells. Furthermore, the degradation of Lyp may be further affected in RA patients by auto-antibodies against calpostatin (543). Calpostatin is a natural calpain inhibitor, and levels are elevated in RA patients implying that calpain activity may be elevated in these patients. In concert, these factors may contribute to ones risk for developing autoimmunity; whether these factors modulate effector T-cell phenotype remains to be determined. The majority of data in humans indicates that the C1858T SNP confers a gain-of-function mutation; however, peptide availability and function can be modulated by a number of factors, highlighting the importance of assessing the protein rather than transcriptional products.

4.5.5 Conclusion

I investigated how an autoimmune associated genetic polymorphism, which is thought to decrease TCR signalling in human T-cells, might affect human Th17 cell responses. The data obtained revealed interesting trends indicating that the presence of the risk T-allele may marginally enhance the proportion and number of IL-17^{+} and IL-17^{+}/IFNy^{+} cells at a Tint or TLo stimulus. Further investigations are required to increase sample sizes but some interesting and potentially novel trends associated with Th17 cell responses have been observed. More investigations are required to reveal how the SNP affects Lyp/Pep function; however it remains interesting to assess if and how an autoimmune associated genetic polymorphism may affect immune function and contribute to disease pathogenesis.

5 Chapter 5. Regulation of Th17 cell responses by T-cell density

5.1 Introduction

In Chapters 2 and 3 I established that *in vitro* human Th17 cell responses were promoted by low strength TCR stimulation. The mechanism for this was most likely due to the induction of inhibitory levels of Ca^{2+} and a failure of NFATc1 to bind to the *IL-17* promoter under high strength stimulating conditions. *In vitro* T-cell responses are modulated not only by the cytokine *milieu* and TCR signal strength but also by the interactions with the surrounding cells. The *in vitro* culture density of T-cells can modulate a number of parameters, including autocrine and paracrine cytokine responsiveness, cell-cell contacts via adhesins and notch molecules, and cell viability (Figure 66). I became interested in the effect that T-cell density may have on Th17 cell responses.

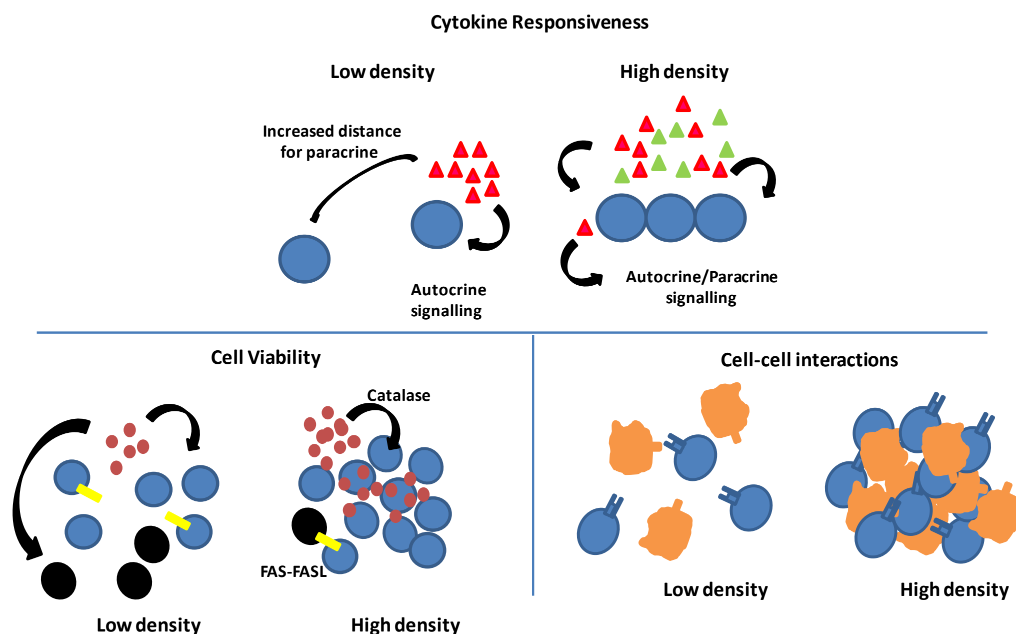


Figure 66. The mechanisms by which T-cell density may modulate T-cell responses

Studies investigating the effect of T-cell density on T-cell responses are limited. Cell density can have a critical role on *in vitro* T-cell survival (544). Resting T-cells rapidly died via apoptosis at low density, but survived at high density due to increased secretion of T-cell survival factors. In a further study, *ex vivo* expansion of T-cells in a

PBMC population was also affected by cell density (545). T-cell expansion in the presence of soluble anti-CD3-Fc was greatest at a high cell density of $1x10^5$ cells/mL compared to a low density of $1x10^3$ cells/mL. Anti-CD3-Fc needs to be cross-linked to Fc receptors on monocytes to be active. At reduced cell densities the T-cell-monocyte contacts were reduced and resulted in decreased availability of TCR stimulation, which in turn reduced T-cell expansion. This study also revealed increased apoptosis at low cell densities which was attributed to reduced expression of a survival factor catalase. These data were also supported by a further study (546). To my knowledge only one study has assessed how memory T-cell responses are regulated by cell density (547). A clonal Th1 cell population was found to be more susceptible to the apoptotic effects of low cell density than a clonal Th2 cell population. However, this study observed a poor association between T-cell density and apoptosis Together, these data indicate that *in vitro* density of T-cell culture can affect T-cell proliferative responses; however, no investigations as yet appear to have addressed the effect that cell culture density has upon the capability of T-cells to generate specific effector cytokine responses.

The influence of T-cell density in modulating T-cell responses may not purely be an *in vitro* phenomenon. The T-cell pool is surprisingly stable throughout life and is maintained by T-cell homeostasis, which precisely regulates T-cell survival, proliferation and apoptosis. However, different densities of T-cells can occur *in vivo* depending on the cellular location (548). The lymph nodes and spleen contain higher densities of T-cells than peripheral sites. In addition, peripheral sites which contain replicating pathogens, such as the gut and skin, have markedly greater T-cell numbers than that at sites such as the liver. Certain T-cell densities created *in vitro* may be more representative of the situation at one immune site compared to another. Investigating how T-cell responses are regulated by density will increase our understanding and may lead to the development of better therapeutics. In a recent review O'Garra, Gabrysova & Spits discussed the necessity to investigate the influence of *in vitro* T-cell density upon T-cell phenotype (549); commenting that different *in vitro* T-cell densities may contribute to the variation in T-cell response observed to certain stimuli. Addressing if T-cell density affects T-cell responses will potentially provide greater understanding of how Th17 cell responses are controlled and may have implications for the

interpretation of *in vitro* T-cell investigations. As yet no investigations assessing the effect of *in vitro* T-cell density on Th17 cell responses have been performed. I therefore assessed if and how T-cell density can modulate Th17 cell responses *in vitro*.

5.2 Aims

The aims of this Chapter were to:

- Assess if T-cell density affects Th17 cell responses both proportionally and absolutely
- Investigate if Th17 cells are specifically affected by T-cell density or if this phenomenon extends to Th1 cells
- Investigate the mechanism by which T-cell density promotes Th17 cell responses

5.3 Experimental Approach

To address the hypothesis that T-cell density would affect human Th17 cell responses and following an initial titration, I compared the IL-17 response of human $CD4^{+}CD45RO^{+}$ T-cells cultured at a 1:1 bead:T-cell ratio at either $1x10^{6}$ (HiD) or $0.0625x10^{6}$ (LoD) cells/mL. Memory $CD4^{+}$ $CD45RO^{+}$ T-cells were isolated by magnetic separation to a purity of >95% (Figure 91). T-cell cultures were performed in IMDM containing 5% serum replacement, with one well of 24 well culture plate containing 1 mL of media. Cultures were performed in the presence of pro-Th17 cytokines IL-1β, IL-23, and TGFβ each at a concentration of 10ng/mL unless otherwise stated. At day 3 of culture 10 U/mL of IL-2 and 10 ng/mL of IL-23 were given to each culture well. Splitting of the different high density and low density culture conditions is outlined below. The effect of T-cell density upon Th17 cell responses was assessed at Day 6. T-cells were restimulated with PMA/Ionomycin for one hour and then cultured in the presence of Brefeldin A for a further 4 hours. Intracellular IL-17 and IFNγ expression was determined by the gating strategy outlined in Figure 11.

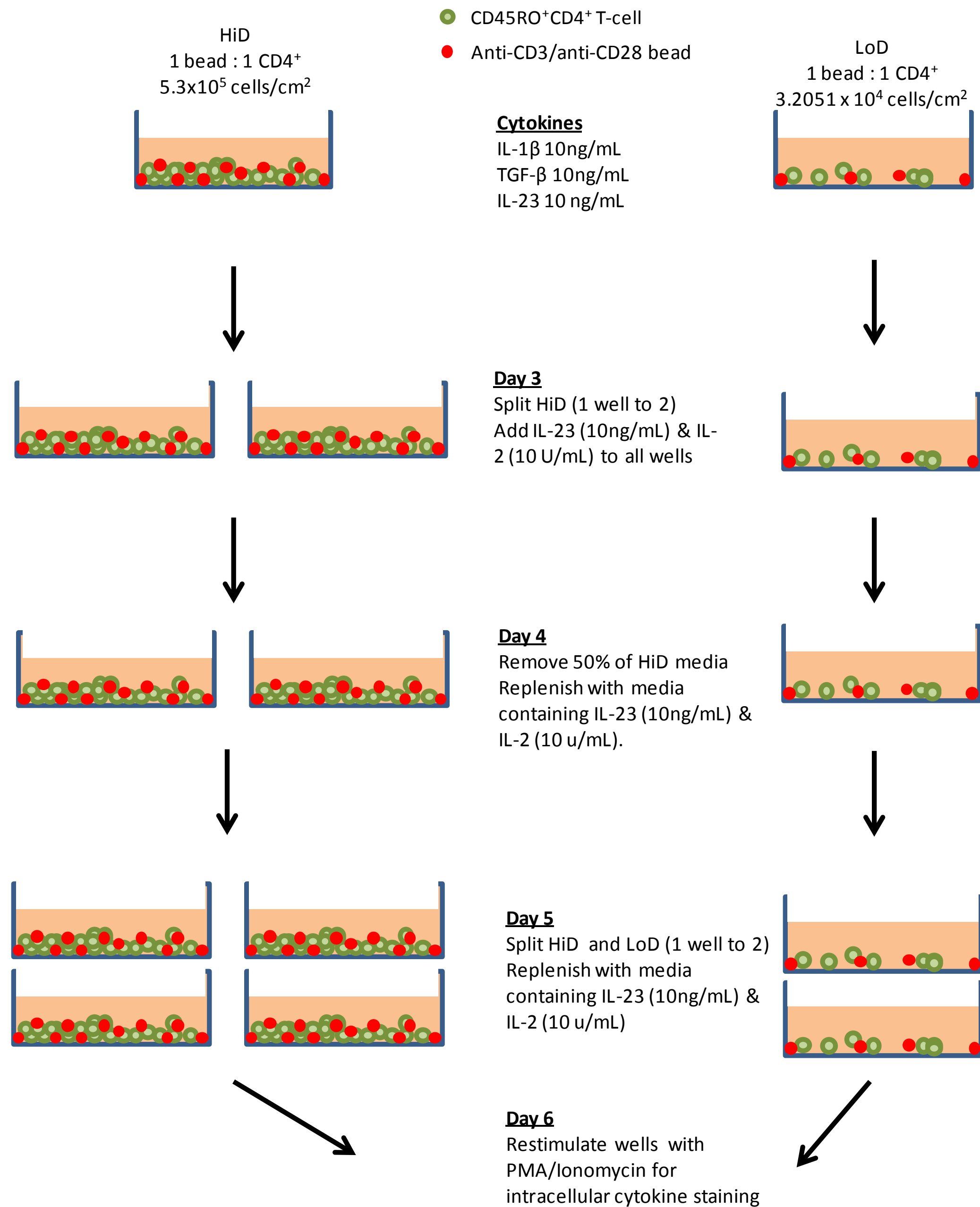


Figure 67. The HiD/LoD culture system. CD45RO$^+$CD4$^+$ T-cells were cultured in IMDM with 10% serum replacement in a 1mL volume on a 24 well plate at a density of 5.3x10^5 cells/cm^2 (HiD) or 3.2051x10^4 cells/cm^2 (LoD). With anti-CD3/antiCD28 beads at a 1:1 bead:T-cell ratio in the presence of proTh17 cytokines IL-1β, IL-23 and TGF-β. T-cells were incubated at 37˚C 5% CO2 for 6 days. During the 6 day culture cells were split as indicated above. Splitting of wells was based upon lightening of the media and a high cell confluency within the well. Day 4 and 5 media was replenished to a volume of 1 mL with IMDM + 10% serum replacement containing 10ng/mL of IL-23 and 10 U/mL of IL-2. At day 6 T-cells were restimulated with PMA/Ionomycin as described in section 7.5.2 and results were analysed as indicated in Figure 11.

5.4 Results

5.4.1 Does density affect Th17 cell responses?

To address if T-cell density affects Th17 cell responses *in vitro*, the number of memory CD4$^+$ T-cells was titrated, decreasing from $2x10^6$ to $0.0625x10^6$ T-cells/mL in two-fold serial dilutions. Each cell density was cultured at a 1 bead: 1 T-cell ratio (equivalent of a THi) for 6 days in the presence of pro-Th17 cell cytokines.

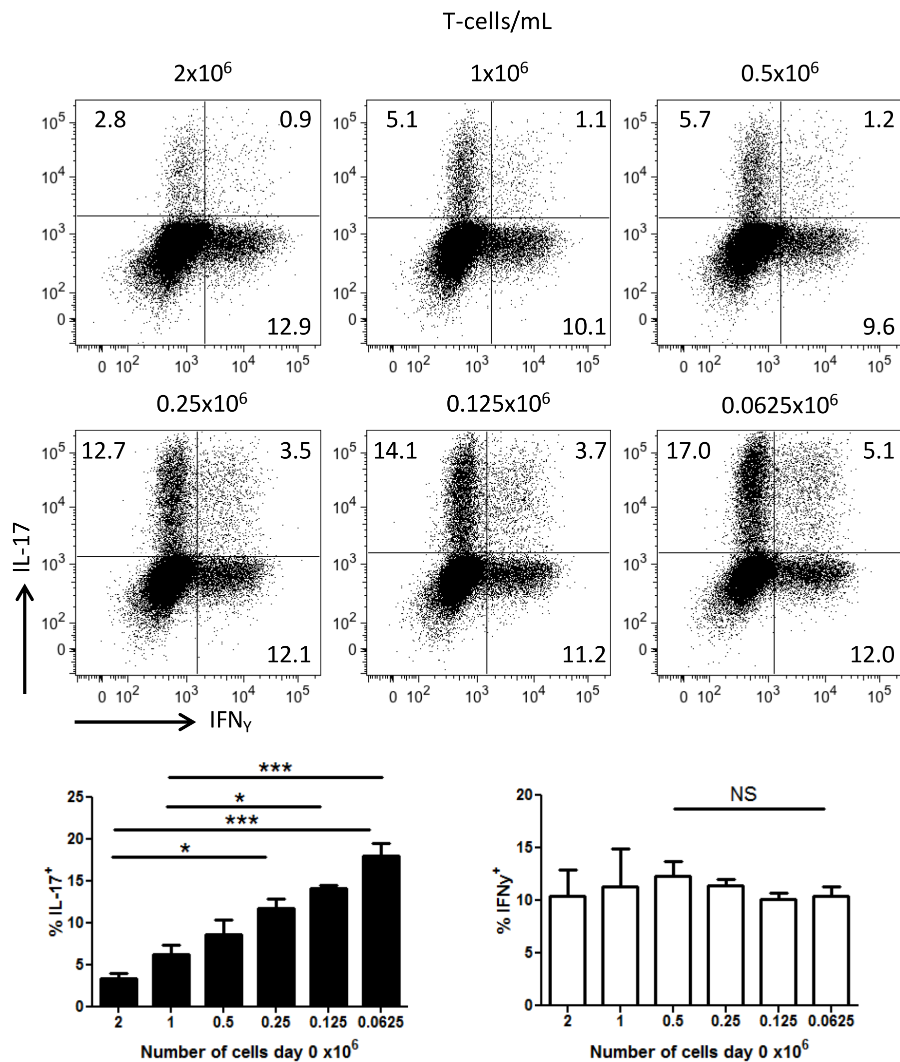


Figure 68. Low cell density promotes IL-17 responses. CD45RO$^+$CD4$^+$ T-cells were cultured with anti-CD3/CD28 beads (1:1 ratio) at two-fold decreasing densities ($2x10^6$ – $0.0625x10^6$) with pro-Th17 cytokines. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNγ was determined by intracellular staining followed by flow cytometry. Plots are representative of 3 independent experiments and the histograms below the plots demonstrate the percentage of cells expressing IL-17 or IFNγ from the 3 independent experiments. Error bars represent SEM, P-values calculated using One-way ANOVA with Bonferroni post test; *P<0.05 ***P<0.001.

On day 6 the signature cytokines of Th17 and Th1 cells, IL-17 and IFNγ respectively, were assessed by intracellular flow cytometry. Interestingly, I found that as the T-cell density decreases the proportion of IL-17^+ cells increases (Figure 68). In addition, the proportion of IL-17^+/IFNy$^+$ was also observed to increase as density decreases, whereas the proportion of IFNy$^+$ cells was unaffected by cell density. Although all conditions received a high strength stimulus (1bead: 1T-cell), a low T-cell density appears to permit Th17 cell responses.

5.4.2 Cytokine availability

A possible explanation for these data might be that cells cultured at a low cell density have a greater concentration of the pro-Th17 cell cytokines on a per cell basis. To assess this, 4-fold decreasing T-cells densities were cultured in the presence of either 10 ng/mL of pro-Th17 cell cytokines, or the same per cell cytokine concentration of 10^{-5}ng/cell. Interestingly, despite reducing the total cytokine concentration within the low density culture increased Th17 cell responses were still observed, and were only marginally reduced compared to the 10 ng/mL culture (Figure 69). The proportion of IFNy$^+$ cells increased as the cytokine concentration decreased, which is potentially due to reduced TGF-β which can suppress Th1 cell responses (329). These data indicate that the increased Th17 cell response resulting from low density culture is not likely to be due to increased cytokine availability.

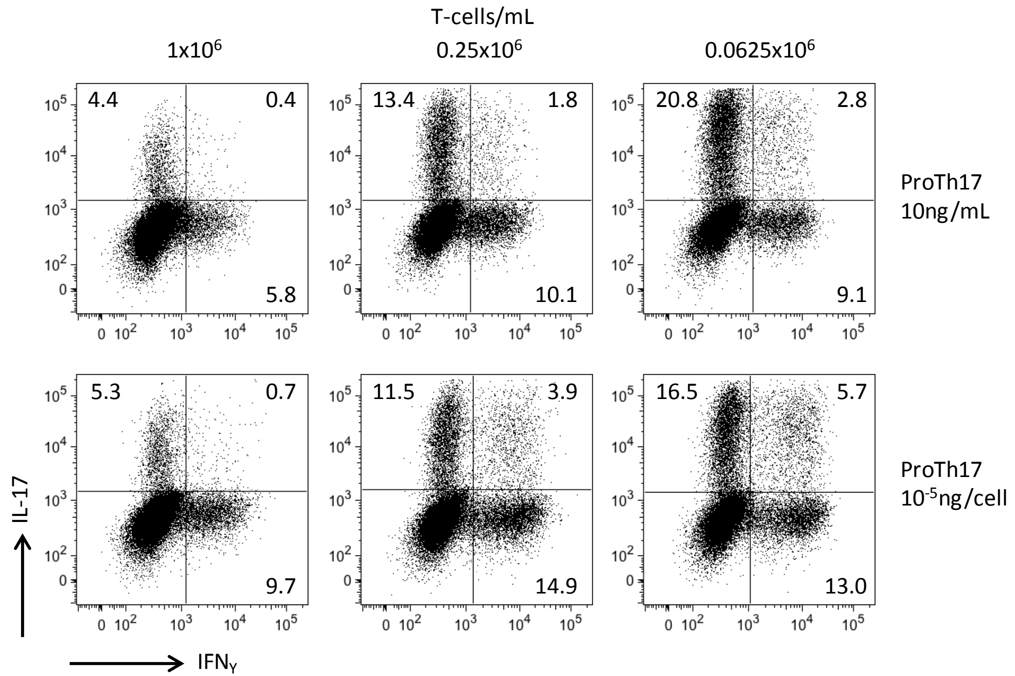


Figure 69. Cytokine availability is not a limiting factor. Memory $CD4^+$ T-cells were cultured with anti-CD3/CD28 beads (1:1 ratio) at four-fold decreasing densities ($1x10^6$ – $0.0625x10^6$). Cells were cultured for 6 days in the presence of either 10ng/mL (top row) or 10^{-5}ng/mL (bottom row) of pro-Th17 cytokines At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNy was determined by intracellular staining followed by flow cytometry. Plots are representative of 3 independent experiments.

5.4.3 Does density affect Th1 cell responses?

I next assessed if the affect of cell density was specific to Th17 cell responses or if Th1 cell responses were also influenced. T-cells were cultured at decreasing T-cell densities in the presence of pro-Th17 cell or pro-Th1 cell cytokines, or in the presence of IL-2 alone (Th0). IFNy responses resulting from pro-Th1 cell culture appeared to be unaffected by T-cell density (Figure 70). In addition, under Th0 cell conditions the proportion of IFNy also remained constant, yet even in the absence of pro-Th17 cell cytokines a slight increase in IL-17 response was observed at lower cell densities. These data indicate that low T-cell density seemed to preferentially promote Th17 cell responses and that this is most striking in the presence of pro-Th17 cell cytokines.

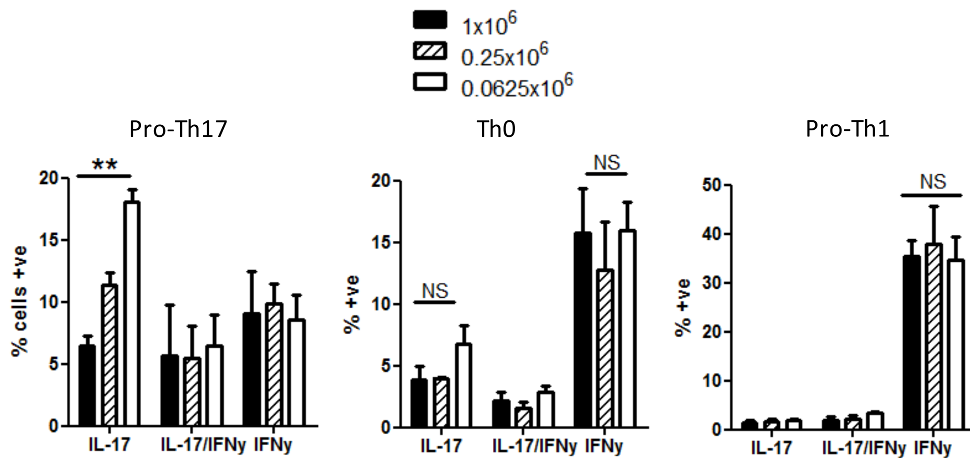


Figure 70. T-cell density does not affect memory Th1 cell responses. Memory $CD4^+$ T-cells were cultured with anti-CD3/anti-CD28 beads (1:1 ratio) at four-fold decreasing densities ($1x10^6$ – $0.0625x10^6$ in the presence of pro-Th17, pro-Th1 or Th0 cell cytokines. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNy was determined by intracellular staining followed by flow cytometry. Data of 4 independent experiments are shown; error bars represent SEM. P values were calculated using a Two way ANOVA with Bonferroni post test **=P<0.01.

I focussed subsequent experiments on a high density culture of $1x10^6$ c/mL (HiD) and a low density culture of $0.0625x10^6$ c/mL (LoD). I performed repeat experiments at these densities under pro-Th17 cell cytokine conditions to study the effect of T-cell density on IL-17 and IFNγ responses. Figure 71 demonstrates that significantly greater proportions of $IL\text{-}17^+$ cells occur as a result of LoD culture compared to HiD culture. Although high variation was observed (most likely due to donor-to-donor variation), matched-pair analysis revealed a significant difference between HiD and LoD Th17 cell responses. The proportions of $IFN\gamma^+$ and $IL\text{-}17/IFN\gamma^+$ did not differ significantly as a result of T-cell density. High variation in the IFNy response between donors was also observed. As memory T-cells were used this may be due to variations in the starting population of Th1 cells within the isolated $CD45RO^+$ $CD4^+$ population.

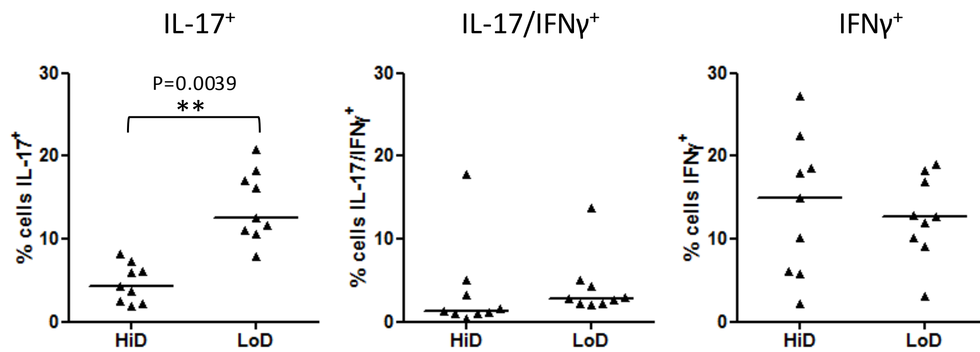


Figure 71. LoD favours IL-17 responses compared to HiD. Memory CD4$^+$ T-cells were cultured with anti-CD3/anti-CD28 beads (1:1 ratio) at either 1x10^{6}c/mL (HiD) or 0.0625x10^{6}c/mL (LoD) in the presence of pro-Th17 cytokines. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNγ was determined by intracellular staining followed by flow cytometry. Percentages of IL-17 and IFNγ single and double producers from 9 independent experiments are shown. Bar represents median. P-values calculated using a Wilcoxson matched pairs test **P<0.01.

5.4.4 Modulating T-cell density with APC derived stimulation

I next assessed if cell density would modulate Th17 responses when T-cells were activated with a physiologically relevant stimulus. Autologous PGN and C.*albicans* activated DC were cultured at a 1 DC: 1 T-cell ratio at either a high density of 1x10^6 or a low density of 0.25x10^6 T-cells/well. PGN activates DC via the TLR2 pathway inducing secretion of pro-Th17 cell cytokines and C.*albicans* hyphae are well documented as Th17 cell inducing antigens (550). Figure 72 demonstrates that APC stimulation at a low T-cell density also resulted in a significant increase in the proportion of IL-17$^+$ cells compared to a high T-cell density. Furthermore, a significant decrease in IFNγ expression was observed as a result of low density culture compared to high density. Considering that the nature of stimulation in an autologous setting should be antigen specific and the the proportion of cells capable of responding is likely to be below 5% the percentages of IL-17$^+$ cells is surprisingly high. This finding may potentially be explained by bystander activation and/or selective expansion of antigen specific T-cells. In summary these data confirm that T-cells cultured at low density activated by either anti-CD3/anti-CD28 beads or DC promote Th17 cell responses.

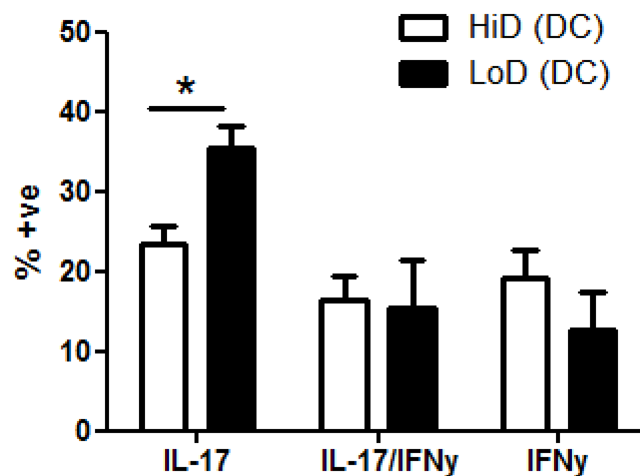


Figure 72. T-cells stimulated with APC at low density promote IL-17 responses. Memory CD4$^+$ T-cells were cultured with autologous DC activated with PGN and C.*albicans* (1:1 ratio) at either 1x10^6 T-cells/mL or 0.25x10^6 T-cells/mL. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNy was determined by intracellular staining followed by flow cytometry. Data of 3 independent experiments are shown; error bars represent SEM. P-values calculated using a Two way ANOVA with Bonferroni Post Test *P<0.05 .

5.4.5 Activation and cell death

I next addressed the kinetics of HiD and LoD activation by measuring CD25 expression. CD25 is the α-chain of the IL-2 receptor and is expressed by human T-cells when they have been activated via the TCR (551). To assess if HiD and LoD cultured T-cells had been activated, cell surface expression of CD25 was assessed at 0, 48, and 144 hours of culture. Data in Figure 73 demonstrates that CD25 expression in both HiD and LoD culture populations had increased within 48 hours of culture, and expression was maintained until day 6 of culture. Pictures of HiD and LoD culture in Figure 74 clearly demonstrate differences in the kinetics of T-cell expansion in HiD and LoD T-cell cultures. T-cell clustering was observed in both conditions by 48 hours, although clusters are far smaller at a low density. At 72 hours HiD cells were split explaining why smaller clusters appear to have formed at 96 hours. Together these data indicate that T-cells in both HiD and LoD cultures cluster and proliferate. The possibility that HiD or LoD culture resulted in increased cell death was also investigated. Day 6 HiD and LoD cells were stained with viability dye, Viaprobe. Data depicted in Figure 75 demonstrates that comparable levels of cell death were observed within both culture densities. However, although the mean values are comparable, high variability in cell

death is also observed, therefore more repeats including the early apoptosis marker, annexin V, would be of benefit.

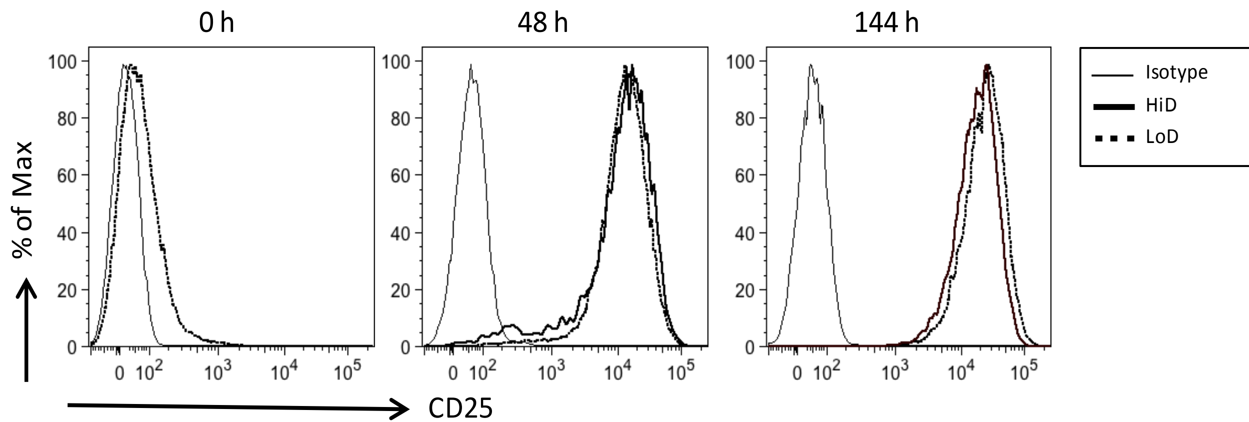


Figure 73. HiD and LoD T-cell expression of activation marker CD25. Memory CD4$^+$ T-cells were cultured under HiD or LoD conditions. Cell surface expression of CD25 was determined prior to culture (0 h), 48 h, and 144 h by flow cytometry. Thin solid line represents isotype control; thick solid line represents HiD, and thick dotted line represents LoD. Plots are representative of 3 independent experiments.

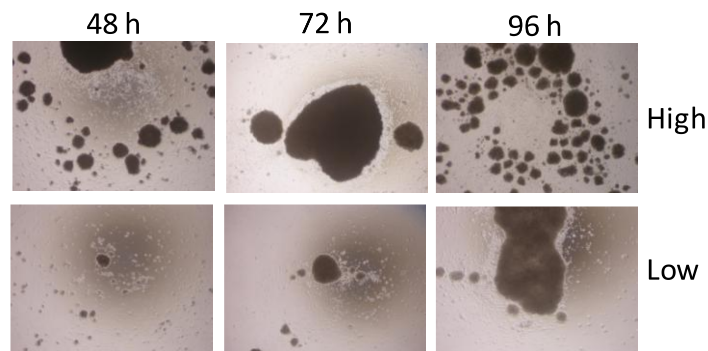


Figure 74. HiD and LoD culture kinetics. Memory CD4$^+$ T-cells were cultured under HiD or LoD conditions. At 48, 72 and 96 hours pictures of cultures were taken at 4 x magnification. Data are representative of 15 independent experiments.

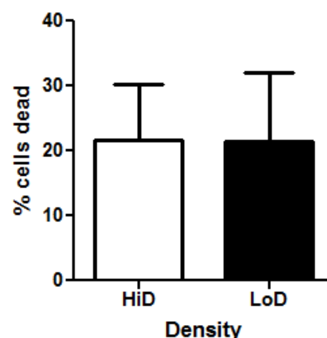


Figure 75. HiD and LoD cell death. Memory CD4$^+$ T-cells were cultured under HiD or LoD conditions. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 in the presence of Brefeldin A. Cells were viability stained with Viaprobe prior to intracellular cytokine staining. Data are representative of 3 independent experiments of percentage of Viaprobe$^+$ cells. Error bars represent SEM.

5.4.6 Are responses proportional and absolute?

The possibility remained that the effect of LoD culture on Th17 cell responses was proportional rather than absolute. The absolute number of live T-cells at day 6 was assessed by trypan blue staining and together with day 6 flow cytometry data the absolute cell counts were determined. Figure 76 reveals that significantly more IL-17^+ cells resulted from LoD culture compared to HiD culture, this was despite the higher absolute number of IL-17^+ cells within HiD conditions. In comparison HiD culture yielded greater numbers of IFNy$^+$ cells than LoD culture. The total number of IL-17^+IFNy$^+$ cells was unaffected by cell density. However, these results do not take into account the difference in the starting number of IL-17^+ and IFNy$^+$ cells within the different densities. I therefore next compared the expansion of HiD and LoD cell populations from day 0 to day 6. The absolute number of IL-17^+ and IFNy$^+$ cells was determined in the starting population of memory CD4$^+$ T-cells. These data were used to calculate the total cell expansion, and the expansion of individual cytokine secreting populations by comparing the fold change from day 0-6 (calculated as above). Figure 77 demonstrates that the total cell expansion was significantly increased in LoD cultures compared to HiD. Interestingly very little difference was observed in the expansion of IFNy$^+$ cells between HiD and LoD cultured cells. However, like the total cell expansion, a striking increase in the expansion of the IL-17^+ and IL-17^+/IFNy$^+$ populations was observed in LoD culture compared to HiD culture. It is also striking to note that IFNy expression is unaffected by this parameter. These data potentially indicate preferential expansion of the IL-17 population in LoD conditions, although it is not possible to confirm this from these data.

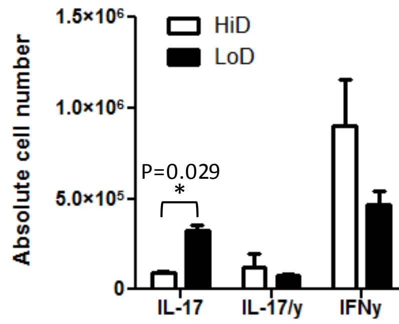


Figure 76. LoD culture increases the absolute number of IL-17^{+} cells. Memory CD4^{+} T-cells were cultured under HiD or LoD conditions. At day 6 T-cells were counted with trypan blue. The absolute number of IL-17 and IFNγ single and double producers was determined by restimulating cells with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNγ was determined by intracellular staining followed by flow cytometry. Data are representative of 3 independent experiments. Error bars represent SEM. P values were calculated using Paired T-test *P<0.05,

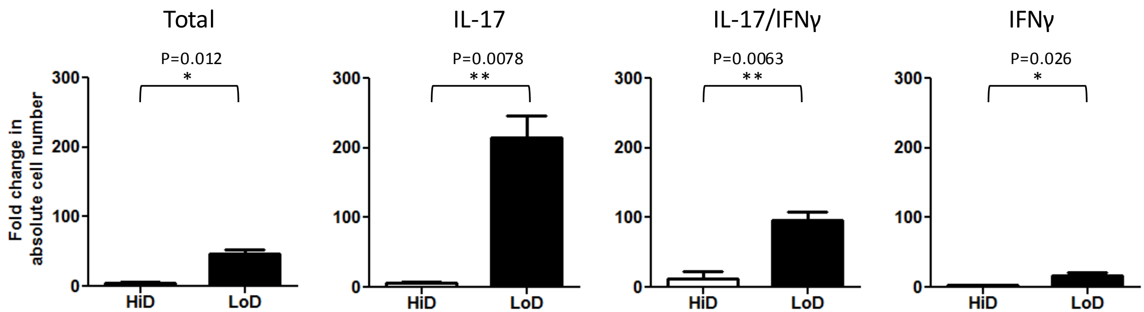


Figure 77. Increased T-cell expansion is observed in LoD cultures. Day 0 T-cells were stimulated with PMA/Ionomycin stimulation for 5 hours the last 4 in the presence of Brefeldin A and expression of IL-17 and IFNγ was determined by intracellular staining followed by flow cytometry. The absolute number of IL-17 and IFNγ single and double producers at day 0 per density was calculated Memory CD4^{+} T-cells were cultured under HiD or LoD conditions. At day 6 T-cells were counted with trypan blue. The absolute number of IL-17 and IFNγ single and double producers was then determined by restimulation with PMA/Ionomycin for 5 hours and intracellular IL-17 and IFNγ staining. The fold change in T-cell expansion was then determined using the absolute cell numbers at day 0 and 6. Data are representative of 3 independent experiments. Error bars represent SEM. P values were calculated using Paired T-test *P<0.05, **P<0.01.

5.4.7 Naive T-cell response to cell density

To address if LoD culture caused preferential expansion of Th17 cells rather than induction, the effect of cell density was assessed in naive CD4^{+} T-cells. Naive CD4^{+} T-cells were cultured at HiD and LoD in the presence of pro-Th17 or pro-Th1 cell cytokines. Pro-Th17 cell culture resulted in poor IL-17 responses although IL-17 responses were increased within LoD conditions (Figure 78). LoD culture of naive T-cells in pro-Th1 conditions is inhibitory to Th1 cell responses, which is more in line with previous investigations into T-cell density (547) than the memory Th1 cell response

observed above (Figure 70). IL-17 expression was increased at LoD, but proportions were substantially reduced compared to those observed in memory T-cell cultures. This may be due to the incorrect cytokine *milieu* or an inability of human naive T-cells to directly differentiate into Th17 cells. These data suggest, but do not confirm, that the effect of cell density is due to preferential expansion rather than induction, as the final Th17 cell proportion appears to be dependent on the day 0 proportion of IL-17^{+} cells.

5.4.8 Secreted factor

I wanted to establish why a low T-cell density would promote Th17 cell responses compared to a high T-cell density, but not affect Th1 cell responses. I firstly assessed if an inhibitory/promoting factor was secreted in to the media of HiD/LoD cells by performing a conditioned media experiment. Figure 79 shows data, similar to those observed in Chapter 3, revealing no repressive or enhancing affect (beyond those most likely explained by decreased media quality) of HiD/LoD conditioned media on IL-17 or IFNγ responses. Based on these data it was decided to assess whether other parameters were affected by cell density.

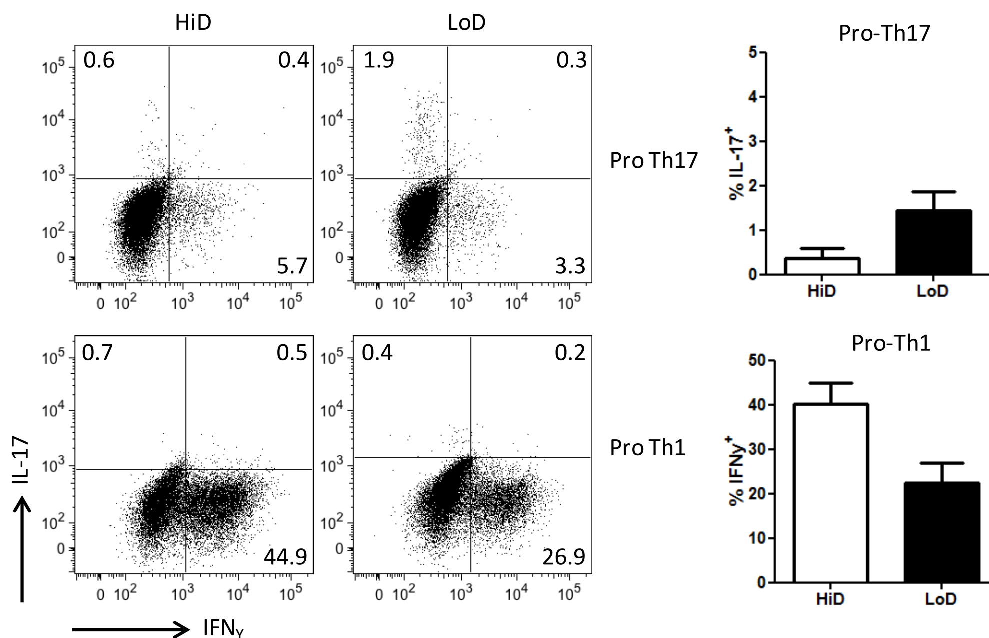


Figure 78. Naïve CD4^{+} T-cell response to HiD/LoD culture. Naive CD4^{+} T-cells were cultured under HiD or LoD conditions for 6 days in the presence of pro-Th17 or pro-Th1 cytokines. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours the last 4 in the presence of Brefeldin A and expression of intracellular IL-17 and IFNγ was determined by intracellular cytokine staining followed by flow cytometry. Plots are representative of 2 independent experiments and the histograms adjacent demonstrate the percentage of cells expressing IL-17 or IFNγ from the 2 independent experiments. Error bars represent SEM.

5.4.9 Cell contact

I hypothesised that cell-to-cell contacts might play a role in determining T-cell phenotype. Notch signalling is a cell contact dependent mechanism that determines cell fate and lineage commitment (552) and can determine CD4$^+$ T-cell effector phenotypes (553, 554). Recent investigations demonstrate that Notch signalling affects human Th17 cell responses (555). Notch ligand Delta like ligand (DLL)-4 has been found to promote Rorc activation and IL-17 production. I hypothesised that cell-cell contacts would be modulated by the differences in cell density and that Notch signalling may be modulated and affect density dependent Th17 cell responses. I therefore firstly assessed if cell-cell contacts were affecting density dependent Th17 cell responses. LoD conditions most likely result in reduced T-cell-T-cell and/or T-cell-bead contacts as the cells are initially more spread out. To assess if increased cell contacts were inhibitory to Th17 cell responses, HiD and LoD cells were cultured on U-bottom 96-well

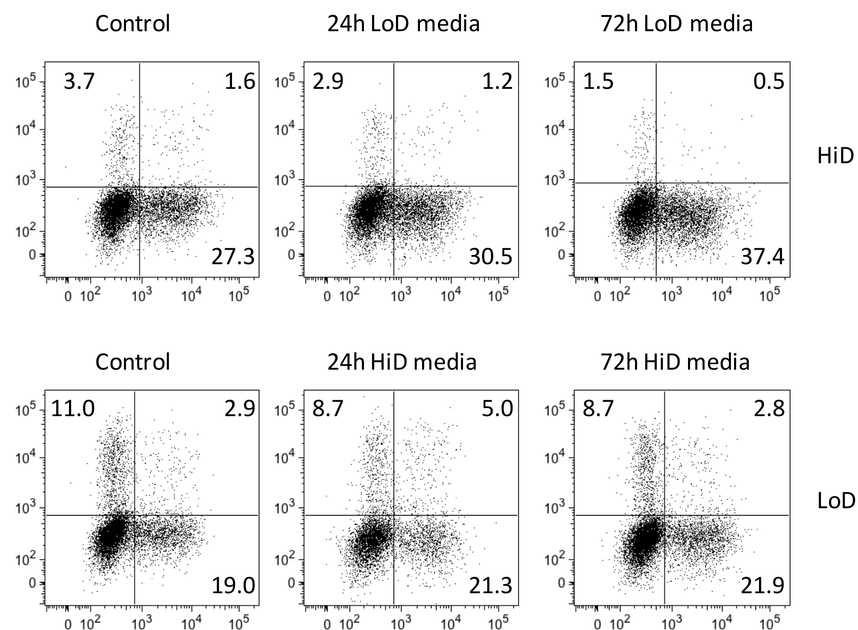


Figure 79. Th17 inhibiting/promoting factors are not contained within HiD/LoD conditioned media. Memory CD4$^+$ T-cells were cultured under HiD or LoD conditions. At 72 hours conditioned media was removed from HiD and LoD cells. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNy was determined by intracellular staining followed by flow cytometry. Plots are representative of 2 independent experiments.

plates (increasing cell contacts) or flat-bottom 96-well (control) plates (461). Figure 80 demonstrates that increased cell-cell contact as a result of U-bottom culture had no

significant effect on LoD mediated IL-17 response when compared to the flat bottom plate. Moreover, on either flat-bottom or U-bottom plates LoD culture favoured Th17 cell responses compared to HiD. The fact that U-bottom plates did not prevent Th17 cell responses indicated that Notch signalling may be unlikely to affect Th17 cell responses in this system. Notch signalling occurs when one of four Notch receptors (N1-4) is bound by a cell bound Notch ligand. There are five notch ligands (DLL 1, 3, and 4 and Jagged 1 and 2) which upon binding allow Notch to undergo proteolytic cleavage by γ-secretase and the translocation of the intracellular domain into the nucleus (552). If Notch signalling were important to HiD or LoD Th17 cell responses, inhibition of Notch signalling would provide an initial indication. I therefore assessed the effect of Notch signalling using a gamma-secretase inhibitor (GSI) which inhibits cleavage of the Notch intracellular domain (N-ICD) by gamma-secretase, in turn preventing N-ICD nuclear translocation (556). The GSI was added to HiD and LoD culture at either day 0, 3 or 0 and 3. Figure 81 demonstrates a small decrease in both IL-17 and IFNγ expression in both HiD and LoD conditions; however the effect was not striking, indicating that Notch signalling may not be important to T-cell responses within this system. However, no positive control was included and so the inhibitor may not be functional. An assessment of the inhibitors ability to prevent gamma-secretase cleavage in a population of cells known to function via Notch signalling would provide a positive control for this. Nevertheless as cell contact and Notch did not look like probable causes of differences in HiD and LoD T-cell responses, the research direction changed course.

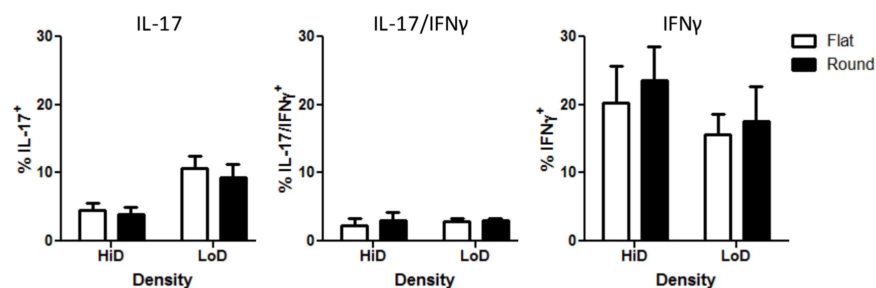


Figure 80. Increased T-cell contacts do not prevent LoD culture promoting Th17 responses. Memory $CD4^+$ T-cells were cultured under HiD or LoD conditions on either a flat-bottom or U-bottom 96 well plate. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNγ was determined by intracellular staining followed by flow cytometry. Data are of 3 independent experiments. Error bars represent SEM.

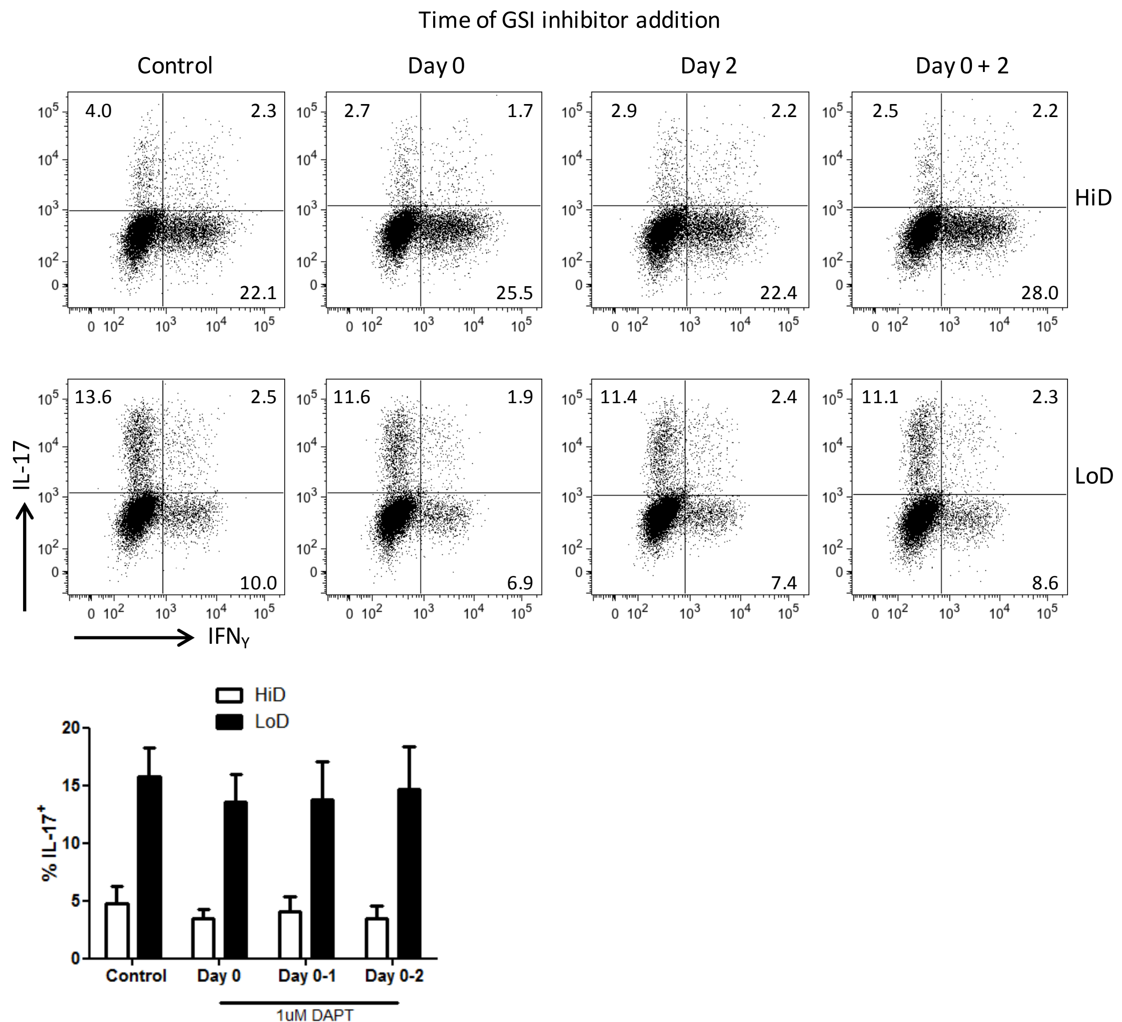


Figure 81. Inhibition of Notch signalling does not affect HiD or LoD culture. Memory CD4^{+} T-cells were cultured under HiD or LoD conditions. DAPT γ secretase inhibitor was added to cultures at Day 0, 3 or 0 and 3 (1μM). At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNγ was determined by intracellular staining followed by flow cytometry. Plots are representative of 2 independent experiments and the histograms below the plots demonstrate the percentage of cells expressing IL-17 from the 2 independent experiments. Error bars represent SEM.

5.4.10 Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor is a highly conserved transcription factor that responds to small synthetic and natural chemicals (557). AhR is associated with Th17 cells and can promote Th17 cell responses (216). IMDM media is widely used for the *in vitro* generation of Th17 cells and contains 4-fold greater levels of tryptophan, which acts as a ligand for the AhR causing its activation and nuclear translocation (215). Previous investigations have revealed that AhR nuclear localisation is dependent on cell density in a keratinocyte cell line (558). The investigation found AhR expression was

predominantly nuclear at sparse cell densities, nuclear and cytoplasmic at subconfluence, and cytoplasmic at confluence. I hypothesised that the AhR may be located in the cytoplasm rather than nucleus of HiD cells preventing *IL-17* expression. A time course assessing *AhR* expression by real time PCR indicated that LoD resulted in greater AhR expression at 96 hours compared to 48 (data not shown) and prompted further investigation into AhR regulation. To assess if differences in IL-17 expression between HiD and LoD cultures occurred post 48 hours I assessed IL-17 expression over a time course. Figure 82 demonstrates that following 72 hours, differences in the IL-17 response began to occur. IL-17 expression from LoD cells steadily increased, peaking at day 6 when the cells are usually phenotyped, whereas, HiD IL-17 responses remained low across all time points. I therefore assessed AhR protein expression at 48, 72 and 96 hours. HiD and LoD cells were cultured in the presence or absence of pro-Th17 cell cytokines. Total cell lysates were taken at each time point and assessed for AhR expression by western blot.

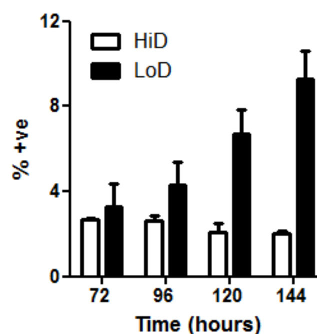


Figure 82. Time course of HiD and LoD IL-17 expression. Memory $CD4^+$ T-cells were cultured under HiD or LoD conditions. At 72, 96, 120 or 144 hours were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 was determined by intracellular staining followed by flow cytometry. Bars represent 2 independent experiments. HiD white bars LoD black bars. Error bars represent SEM.

Although both cell densities had increased AhR expression in the presence of pro-Th17 cell cytokines, LoD culture resulted in greater AhR expression, particularly at 72 hours. To investigate the cellular location of AhR expression in HiD and LoD cultured cells, cytoplasmic and nuclear lysates were taken at 72, 96 and 120 hours.

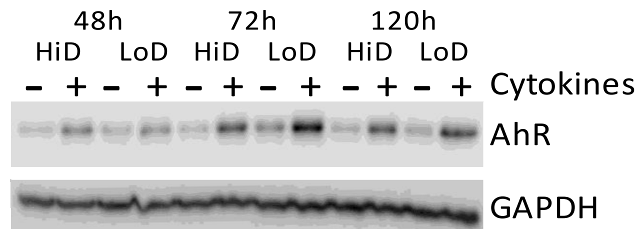


Figure 83. Expression of AhR. Memory CD4⁺ T-cells were cultured under HiD or LoD conditions in the presence or absence of pro-Th17 cell cytokines. At 48, 72, or 96, whole cell lysates were prepared and expression of AhR determined by Western blot. Loading control GAPDH. Blots are representative of 2 independent experiments.

Figure 84 indicates that in the presence of pro-Th17 cell cytokines the majority of AhR is present within the cytoplasm of HiD and LoD cells. Expression of AhR is again increased in LoD conditions compared to HiD. Interestingly over time the expression of AhR in the nucleus increases in LoD cells, peaking at 120 hours, though expression remains low compared to cytoplasmic expression. These data indicate that within LoD culture AhR expression is increased, however striking differences in AhR location are not observed. Although AhR is suggested to be important for Th17 cell responses, how AhR mediates this remains poorly understood. Increased expression of AhR in LoD cells may be due to increased availability of AhR ligands present in IMDM media on a per cell basis. Moreover increased expression of AhR may be an effect of increased proportions of Th17 cells with LoD culture rather than a cause. Further investigations are therefore required to determine if and how the AhR may affect density dependent Th17 cell responses.

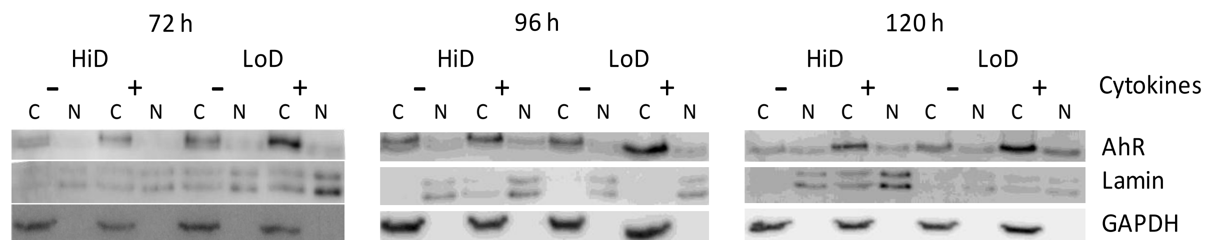


Figure 84. Localisation of AhR. Memory CD4⁺ T-cells were cultured under HiD or LoD conditions in the presence or absence of pro-Th17 cell cytokines. At 72, 96 or 120 hours, cytoplasmic and nuclear lysates were prepared and expression of AhR determined by Western blot. Loading controls for cytoplasmic and nuclear loading were GAPDH and Lamin. Blots are representative of 3 independent experiments.

5.4.11 HiD and LoD activation of STAT3

STAT3 is an important transcription factor for mediating Th17 cell responses (559). STAT3 signalling may be initiated by binding of cytokines including IL-6, IL-23 and IL-21 to their respective receptors, which induces receptor dimerisation and allows JAKs to bind (348). JAKs create phosphorylation sites on the dimerised receptors allowing STAT binding. JAKs are then able to phosphorylate the receptor bound STATs, causing STAT activation, dimerisation, and subsequently nuclear translocation. STAT3 is a key transcription factor in Th17 cell responses. Recent ChIP sequencing studies demonstrated STAT3 binding to promoter sites of IL-17 response genes including *Roryt, Rora, Il-17a and f and Il-21* (339). Three investigations have indicated that STAT3 activation can be determined by cell density (560–562). These investigations have been conducted in non-T-cell lines and although the data are conflicting they do demonstrate that STAT3 can be regulated by cell density. I therefore proposed that cell density was affecting the activation of STAT3 in HiD and LoD cells and that this would in turn affect Th17 cell responses. I therefore assessed the activation of STAT3 in HiD and LoD cells at 72 hours (Figure 85). Active STAT3 is measured by a phosphorylation event at Tyr705. Total STAT3 expression is consistent between HiD and LoD conditions in the presence or absence of cytokines. STAT3-Try705p activation is increased in LoD conditions in the presence of pro-Th17 cell cytokines compared to HiD. The increased activation of a pro-Th17 cell transcription factor may account for the difference in Th17 cell response.

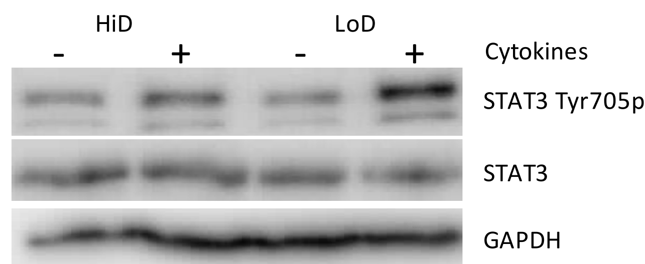


Figure 85. Activation of STAT3 in HiD and LoD culture. Memory CD4$^+$ T-cells were cultured under HiD or LoD conditions in the presence or absence of pro-Th17 cytokines. At 72, 96 or 120 hours whole cell lysates were prepared and expression of STAT3Tyr705p (top) STAT3 (middle) and GAPDH loading control (bottom) were determined by Western blot. Blots are representative of 3 independent experiments.

HiD cultures potentially have reduced Th17 cell responses because of reduced availability of STAT3 activating cytokines. Despite addition of IL-23 to HiD and LoD cultures at days 0 and 3, the possibility remained that IL-23 may be limited in HiD cultures. IL-21 is also a STAT3- activating cytokine and is associated with a Th17 cell phenotype. IL-21 can be endogenously produced by T-cells to initiate a positive feedback loop that enhances Th17 responses (348, 349). Although IL-21 expression has not been assessed in HiD/LoD cultures I also addressed if the addition of IL-21 would enhance HiD Th17 cell responses. Therefore STAT3 activating cytokines IL-23 and/or IL-21 were added to HiD cultures daily until 72 hours. Figure 86 demonstrates that despite increased addition of IL-23 and/or IL-21, HiD cells still fail to increase Th17 cell responsiveness. Therefore IL-23 and/or IL-21 availability does not appear to be a limiting factor for HiD Th17 cell responses. It would also be interesting to assess the expression of IL-23 and IL-21 receptors on HiD and LoD cells to investigate if differences in STAT3 activation were due to cytokine responsiveness.

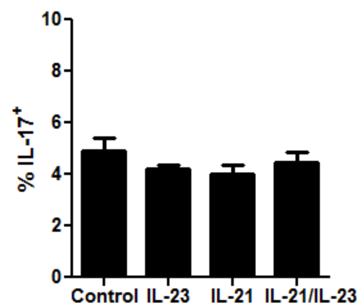


Figure 86. Increase IL-23 or IL-21 availability does not increase HiD IL-17 responses. Memory CD4+ T-cells were cultured under HiD or LoD conditions. Daily IL-23 (10ng/mL) and/or IL-21 (10ng/mL) were added to cultures for the first 3 days of culture. At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 was determined by intracellular staining followed by flow cytometry. Data are of 2 independent experiments. Error bars represent SEM.

In order to assess if the increased STAT3 expression is essential to LoD Th17 cell responses, the effect of STAT3 inhibition was assessed. Inhibition of STAT3 was performed with STAT3 inhibitor S3I-201 (NSC74859). S3I-201 is a chemical probe inhibitor that prevents STAT3 dimerisation and STAT3 DNA binding (563). Addition of the STAT3 inhibitor to LoD cells was performed at 72 hours the time at which STAT3 activation was observed to increase in LoD cells. The inhibitor was added at either double the IC_{50} (200nM) or the IC_{50} (100nM). The preliminary experiment (Figure 87 A) yielded promising results. STAT3 inhibition at 200nM (double the IC_{50}) resulted in substantially reduced IL-17 responses compared to either LoD or DMSO control populations. However a repeat experiment in a different donor did not yield the same response (Figure 87B). At 200nM the inhibitor had little effect on Th17 cell responses, whereas 100nM did cause inhibition of Th17 cell responses compared to LoD and DMSO controls. However, in both donors the IFNy response was substantially decreased by the STAT3 inhibitor. During both cultures I observed that T-cell proliferation was stunted and that cell death increased in the presence of the inhibitor.

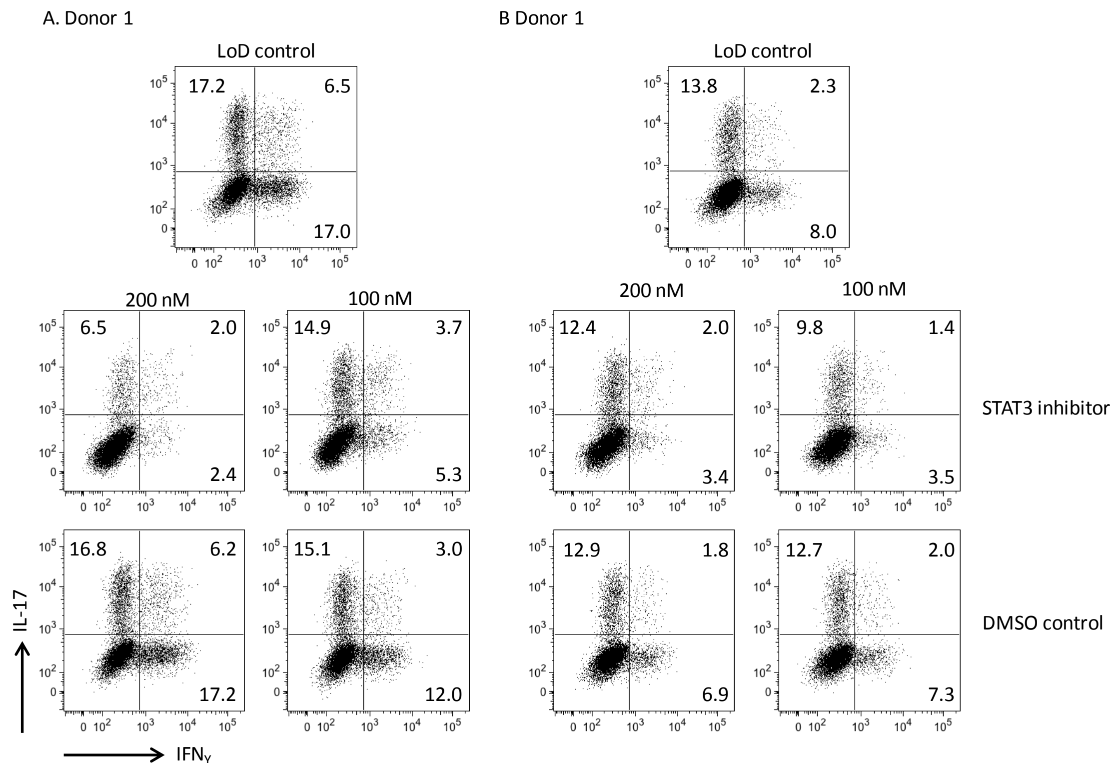


Figure 87. Inhibition of STAT3 activation in LoD cultures. Memory CD4^{+} T-cells were cultured under LoD conditions. STAT3 inhibitor S31-201 or DMSO control were added to cultures at 72 hours (200nM). At day 6 T-cells were restimulated with PMA/Ionomycin for 5 hours, the last 4 hours in the presence of Brefeldin A. Expression of IL-17 and IFNγ was determined by intracellular staining followed by flow cytometry. Plots are of 2 independent experiments, (A) Donor 1 (B) Donor 2.

These effects have been previously described on tumour cells with preferentially activated STAT3 (564). Viability markers were not included in these experiments but would have been beneficial to gate out dead cells which may have contributed to the differences in donor responses. STAT3 is an important regulator of cell cycle progression (565), which most likely explains the observed effect on cell growth. Future experiments should also assess the effect of STAT3 inhibition on HiD cell growth as a control. In summary, although low cell density has been found to promote Th17 cell responses, the mechanism for this finding remains elusive; however data within this Chapter provide promising future lines of investigation to determine how T-cell density affects Th17 cell responses.

5.5 Discussion

The data presented within this Chapter provide a novel insight into the regulation of human Th17 cell responses by cell density. Data demonstrate that low density culture of T-cells results in increased Th17 cell responses compared to high density culture with either anti-CD3/anti-CD28 bead or APC stimulation. Conversely modulating T-cell density had no effect on memory Th1 cell responses. Present data indicate that increased STAT3 activation may be the mechanism promoting Th17 cell responses in LoD conditions although further investigations are required. These data provide a novel and interesting insight into the potential of regulation of human Th17 cell responses by T-cell density.

5.5.1 T-cells and cell density

Very few investigations have assessed the effect of *in vitro* T-cell density on T-cell responses. Prior investigations, largely focussed on how density affects T-cell proliferation (546). Generally high cell densities promote proliferation and prevent apoptosis in resting PBMC populations. However the conditions required for optimal proliferation and cell survival may not be the same conditions required for a specific effector T-cell response. The importance of considering *in vitro* T-cell density was recently highlighted in an investigation into the cytokine release syndrome (CRS) (566). CRS was induced with detrimental effects as a result of the TGN1412 antibody, a CD28 superagonist, which was intended to treat RA. TGN1412 however caused massive cytokine release in phase I trials, but had not done so within *in vitro* trials from freshly isolated PBMC (567). Romer *et al* found that previous *in vitro* trials would have revealed the potential for CRS by culturing PBMC at a high density for two days which restores antibody sensitivity by up-regulating the functional activity of monocytes that increase $CD4^+$ T-cell activity (566). Data within this Chapter indicate that *in vitro* T-cell density can influence cytokine secretion patterns and the phenotype of effector T-cells. Low cell density preferentially promotes Th17 cell responses compared to Th1 cells although it would also be interesting to explore further T helper cell phenotypes including Th2, Treg, and Th9 cells. By gaining a greater understanding of how cell density contributes to *in vitro* T-cell responses this may increase our understanding of

T-cell regulation in disease and improve the *in vitro* to *in vivo* transition in therapeutic trials.

5.5.2 STAT3

My initial data indicate that STAT3 activation may be density-dependent, although as with AhR expression this may be an effect rather than a cause of density-dependent Th17 cell responses. Low cell density correlated with increased STAT3 activation whilst reduced STAT3 activation was observed in high density culture. STAT3 is an important regulator of a number of Th17 response genes (339). Hyper IgE Syndrome patients have a dominant negative STAT3 mutation and are deficient in Th17 cells, highlighting the importance of STAT3 in Th17 cell responses (559). Prior investigations have observed that cell density can modulate STAT3 activation. Within a breast epithelial cell line, STAT3 activation increases as cell confluence increases but diminished post confluence (562). Similar effects were observed within both a Caco-2 cell line and a squamous carcinoma cell line (560, 561). These data appear to contradict the data observed in
Figure 85. There are however striking differences between these and my experiments which may account for the contradiction. These investigations have used non-immune immortalised cell lines which may have different regulation of STAT3 activation. Furthermore, different mechanisms were found to regulate density dependent STAT3 activation in each of these models. One investigation demonstrated that increased squamous cell confluence caused the down-regulation of cyclin dependent kinase 2 (cdk2), a negative regulator of STAT3 (561). In comparison, in a breast carcinoma cell line, density dependent STAT3 activation was due to cell-cell adhesions (562). These studies indicate that mechanism by which STAT3 is regulated by cell density may be cell-specific.

A further difference is that STAT3 activation in T-cells is partly controlled by the cytokine *milieu*. In the absence of pro-Th17 cell cytokines STAT3 activation was reduced. A density dependent mechanism may provide conditions allowing cytokine responsiveness. It may therefore be interesting to further analyse differences in cytokine receptor expression between HiD and LoD cells. In particular the IL-23R may

be interesting to assess. IL-23 is part of the pro-Th17 cell *milieu* and signalling via the IL-23R is capable of activating STAT3, although IL-23R expression on HiD and LoD cells remains unknown. Initial data has revealed that STAT3 inhibition reduces Th17 cell responses at LoD indicating that STAT3 may be important in mediating the density dependent effect on Th17 cell responses. High variability was observed in the response to STAT3 inhibitors. This may in part be due to the affect on cell proliferation and viability. As proliferation was affected, future experiments should compare the effect of STAT3 inhibition on HiD proliferation. Furthermore, viability staining would allow dead cells to be gated out, which may minimise variation. These investigations are still in their infancy and further repeats of STAT3 inhibition are required. More extensive signalling analysis could include assessment of Socs3 expression, a negative regulator of STAT3 and Th17 responses (369). Future experiments also need to address if activated STAT3 is a cause or an effect of density dependent Th17 cell responses, and explore how density may modulate STAT3 activation (568).

5.5.3 Aryl hydrocarbon receptor

The AhR transcription factor has been described to be important to Th17 cell responses and is associated with *in vivo* Th17 cell phenotype (216). Prior data had indicated that AhR location and activation could be determined by cell density. AhR expression was enhanced in LoD conditions compared to HiD, however only minimal differences were observed in AhR localisation at the two densities. Only a small increase in AhR nuclear localisation was observed in LoD cells compared to HiD. Further investigations are required to determine if differences in AhR expression contribute to density dependent Th17 cell responses. However, as differences, particularly in nuclear localisation, are not striking it seems unlikely that AhR is the major cause of density dependent Th17 cell responses. One potential mechanism by which AhR may modulate Th17 cell responses from within the cytoplasm is by STAT binding. STAT1 and STAT5 are capable of repressing Th17 cell responses (377, 569). AhR has recently been demonstrated to be capable of binding to STAT1 and STAT5, and inhibits the repressive effects that these STATs have on Th17 cell responses (377). It is possible that the increased AhR present within the cytoplasm of LoD cells prevents STAT1 from repressing Th17 cell responses. Assessing the difference in STAT1

activation and location in HiD and LoD cells would be interesting to assess, and if differences were observed then performing a STAT1 co-immunoprecipitation with AhR would indicate if AhR was capable of sequestering STAT-1.

5.5.4 Cell-cell contact and Notch signalling

Previous studies indicated that density modulates cell-cell contact and contributes to density dependent effects. In a prior investigation T-cells were cultured in U-bottom 96 well plates to increase cell-cell contacts (461). Repeating this experiment did not indicate that cell contact contributed to the density dependent differences in T-cell responses. Another mechanism that has previously been demonstrated to block cell contacts is to culture cells in the presence of anti-LFA-1 and anti-CD18 antibodies (547). These antibodies are capable of preventing T-cell interactions by blocking T-cell aggregation which occurs during T-cell activation. A further mechanism to explore cell-cell contacts is by culturing cells in the presence of a Ca^{2+} chelator. Notch signalling is capable of determining Th17 cell responses and is dependent on DC expression of Notch ligands in a cell contact dependent manner (554). However my data indicated that Notch signalling was unlikely to contribute to density dependent Th17 cell responses, as gamma-secretase inhibition had no effect on T-cell responses; although the conclusions drawn from these experiments are limited due to the absence of a positive control. T-cells are capable of expressing Notch ligands, but in previous studies the Notch ligands that regulated Th17 cell responses were provided by APC (555) and may therefore explain why Th17 cell responses in this model appear to be Notch signalling independent.

5.5.5 T-cell viability

Prior investigations into the effects of density upon T-cell responses have demonstrated that apoptosis is increased at low cell density (544, 545). The effect of apoptosis upon density dependent Th17 cell induction may therefore merit further investigation than conducted within this Chapter. High cell density culture is thought to reduce T-cell apoptosis via secretion of catalase. Catalase present in high density conditioned media can reduce apoptosis in low density cells by protecting against hydrogen peroxide present in the low density extracellular *milieu* (546). These

investigations were conducted in serum free conditions, therefore the use of serum replacement in HiD and LoD culture potentially provides factors capable of preventing apoptosis and supporting low density culture (546). Investigations into the effect of cell density upon Th1 and Th2 cell clones have demonstrated that Th1 cells are more susceptible to the effects of T-cell density than Th2 cells (547). Recent studies have demonstrated that Th17 cells are more resistant to Fas mediated apoptosis than Th1 cell, but more susceptible than Th2 cells (456). Data within this Chapter however demonstrate that memory Th1 cell responses were not modulated by T-cell density. If density dependent apoptosis were occurring, then Th1 cell responses, which are highly susceptible to apoptosis, would be expected to be affected by density. Furthermore, initial analysis of viability did not indicate vast differences between HiD and LoD culture, which may explain why Th1 cell responses were not affected by density. However, assessing if density affects T-cell apoptosis and provides a selective pressure on the population would be interesting to assess.

5.5.6 *In vivo* T-cell density

Gorak-Stolinska *et al* comment that higher effector cell densities are analogous to sites of inflammation *in vivo* such as the rheumatoid joint or *Mycobacterium* infections (547). Regarding Th17 cells, recent literature indicates that Th17 cell are particularly important in the pathogenesis of early RA driving chronic joint destruction (253). Furthermore, Th17 cell fate mapping studies have demonstrated that chronic infection causes IL-17$^+$ cells to switch to the production of IFNγ. In comparison, the clearance of an acute *C.albicans* infection was associated with IL-17$^+$CD4$^+$ T-cells. By day 5 when the infection was cleared, IL-17 expression had switched off (337). In these situations it is possible that cell density is one of many factors that contribute to the limitation of Th17 cell responses. Low cell density at either early infection or the initial stages of autoimmunity may allow the expansion of Th17 cells, as density increases this may constrain/inhibit the Th17 cell response, prompting a change in T-cell phenotype. Analysing the effect of cell density upon T-cell phenotype *in vivo* would however be more difficult to address; but harnessing fate mapping studies along with immunohistochemistry or *in vivo* imaging may facilitate these investigations (337, 570).

5.5.7 Conclusion

In summary, these data provide a novel insight into the regulation of human Th17 cell responses by T-cell density. Low cell density promotes Th17 cell responses *in vitro* and these effects may be regulated by T-cell density dependent activation of STAT3. The potential remains that these effects are an *in vitro* phenomenon. However, simple cell culture parameters such as cell density can clearly have a profound influence on T-cell effector phenotype. This has implications when data derived from *in vitro* culture are extrapolated to the *in vivo* setting. Increased investigation within this area may increase the consistency of data assessing the factors that regulate T-cell responses and provide greater understanding of how T-cell responses are regulated.

6 Chapter 6. General Discussion

Within this thesis I aimed to investigate factors, other than the cytokine milieu, that were capable of regulating the human Th17 cell response. Prior to the identification of Th17 cells, studies established that T-cell stimulation strength could determine Th1 and Th2 cell responses (394). In recent years, studies in mice have indicated that murine Th17 cell responses can be affected by stimulation strength (398–400) and therefore, I hypothesised that stimulation strength would also influence human Th17 cell responses. Having found an effect of stimulation strength upon Th17 cell responses I also sought to assess an *in vivo* situation that may permit stimulation strength dependent Th17 cell responses associated with autoimmunity. In addition, I finally assessed the influence of T-cell density on Th17 cell responses, a parameter largely ignored in *in vitro* culture conditions. The major finding within this thesis are summarised in Figure 88.

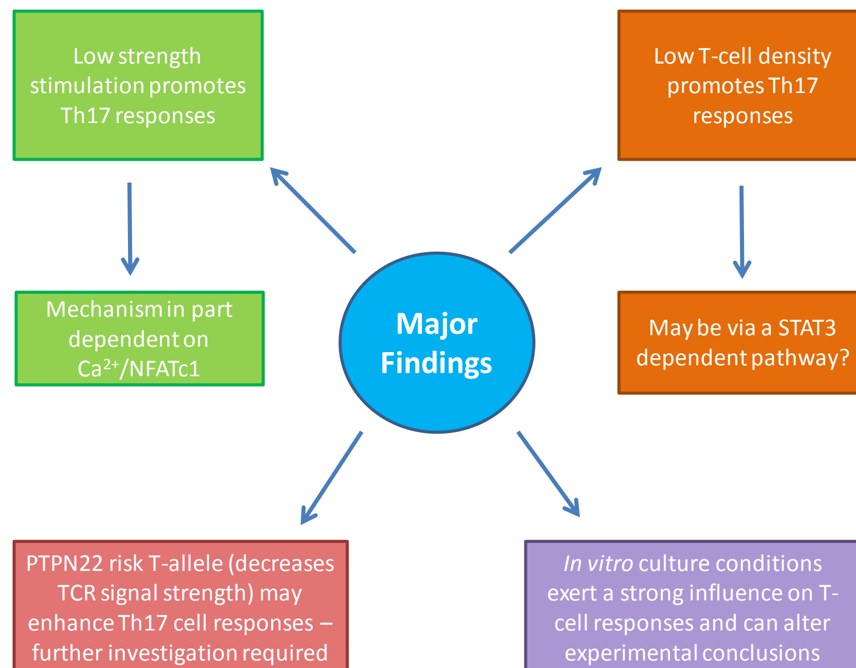


Figure 88. Major findings from thesis entitled: Regulation of human T helper 17 cell responses.

6.1 Summary of Findings

6.1.1 Chapter 2

The initial focus of my thesis was to investigate how stimulation strength may affect human Th17 cell responses. T-cells were activated with different ratios of anti-CD3/anti-CD28 beads, in the presence of pro-Th17 cytokines (IL-1β, TGF-β, and IL-23). It was established that low strength TCR stimulation promoted human Th17 cell responses both proportionally and absolutely. The availability of CD28 co-stimulation and the presence of pro-Th17 cytokines are both essential factors for low strength Th17 cell responses. Moreover, these data counter prior claims that the cytokine *milieu* is capable of reversing the effect of stimulation strength (67): THi stimulated cells in the presence of pro-Th17 cytokines did not generate IL-17^{+} T-cells. Furthermore, the use of a more physiologically relevant stimulus of moDC also demonstrated that lower strength stimulation provided by APC promotes Th17 cell responses. The strength of stimulation was reduced either by decreasing the allogeneic moDC:T-cell ratio or by decreasing the concentration of super-antigen at a fixed ratio of autologous moDC and T-cells; both demonstrated that reduced stimulation strength promotes Th17 cell responses. Figure 89 depicts the stimulation strength requirements for some of the CD4^{+} T helper subsets, and correlates where my finding that human Th17 cell responses are favoured by low strength stimulation with these. However the diagram forms only an estimate as the direct investigative comparisons have not been made and in addition much of the data regarding the other subsets has been performed in mice.

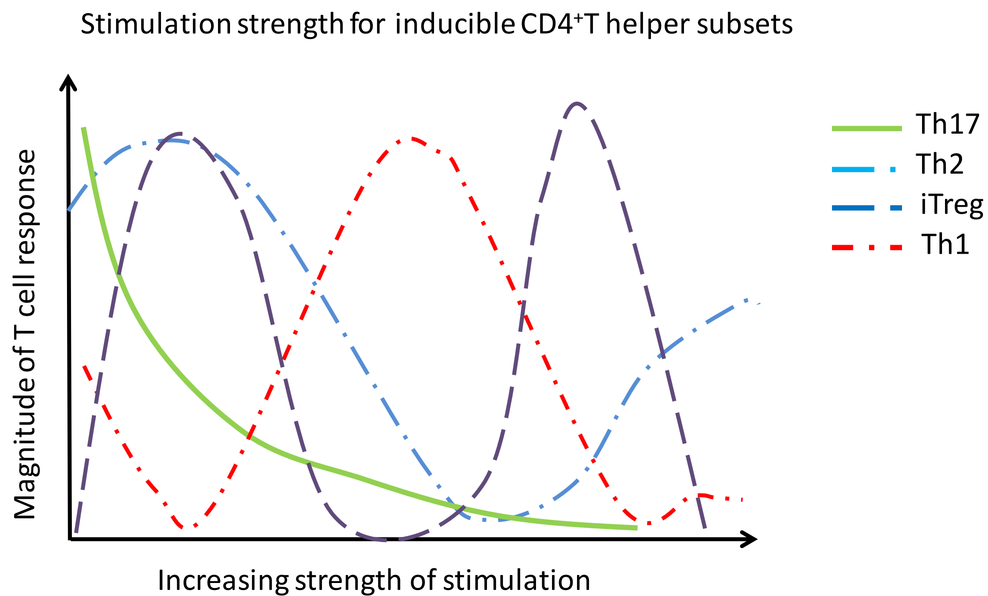


Figure 89. The stimulation strength requirements of CD4+ T helper subsets. My data indicate that human Th17 cell responses are favoured by low strength stimulation (514). iTreg are favoured by low doses of strong agonists (425) but also *in vivo* generation of IL-10 producing iTreg are promoted by high affinity and high peptide dose (474). Th2 cells may like human Th17 be favoured by low affinity peptides or low antigen doses (402, 403) but can also be induced at very high doses depending on the antigen (404). Th1 cells are induced by high affinity peptide or high dose antigen (402, 403).

6.1.2 Chapter 3

Having established a role for stimulation strength in human Th17 cell responses a number of hypotheses were explored to explain the mechanism by which this occurred. Although markers of regulatory T-cells including IL-10, TGF-β, and Foxp3 were elevated in THi conditions compared to TLo, further analysis did not reveal that these factors were capable of inhibiting THi Th17 cell responses. However, investigations assessing NFATc1, a TCR/Ca^{2+} regulated transcription factor important to *IL-17* expression (492), revealed that NFATc1 binding to the *IL-17* promoter was enhanced in TLo stimulated cells compared to THi cells. Furthermore, the addition of a Ca^{2+} ionophore, ionomycin, inhibited TLo IL-17 responses by ~50%, indicating that high Ca^{2+} signalling, such as that induced by high strength TCR signalling, is potentially inhibitory to human Th17 cell responses. However, the difference in NFATc1 binding to the promoter was not due to differences in NFATc1 translocation to the nucleus, indicating that THi culture alters the capacity of NFATc1 to bind to the *IL-17* promoter. Potential mechanisms may include chromatin regulation or the binding of repressive transcriptional elements. The data within Chapter 3 currently indicates that in part low strength stimulation favours Th17 responses via a NFATc1/Ca^{2+} dependent mechanism.

6.1.3 Chapter 4

The findings from Chapters 2 and 3 raised the question of how low strength stimulation may affect Th17 responses *in vivo*, and may promote Th17 responses as associated with autoimmune diseases, including RA (261, 266, 269, 571). Interestingly, many of the genes associated with increased risk of developing RA are genes that function as part of the TCR signalling pathway and stimulation strength (516). For example, a SNP within the PTPN22 gene encoding the protein tyrosine phosphatase Lyp is highly associated with the pathogenesis of RA (517). Lyp is a negative regulator of TCR signalling and expression of the PTPN22 1858T allele is thought to confer a gain-of-function mutation resulting in enhanced inhibition of TCR signalling (521). I investigated if 'healthy' human memory T-cells from T-allele carriers would have enhanced Th17 responses due to reduced TCR signalling conferred by the PTPN22 C1858T SNP. I genotyped 20 individuals for the C1858T SNP and then assessed IL-17, IFNy and IL-10 responses, that resulted from memory T-cells stimulated with anti-CD3/anti-CD28, by ICS and/or ELISA. Although the proportion and absolute IL-17 and IL-17/IFNy response was marginally higher in the T-allele carriers than C-allele homozygotes, and the IL-10 secretion was lower, the number of genotyped individuals was insufficient to draw statistical significance. Sample size analysis revealed that for statistical significance to be drawn from more striking observations an excess of 12 donors per group would be required.

6.1.4 Chapter 5

I lastly investigated the role that T-cell density may have in determining Th17 cell responses. Cell density is a variable of *in vitro* tissue culture that has been largely ignored, evident by the absence of publications within this field. In addition, although the majority of publications may state cell number or ratio, they do not refer to the plate well size and therefore cell density can be a further variable between experiments. Data generated within Chapter 5 demonstrated a profound role for cell density in modulating Th17 effector responses, where a low T-cell density promoted both absolute and proportional Th17 responses despite receiving a high strength stimulus. Furthermore the proportion of IFNy$^+$ T-cells, when cultured in the presence of pro-Th1 cytokines, were unaffected by T-cell density indicating that the effect may

be more specific to Th17 cell responses. The mechanism for density dependent Th17 cell responses was explored, addressing factors including: excess cytokine availability, cell-contact, Notch signalling and AhR translocation, though investigations did not indicate that these were responsible. STAT3, a transcription factor important to Th17 cell responses (559), has been demonstrated to have altered activation depending on the density of non-immune cells (560–562). I found that STAT3 activation was indeed higher in LoD cells compare to HiD, though inhibition of STAT3 in LoD cells had variable effects. Although the mechanism determining density-dependent Th17 cell responses is still being explored, data thus far indicate that STAT3 signalling may play a role in the low density mediated Th17 cell responses.

The findings outlined above are interesting in themselves; however, collectively this thesis further highlights the importance of assessing simple cell culture factors such as *in vitro* T-cell density, and the type and/or strength of stimulation employed during an investigation, which can have dramatic effects on results. Within the Th17 cell field cell culture medium has also been demonstrated to affect T-cell responses, with IMDM favouring Th17 responses compared to conventional RPMI (215), but the question remains which condition better represents the *in vivo* situation? Furthermore, within *in vivo* mouse models it is acknowledged that certain mouse models more readily lead to a specific T-cell response. For example the DBA/2 mouse strain is biased towards a Th2 response, whereas the C57BL/6 is biased to Th1 (572), and therefore the strain of mouse chosen could alter the interpretation of an investigation. Together these findings emphasise the need to further investigate *in vitro* culture conditions/experimental models, which may allow the development of more standardised techniques that will hopefully permit better comparison to the human *in vivo* system in the future. However, although the *in vitro* systems currently used are artificial, they are useful for delineating mechanisms capable of controlling Th17 development. For example, although there is no physiological relevance of IMDM and RMPI *in vitro* culture media, the comparison of these media highlighted that AhR signalling is an important mechanism in Th17 development, and may be important *in vivo*.

6.2 Future investigations

6.2.1 T-cell density

There are many future lines of investigation that can stem from the research performed within this thesis. Primarily the mechanism underpinning the promotion of IL-17^{+} cells by low density culture needs to be confirmed. The data depicted in Chapter 5 indicate that enhanced STAT3 activation may explain the difference in response. Although the importance of STAT3 in this system remains to be determined, data with the literature do clearly indicate that STAT3 is important for Th17 cell responses. It may therefore be of more benefit to establish how cell density in the model leads to differential STAT3 activation. One study demonstrated that cell-contact dependent mechanisms can contribute to density-dependent STAT3 activation which may require further exploration in the HiD/LoD system (562). In addition, the requirement of pro-Th17 cytokines for low density-dependent Th17 cell responses may indicate that differences in cytokine receptor expression may confer the observed difference in STAT3 activation. In the event that enhanced STAT3 activation in LoD cells is a marker of Th17 cell induction rather than the cause of density-dependent responses there are further lines of investigation that may be pursued. For example, IL-2 has been demonstrated in murine models to inhibit Th17 cell responses by inducing STAT5 activation which binds to the *Il-17* promoter to block STAT3 binding (573). One investigation has revealed that in mouse T-cells stimulated with the same peptide dose, only T-cells cultured at a high density where able to sense IL-2 and phosphorylate STAT5 (146). The high and low densities assessed were comparable to the HiD and LoD densities used in my investigation, furthermore data observed within our laboratory indicates that anti-CD3/anti-CD28 stimulation enhances T-cell IL-2 secretion. Therefore a further possibility is that IL-2 production and T-cell responsiveness at high density inhibits Th17 responses.

Once the mechanism of density-dependent Th17 cell responses has been established it would be interesting to assess if this is an *in vitro* phenomenon or if it has *in vivo* relevance. However, assessing the affect of T-cell density *in vivo* may be difficult. Although not *in vivo*, a simple experiment to create a more physiological situation

would be to perform T-cell:DC co-cultures upon epithelial cell layers, which may alter density dependent T-cell responses by changing cell-cell contacts. One potential method of assessing density *in vivo* may be to employ the novel nano-magnetic labelling technology which has been used to guide stem cells to damaged tissue and DC cell therapies to target sites in mice (574, 575). To assess the effect of density on T-cell responses *in vivo*, T-cells could be labelled with nano-magnets and injected into mice, an external magnet allows the T-cells to be guided or held to a certain tissue area which can be assessed by MRI. Depending on the number of labelled cells injected this would increase or decrease the density of T-cells in a single locale. APC primed with Th17 promoting stimuli could also be magnetically labelled to allow the differences in the immune response conferred by T-cell density to be assessed.

6.2.2 Influence of stimulation strength

The strength of T-cell stimulation as discussed in Chapters 2 and 3 can be determined by a number of factors including the rate and duration of TCR triggering, the affinity of TCR for pMHC, the number of TCR activated, and the signal threshold which can be modulated via CD28 signalling (388). Within my investigations the strength of stimulation was modulated by altering the number of expander beads/DC, or by altering the amount of anti-CD3/super-antigen present per bead/DC respectively. In addition the effect of the duration of stimulation on Th17 responses was also briefly explored. In each model varying stimulation strength it was found that low strength stimulation favoured Th17 cell responses.

Further investigation into the regulation of Th17 cell responses by low strength stimulation in an antigen-specific manner would also be interesting. By using a TCR transgenic mouse model, the strength of stimulation may be modulated by altering the quantity of peptide loaded, or by using altered peptide ligands which reduce the quality of MHC to peptide binding or T-cell to pMHC binding (400). These experiments would allow the T-cell response to be assessed in an antigen-specific manner, which is not possible when using human T-cells due to the TCR polyclonality. Compared to T-cell activation by an antibody, antigen-specific T-cell activation of TCR-transgenic mouse T-cells provide a more physiologically relevant stimulus. The physiological

relevance of a fully TCR-transgenic T-cell response is also unclear (576), but these investigations would improve and compliment our understanding of Th17 cell regulation and allow comparison to *in vitro* induced human Th17 cell responses.

Differences in NFATc1:*IL-17* promoter binding were observed in THi and TLo cultures and the addition of ionomycin reduced Th17 cell responses by 50%. These results, however, do not comprehensively establish if differences in Ca^{2+} signalling are responsible for the difference in Th17 response. Future analysis of stimulation strength dependent T-cell responses may be benefitted by including analysis of Ca^{2+} signalling, to confirm that Ca^{2+} flux does indeed differ. Furthermore, conclusions would have been strengthened by assessing if NFATc1:*IL-17* promoter binding was decreased in TLo cultures following the addition of Ionomycin, which reduced IL-17 responses by 50%. In addition, as loss/gain-of function effect of the SNP is still uncertain, the data within Chapter 4 concerning the PTPN22 SNP would also be strengthened in future by assessing differences in Ca^{2+} signalling. The effect of the risk T-allele has previously been demonstrated to attenuate Ca^{2+} signalling in memory T-cells (528), though there is still some debate over whether the SNP confers a gain or loss-of-function mutation (519). By establishing in healthy donors if Ca^{2+} signalling is attenuated, this would confirm if the T-allele was able to reduce stimulation strength and allow the SNP effect to be quantified. Methods to assess Ca^{2+} signalling in these situations could include confocal microscopy or flow cytometry.

Although my investigations have established that low strength stimulation promotes Th17 cell responses I could not contextualise these findings with the other human T-cell effector phenotypes. To achieve this, the effect of stimulation strength on the broad spectrum of T helper subsets is required. One means by which this could be achieved is by culturing T-cells under Th1, Th2, Treg inducing conditions, assessing the effects of different stimulation strengths on phenotype by using a broad panel of defined cytokine markers. Furthermore, it is not only the strength of T-cell activation that is important to determining T-cell phenotype: different molecules comprising the immune synapse can also contribute to ‘stimulation strength’. Different signalling

pathways are activated depending on the stimulatory molecule bound (e.g. CD3, CD5, CD28), which can result in differences in transcription and therefore T-cell response. An example of this was recently highlighted where CD5 co-stimulation was found to have a novel role in naive Th17 cell induction (577). In the presence of anti-CD3, anti-CD5 co-stimulation was observed to be favourable in comparison to CD28 stimulation at inducing a Th17 cell response, thought to be due to elevated IL-23R expression on T-cells by CD5. However, although the secretion of IL-17 is high, the proportions of Th17 cells remained low (on average 3.7%), as observed in previous studies assessing human Th17 induction from naive T-cells. Furthermore, induction of IFNy^{+} populations was greater than IL-17^{+} under CD3/CD5 stimulation compared to CD3/CD28 stimulation. In addition, cell adhesion molecules/integrins such as CD2, ICAM-1 and LFA-1 contribute to the strength of immune synapse formation, modulate TCR signalling thresholds, and can modulate Th1 and Th2 responses (106, 578, 579). Therefore, addressing the effect of different stimulation strengths and routes of modulation upon T-cell responses may reveal novel mechanisms by which naive and/or memory Th17 cell responses are induced or regulated.

6.2.3 Potential role of IL-2

IL-2 is mainly a T-cell derived growth factor that supports both T-cell proliferation and survival (1). Activation of T-cells via TCR and CD28 results in rapid secretion of IL-2 proportional to the strength of stimulation via an AP-1/NFAT dependent pathway (2, 3). However, investigations have found IL-2 to have an inhibitory effect upon murine Th17 cell induction (4). The inhibitory effect of IL-2 is thought to be due to the activation of the STAT5 signalling pathway (5). STAT5 can directly repress the *Il-17A* locus, but in addition, STAT3 and STAT5 compete for the same *Il-17A* binding sites and the balance of these two transcription factors has been found to determine the capability of IL-2 to repress Th17 cell induction. Furthermore, IL-2 signalling has also been demonstrated to decrease expression of gp130, a subunit within the IL-6R family, preventing IL-6 signalling and thereby decreasing STAT3 activation (6).

Until recently, IL-2 had not been observed to inhibit human Th17 cell responses (7). The presence of IL-2 in combination with IL-1β was found to enhance human Th17 cell

responses, and blockade of IL-2 signalling prevented IL-17 expression (8). Furthermore, the combination of IL-1β and IL-2 was been found to be sufficient to convert human nTreg cells into Th17 cells, indicating a further discrepancy between the human and murine differentiation systems (9). Human Th17 cell differentiation does not require IL-6 and therefore this may be one reason for the difference in the murine-human observation. However, very recently IL-2 was observed to reduce IL-17 expression in human memory Th17 cell clones, potentially suggesting parity between the murine and human systems (10).

Within this thesis, the role of IL-2 upon both high stimulation strength (THi) and high density (HiD) conditions was not explored; this was mainly due to the historic support for the use of IL-2 in human Th17 cell cultures. However, as IL-2 is an early effector molecule induced by TCR stimulation and increases as a result of increased stimulation strength it is a limitation of this thesis that the contribution of IL-2 has not been addressed within these systems. Nevertheless, recent experiments, outside of this thesis, have explored the potential inhibitory role of IL-2 within the HiD/LoD model. The addition of an anti-IL-2 neutralising antibody at 10 ug/mL at days 0 and 3 was not observed to enhance the proportion of IL-17^{+} cells, but did reduce T-cell proliferation. Furthermore, the addition of 2000 U/mL of IL-2 daily to LoD cultures was also observed to have no inhibitory effect upon the proportion of IL-17^{+} cells, despite LoD cells, like HiD cells, being IL-2 responsive from 24 hours as marked by expression of CD25 (Figure 69). In addition, STAT5 phosphorylation, which mediates the negative effects of IL-2 on IL-17 expression in mouse T-cells, was assessed by western blot in HiD and LoD cells at 72 and 96 hours. STAT5 phosphorylation was found to be enhanced within LoD cell lysates compared to HiD cell lysates at both time points. Together these data suggest that IL-2, if inhibitory to human Th17 cell responses, is not the sole factor contributing to the inability of HiD/THi cultures to generate IL-17^{+} T-cells.

6.2.4 Regulation of Th17 cells by miRNA

The study of immunology is moving towards gaining a greater understanding of the signalling and transcriptional regulation of discrete populations of T-cells. As previously mentioned I think that assessing differences in chromatin regulation conferred by stimulation strength would prove informative. A further area that would also be tempting to investigate is the role and regulation of miRNAs on stimulation strength dependent Th17 cell responses. Recently microRNAs (miRNAs) have been demonstrated to play an important role in immune regulation, including T helper cell subset development (586). miRNAs are non-coding RNAs of 21-24 nucleotides in length that regulate gene expression through different mechanisms, including inhibition of mRNA translation or the induction of mRNA degradation(587). miRNAs have been found associated with a Th17 cell phenotype. miRNA-326 contributes to multiple sclerosis disease progression by promoting Th17 responses through repression of Est-1, a negative regulator of Th17 (588). Another group reported the co-expression of miRNA-146a and IL-17 in RA patients (589) and, more recently, it was demonstrated that expression of miR-133b and miR-206 is co-regulated with IL-17 production in murine and human T cells (590). In addition, studies have demonstrated that TCR signalling and strength can be regulated by miRNA. MiR-181a has been shown to enhance TCR sensitivity by downregulating multiple phosphatases and lowering the TCR signalling threshold (591). Moreover, CD28 stimulation upregulates miRNA-214 which targets *Pten* a negative regulator of TCR signalling (592). Therefore it would be tempting to compare the miRNA profiles of THi and TLo stimulated cells to assess if certain miRNAs are capable of regulating stimulation strength-dependent Th17 responses. The requirement of miRNA for THi and TLo responses could be assessed initially by knocking down dicer, a cytoplasmic ribonuclease essential to miRNA maturation and function (593). If miRNA are found to be important, a miRNA array could be performed to identify potential miRNAs capable of differentially regulating stimulation strength dependent responses. The function of the identified miRNAs could be confirmed by specific miRNA over-expression or knock-down.

6.2.5 PTPN22 C1858T SNP and T-cell regulation

A further project to explore is that of PTPN22 regulation of T-cell responses. Although the data presented within Chapter 4 indicated only a small effect of the minor T-allele on Th17 cell responses, further studies have revealed that alteration to PTPN22 function conferred by the SNP can alter T-cell responses. Moreover, src family kinases (SFKs) are also involved in the regulation of many immune receptor signalling pathways including TLR, chemokine, and Fc Receptors (539). PTPs regulate SFK-dependent pathways and therefore perturbations to SFK signalling conferred by polymorphisms in PTPs are likely to affect the immune response (594). Therefore an interesting project may be to assess the contribution of PTPN22 SNPs to T-cell development and function by taking a broader approach. The capability of Lyp to regulate DC maturation and function and how this changes with the presence of the minor T-allele has not been explored in detail. Alterations to DC function would be predicted to affect T-cell development and phenotype by altering the cytokine milieu and the provision of co-stimulation as seen by Zhang *et al* (519). In order to perform this study the availability of Pep$^{-/-}$ mouse models would be a particularly useful model, as Lyp inhibitors which could be used for human DC and T-cell investigations are less selective (595). Furthermore, the availability of sufficient numbers of healthy human genotyped donors would be essential to finally confirm experimental findings. This study could provide new information on how a SNP in the PTPN22 increases susceptibility to certain autoimmune diseases.

6.2.6 Conclusion

In conclusion, this thesis demonstrates a profound and novel role for stimulation strength and T-cell density in determining *in vitro* human Th17 cell responses. Low strength stimulation was found to promote Th17 cell responses via a Ca^{2+}/NFATc1 dependent pathway: a finding that may have implications for the type of therapies and treatments used to control Th17 cell associated pathologies. Moreover, this research suggests that perturbations to TCR signalling associated with RA may affect the balance of the T-cell response, potentially favouring Th17 cell development. In addition, Th17 cell responses were also favoured by a low T-cell density. Although the mechanism is yet to be determined, this finding provides a novel insight into how Th17

cell responses are regulated *in vitro* and may alter our interpretation of how successful *in vitro* defined therapies against Th17 cells are. In addition to the main findings, this research also serves to highlight the dramatic effect that alterations to *in vitro* culture conditions can have on immune responses: supporting calls for standardisation of human *in vitro* and *ex vivo* T-cell analysis and careful comparison with *in vivo* murine systems.

7 Chapter 6 Materials and Methods

7.1 T-cell isolation and culture

7.1.1 Source of Cells

Buffy coats or Leucocyte Reduction System (LRS) cones were obtained from the National Blood Service. Peripheral blood samples, were obtained with informed consent following approval by the Newcastle and North Tyneside Research Ethics Committee 2.

7.1.2 Isolation of peripheral blood mononuclear cells

PBMC were obtained from Buffy coat, LRS cone, or peripheral blood by density gradient centrifugation over Lymphoprep. Lymphoprep separates the PBMC from red blood cells and granulocytes using a density gradient. The buffy coat was firstly diluted 1:4, (LRS cone 1:2; peripheral blood 1:1) using pre-warmed Hanks Balanced Salt Solution (HBSS; Ca^{2+} and Mg^{2+} free; Sigma; St Louis USA) supplemented with 2mM ethylenediamine tetraacetic acid (EDTA) (Sigma), a metal ion chelator that prevents binding of Ca^{2+} dependent cadherins and the clumping of cells. 20 mL of the diluted buffy coat, cone, or peripheral blood was layered onto 15 mL of Lymphoprep and then centrifuged at 895 g (low acceleration, brake off, room temperature) for 30 minutes. PBMC were then retrieved from the Lymphoprep and plasma interface and were then washed in cold HBSS + 1% FBS (Gibco; Paisley, UK) at 600 g for 7 minutes (4°C) to remove any residual Lymphoprep (Axis-shield PoC; Oslo, Norway) and debris. PBMC were washed a second time in cold HBSS + 1% FBS at 250 g for 7 minutes to remove platelets. PBMC were kept cold throughout to prevent cell adhesion and activation. PBMC were filtered through a 100 µM nylon filter (BD Biosciences; Oxford, UK) to remove any clumps or debris and then cells were counted on a Burker counting chamber.

7.1.3 Isolation of naïve $CD4^+$ T-cells

The EasySep human naïve $CD4^+$ T-cell enrichment kit (StemCell Technologies; Vancouver, Canada) was used to extract naïve $CD4^+$ T-cells from PBMC by negative

selection. The RoboSep was used in conjunction with the kit for the automated isolation of naive CD4⁺ T-cells. The naïve T-cell enrichment kit enriches naïve CD4⁺ T-cells using a cocktail of monoclonal antibodies against CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123, TCRγ/δ, and glycophorin A. These antibodies are complexed to an anti-dextran antibody to form bispecific Tetrameric Antibody Complexes (TAC), which recognise both the cell surface antigen expressed on the unwanted cells and dextran-coated magnetic nanoparticles. A separate biotinylated anti-CD45RO antibody and a bispecific TAC against biotin and dextran, which is included in the enrichment cocktail, is used to remove CD4⁺CD45RO⁺ memory T-cells. The cells labelled by the cocktail and magnetic nanoparticles are removed using a magnet, leaving the pure population of naïve CD4⁺ T-cells in suspension. The procedure was followed according to manufacturer's instructions. Following isolation the cells were counted using a Burker counting chamber. The purity of the naïve CD4⁺ T-cells was measured by cell surface staining for CD4 and CD45RA using the surface staining protocol described below and analysed by flow cytometry. Purity was >90% (Figure 90).

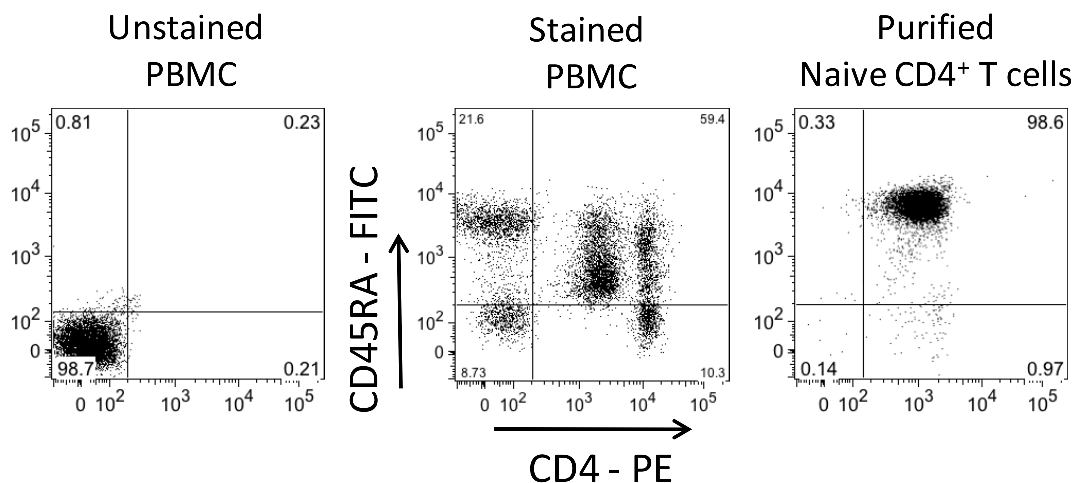


Figure 90. Naive CD4⁺ T-cell purity. Naïve CD4⁺ T-cells were isolated from PBMC by negative selection using the RoboSep naive CD4⁺ T-cell enrichment kit. Naïve CD4⁺ T-cell purity was measured by staining for CD4 and CD45RA. T-cells were gated on forward scatter side scatter to remove cell debris. Data are representative of 3 independent experiments.

7.1.4 Isolation of memory CD4⁺ T-cells

The EasySep human memory CD4⁺ T-cell enrichment kit (StemCell Technologies) was used to extract memory CD4⁺ T-cells from PBMC by negative selection. The RoboSep was used for automated isolation. The memory CD4⁺ T-cell enrichment kit enriches

memory CD4$^+$ T-cells using a cocktail of bispecific TAC recognising CD8, CD14, CD16, CD19, CD20, CD36, CD45RA CD56, CD123, TCRγ/δ, and glycophorin A on unwanted cells, and dextran-coated magnetic microparticles. The cells labelled by the cocktail and magnetic microparticles are removed using a magnet, leaving the remaining pure population of memory CD4$^+$ T-cells in suspension.

The procedure was followed according to manufacturer's instructions. Following the magnetic isolation procedure isolated cells were counted using a Burker counting chamber. The purity of the memory CD4$^+$ T-cells was measured by cell surface staining for CD4 and CD45RO using the surface staining protocol described below and analysed by flow cytometry. Purity was routinely >90% (Figure 91).

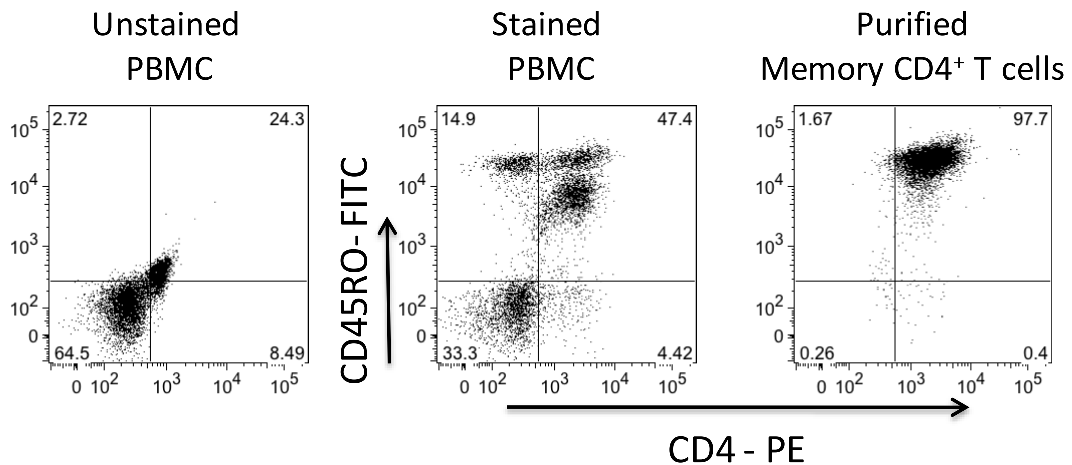


Figure 91. Memory CD4$^+$ T-cell purity. Memory CD4$^+$ T-cells were isolated from PBMC by negative selection using the RoboSep memory CD4$^+$ T-cell enrichment kit. Memory CD4$^+$ T-cell purity was measured by staining for CD4 and CD45RO. Firstly cells were gated on forward scatter and side scatter to remove cell debris. Data are representative of 6 independent experiments.

7.1.5 Isolation of CD4$^+$ T-cells

RosetteSep Human CD4$^+$ T-cell Enrichment Kit (StemCell Technologies) was used to extract CD4$^+$ T-cells from buffy coats or LRS cones. The enrichment antibody cocktail negatively selects CD4$^+$ T-cells by cross linking unwanted CD4$^-$ cells to red blood cells, forming immunorosettes. The mixture is layered over a density gradient media, e.g. Lymphoprep, and centrifuged. The increased density of the rosetted cells causes them to pellet, leaving the enriched CD4$^+$ T-cell population at the interface between the plasma and Lymphoprep layers, allowing easy removal with a Pasteur pipette.

The procedure was followed according to manufacturer's instructions. After washing, the resultant CD4$^+$ T-cells were resuspended in Phosphate buffered saline (PBS) + 2% FBS and counted using a Burker counting chamber. The purity of the CD4$^+$ T cells was measured by cell surface staining for CD3 and CD4 using the surface staining protocol described 7.5.1 and analysed by flow cytometry. Purity was found to be routinely >90% (Figure 92).

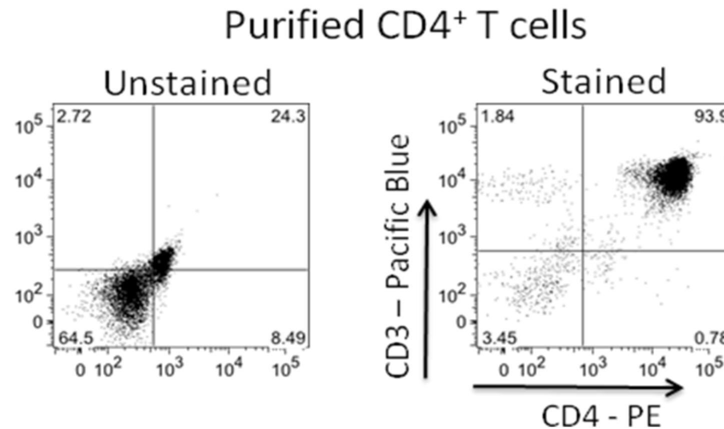


Figure 92. CD4$^+$ T-cell purity. CD4$^+$ T-cells were isolated directly from buffy coats or LSR cones using RosetteSep CD4$^+$ T-cell enrichment cocktail. Purified CD4$^+$ T cells were stained with CD3 and CD4 to measure T-cell purity. Cells were firstly gated on forward scatter vs side scatter in order to remove debris. Data are representative of 3 independent experiments.

7.2 Cryopreservation of T-cells

Following cell separation and isolation, T-cells were either cultured or cryopreserved. Cyropreservation allows long term cell storage of cells for future use. Cyropreservation of cells requires them to be cooled between -80˚C and -197˚C (liquid nitrogen boiling point), which prevents biological processes from occurring, including those controlling cell death. To prevent cellular damage during cryopreservation a cryoprotectant, such as Dimethyl Sulphoxide (DMSO; Sigma), is added. DMSO lowers the freezing temperature and prevents the formation of ice crystals; however DMSO is toxic to cells. Toxic effects of DMSO are minimised when used in a 10% solution with FBS. In addition, ensuring that all reagents are pre-cooled for freezing and pre-warmed for thawing protects cells during cyropreservation. Briefly, ice-cold cells were pelleted at 400g, for 7 minutes, at 4˚C. Following aspiration of the supernatant, cells were resuspended at a concentration of 10x10^6 cells/mL in ice cold FBS + 10% DMSO, and transferred to a pre-cooled cryovial (1 mL/ vial). Cells were then transferred to -80˚C in

a polystyrene box to control the rate of freezing. For long term cell storage after 1 week cells were transferred to liquid nitrogen.

7.3 CD4+ T-cell culture

Isolated CD4+ T-cells were plated to a concentration of $1x10^6$ cells/mL, in 1 mL of IRS10 (Iscoves Modified Dulbeccos Medium (IMDM, Sigma) containing 10% Knockout Serum Replacement (Invitrogen; New York, USA) with Penicillin/Streptomycin (100 µg/mL, Gibco) and L-glutamine (2mM, Cambrex; Wiesbaden, Germany) in a 24 well plate. CD4+ T-cells were activated using anti-CD3/anti-CD28 T cell expander Dynabeads (Invitrogen). To measure the effects of stimulation strength the ratios of anti-CD3/anti-CD28 expander beads and CD4+ T-cells were titrated. Following titration a high and low strength stimulus were chosen for T-cell activation: 1:1 (THi) or 1:50 (TLo) bead:T-cell ratio, respectively. To induce specific effector T-cell responses, day 0 CD4+ T-cells were cultured in the presence of skewing cytokines (Table 5). Cells were cultured at 37°C, 5% CO_2 for 6 days (longer term cultures in which the cells are rested are described below). On day 3 of culture the appropriate cytokines (see Table 5) were added to cells. As the cells proliferated during culture, cells were split as and when required, in part indicated by a change in media colour.

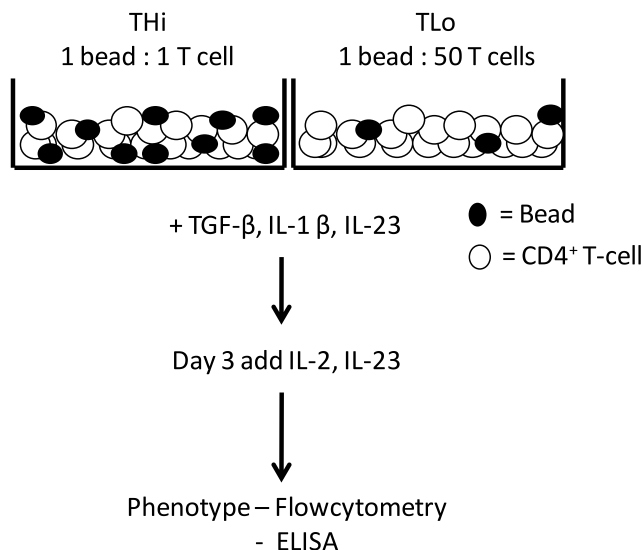


Figure 93. THi/TLo Th17 culture conditions. Human CD4+ T cells are activated with anti-CD3/anti-CD28 expander beads under THi (1:1 bead:cell ratio) or TLo (1:50 bead:cell ratio) conditions in the presence of pro-Th17 cytokines TGF-β, IL-1β, & IL-23. At day 3 IL-2 and IL-23 are added to cultures. At day 6 the T-cells are phenotyped by Intracellular flowcytometry and/or ELISA.

Post day 3 refreshing or splitting was performed using IRS10 containing IL-2 (10 IU/mL) and IL-23 (10 ng/mL) for Th17 conditions or for Th1, Th2 and Th0 cell conditions IRS10 containing 10 IU/mL IL-2. For any additional reagents used in the investigation of Th17 cell responses, their concentrations, and addition times are shown in Table 6. At day 6 the.cells were restimulated with PMA/ionomycin in the presence of BFA (as described 7.5.2) and the T-cell phenotype was assessed by intracellular cytokine staining and flow cytometry (as described 7.5.3) measuring the proportion of IL-17^{+} or IFNy^{+} cells.

T helper cell phenotype	Cytokines added Day 0	Cytokines added Day 3
Th1	IL-12 (10ng/mL; Peprotech; London, UK)	IL-2 (10 IU/mL Roche West sussex, UK)
Th2	IL-4 (10ng/mL; Immunotools; Friesoythe, Germany)	IL-2 (10 IU/mL)
Th17	IL-1β (10ng/mL; Peprotech) TGF- β (10ng/mL; Peprotech) IL-23 (10ng/mL; R&D Systems)	IL-2 (10 IU/mL) IL-23 (10ng/mL)
Th0	IL-2 10 (IU/mL)	IL-2 (10 U/mL)

.
Table 5. Cytokines and concentrations used for T helper cell responses

Factor	Concentration	Addition	Company
Anti-IL-4	20 µg/mL	Day 0 & 3	eBioscience; SanDiego, USA
Rat IgGk Isotype	20 µg/mL	Day 0 & 3	eBioscience
Anti-CDw210 (IL-10R)	20 µg/mL	Day 0 & 3	BD Bioscience
Rat IgG2ak Isotype	20 µg/mL	Day 0 & 3	BD Bioscience
Alk 5 inhibitor SB505124	5, 0.5, 0.05 µM	Day 0 & 3	Sigma
DAPT inhibitor	1 µM	Day 0, 3 or 0 & 3	Enzo Life Sciences (Exeter, UK)
Recombinant human IL-10	10 ng/mL	Day 0 & 3	Peprotech
Ionomycin	500 nM	Days 0-3 Daily	Sigma
S115 STAT3 inhibitor	200-50 µM	48 or 72 hours	Selleck (Boston, USA)

Table 6. Additional reagents used during Th17 cell cultures.

7.3.1 CD4$^+$ T-cell culture: resting cultured CD4$^+$ T-cells

For T-cell culture past day 6, the T-cells were harvested at day 6 into a 5 mL round bottom polystyrene tube. The tube was placed into an EasySep magnet for 3 minutes to remove the anti-CD3/anti-CD28 beads: the magnetic beads are attracted to the magnet leaving the T-cells in suspension. The T-cells were poured out into a universal tube and were then pelleted in PBS + 2% FBS at 400g for 7 minutes (room temperature). Following resuspension of the cell pellet the cell number and viability were assessed by cell counting with trypan blue (Sigma) using a Burker counting chamber. Trypan blue is able to pass through the membrane of dead cells, but not alive cells, making dead cells appear blue when viewed under a microscope. Cells were cultured in IRS10 at a concentration of $1x10^6$ cells/mL in a 24 well plate in the presence of IL-2 (10 IU/mL) and IL-23 (10 ng/mL) for Th17 cell cultures and incubated at 37°C, 5% CO_2 until day 10 or 14. If required, media was refreshed with IRS10 containing 10 U/mL IL-2 and 10 ng/mL IL-23. At day 10 or 14 the cells were restimulated with PMA/ionomycin in the presence of BFA (as described 7.5.2) and the T-cell phenotype was assessed by intracellular cytokine staining and flow cytometry (as described 7.5.3) measuring the proportion of IL-17$^+$ or IFNy$^+$ cells.

7.3.2 CD4$^+$ T-cell culture: anti-CD3 and anti-CD28 titrations using the MACS T-cell activation/expansion Kit

In addition to the anti-CD3/anti-CD28 T cell expander Dynabead titrations described above, the MACS T-cell Activation/Expansion Kit (Miltenyi Biotec; Cologne, Germany) was also used to assess stimulation strength. By using magnetic beads bound with anti-biotin antibody, anti-CD3-Biotin and anti-CD28-Biotin can be conjugated to the anti-biotin beads at a chosen concentration. Anti-biotin magnetic beads were loaded with 40, 10 and 2.5 µg/mL of anti-CD3-biotin +/- 40, 10 and 2.5 µg/mL of anti-CD28-biotin, according to the manufacturer's instructions. Following the conjugation of antibody to bead, $1x10^6$ CD4$^+$ T-cells were cultured in IRS10 with the beads at the manufacturer recommended ratio of 1 T-cell:2 beads. Cells were cultured under pro-Th17 cell cytokine conditions (Table 5). At day 6 for ELISA analysis the cells were harvested and

pelleted at 400g for 7 minutes at 4˚C. Cell culture supernatant was removed and transferred to microcentrifuge tubes and frozen at -20°C for analysis by ELISA (as described below). A separate well of day 6 T-cells were restimulated with PMA/ionomycin in the presence of BFA (as described 7.5.2) and the cells were phenotyped by intracellular cytokine staining and flow cytometry (as described 7.5.3) generally measuring the proportion of IL-17$^+$ or IFNy$^+$ cells.

7.3.3 Memory CD4$^+$ T-cell culture: affect of cell density

Memory CD4$^+$ T-cells were isolated using EasySep Human Memory T-cell enrichment kit as described above. Memory CD4$^+$ T-Cells were cultured in 24 well plates in IRS10 in a total culture volume of 1 mL with anti-CD3/CD28 beads at a 1:1 (THi) ratio. To achieve different densities, T-cells at a starting density of 1x10^6 cells/mL were combined with anti-CD3/CD28 beads at a 1:1 ratio, cells and beads were then serially diluted in IRS10 either two or four fold as indicated. Pro-Th17, pro-Th1, or Th0 cell cytokines were added to each T-cell culture density at the indicated concentration (Table 5). Titration of cytokines in parallel with T-cell density was achieved by adding pro-Th17 cell cytokines at the usual concentration with the 1x10^6 cell density and then serially diluting cytokines in combination with the T-cells and beads. Cells were incubated at 37˚C 5% CO_2 and refreshed as indicated above. At day 6 the cells were restimulated with PMA/ionomycin in the presence of BFA (as described 7.5.2) and the T-cell phenotype was assessed by intracellular cytokine staining and flow cytometry (as described 7.5.3) measuring the proportion of IL-17$^+$ or IFNy$^+$ cells.

7.3.4 Memory CD4$^+$ T-cell culture: duration of T-cell activation

1x10^6 memory CD4$^+$ T-cells were cultured in 24 well plates in IRS10 in a total culture volume of 1 mL with anti-CD3/CD28 beads at a 1:1 ratio (THi) in the presence of pro-Th17 cytokines (Table 5). Cells were cultured for 24h, 48h, or 72 h, after which cells were either harvested into a 5 mL round bottom tube or a universal tube (beads not removed). The T-cells in the round bottom tube were placed into an EasySep magnet for 3 minutes to remove the anti-CD3/anti-CD28 beads. Both T-cell poplulations (+/- beads) were then pelleted in Hanks + 1% FBS at 400g for 7 minutes (room temperature). Following resuspension of both cell populations pellets, the number of

viable cells was determined by trypan blue staining using a Burker counting chamber. Cells were re-cultured at a concentration of 0.5×10^6 cells/mL in the presence or absence of pro-Th17 cell cytokines (Table 5). Cells were cultured for 6 days at 37°C 5% CO_2. At day 6 the cells were restimulated with PMA/ionomycin in the presence of BFA (as described 7.5.2) and the T-cell phenotype was assessed by intracellular cytokine staining and flow cytometry (as described 7.5.3) measuring the proportion of IL-17$^+$ or IFNy$^+$ cells.

7.4 Isolation and culture of monocyte derived dendritic cells

Generation of monocyte derived dendritic cells (moDC) from monocytes was based upon the established method described by Sallusto and Lanzavecchia, and Romani *et al* (596, 597). Culture of CD14$^+$ monocytes with IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF) for 6 days induces an immature DC phenotype without cell division. Maturation of moDC is achieved by 24h culture of immature moDC with inflammatory stimuli, such as lipopolysaccharide, peptidoglycan, or TNFα.

7.4.1 Isolation of CD14$^+$ monocytes from PBMC

Monocytes were separated from the PBMC using CD14 positive selection by magnetic activated cell sorter (MACS, Miltenyi Biotec). The principle of MACS separation is that the magnetic MicroBeads are conjugated to an antibody raised against CD14. When the MicroBeads are added to PBMC, the MicroBeads bind to monocytes expressing CD14. When the labelled PBMC were passed through a MACS column placed within a magnet the unlabelled CD14$^-$ cells pass through the column whereas the labelled CD14$^+$ cells are retained within the column. Once the column is removed from the magnet the CD14$^+$ cells can be eluted.

The procedure was followed according to manufacturer's instructions. Briefly, it was ascertained from previous separations that the PBMC population contains 15-30% CD14$^+$ monocytes. Throughout the separation MACS buffer (Dulbeccos phosphate buffered saline (DPBS, Lonza), + 2 mM EDTA + 0.5% FBS) was used and kept ice cold, and all centrifugation steps were performed at 4°C. Firstly the PBMC were washed in MACS buffer at 400 g for 7 minutes. PBMC were then resuspended in MACS buffer

(800 µL per $100x10^6$ cells) and CD14 MicroBeads (Miltenyi Biotec) were added (100 µL per $100x10^6$ cells) for 15 minutes at 4°C and the solution was gently shaken every 5 minutes to ensure appropriate mixing. PBMC were then washed in MACS buffer at 400 g for 7 minutes to remove unbound CD14 MicroBeads. During centrifugation the MACS magnet was prepared by rinsing an LS MACS column (Miltenyi Biotec) with 3 mL MACS buffer. Following centrifugation the cell pellet was resuspended to a concentration of $100x10^6$ cells/mL in MACS buffer and transferred to the pre-washed LS column. Once the cell suspension had passed through the column the column was then washed a further 3 times with 3 mL of MACS buffer to remove any remaining $CD14^-$ cells. Finally the column was removed from the magnet and placed over a universal tube and the $CD14^+$ monocytes were eluted from the column using 5 mL of MACS buffer. The purified $CD14^+$ monocytes were then centrifuged at 400 g for 7 minutes, 4°C, and resuspended MACS buffer. Cells were then counted using a Burker counting chamber. The required number of $CD14^+$ monocytes were centrifuged at 400 g for 7 minutes in order to remove residual MACS buffer. $CD14^+$ monocytes were then carefully resuspended to prevent activation, in either RF10 (RPMI (Sigma) with Penicillin/Streptomycin (100 µg/mL), L-glutamine (2mM) and FBS (10%)) for future use in allogeneic DC:T cell cultures or in complete cell serum-free media, CellGro DC (CellGenix; Freiburg, Germany), for autologous DC:T-cell cultures.

7.4.2 Culture of monocyte-derived dendritic cells

$CD14^+$ monocytes were cultured at a concentration of $0.5x10^6$ cells/mL in either 1 mL of RF10, or 1 mL of CellGro in a 24 well plate. To each well IL-4 (Immunotools) and GM-CSF (Immunotools) were added to a final concentration of 50 ng/mL. Following plating, monocytes were cultured for 6 days at 37°C in 5% CO_2 to induce monocyte differentiation to an immature DC phenotype. On day 3, IL-4 and GM-CSF were refreshed by carefully removing 450 µL supernatant and replacing with 500 µL pre-warmed RF10 or CellGro containing 50 ng of IL-4 and 50 ng GM-CSF. moDC were activated for 24h on day 6 by the addition of 50 µg/mL of *S.aureus* peptidoglycan (InvivoGen; Toulouse, France), a TLR2 agonist which induces the release of pro-Th17 cell cytokines from dendritic cells, to produce PGN-matured DC. PGN maturation was performed in the presence of Candida albicans allergen extract in Chapter 5 at a final

concentration of 1:5000 w/v (Soluprick, ALK Abello; Berkshire, UK). Alternatively, moDC were left untreated to produce immature DC. On day 7 moDC were incubated on ice for 1 h to reduce cell adherence. The cells were then harvested into a universal tube, washed twice in HBSS + 1% FBS at 400g for 7 minutes (4°C), and resuspended in IRS10. Cell number and viability was assessed by cell counting with trypan blue using a Burker counting chamber. The moDC were used to activate T-cells as described in 7.4.3, 7.4.4, & 7.4.5.

7.4.3 T cell activation: Mixed Lymphocyte Reaction

In order to assess the effects of stimulation strength upon Th17 generation from T-cells using DC activation, a mixed lymphocyte reaction (MLR) was used. The principle of a MLR is that the TCR on allogeneic T-cells will react to the mismatched MHC molecules present on the DC resulting in an alloresponse. Day 7 PGN-matured DC were co-cultured with allogeneic T-cells (either at: $1x10^6$:$1x10^6$, $1x10^5$:$1x10^6$, $2x10^4$:10^6 DC:T-cell ratio) in 1 mL IRS10 for 6 days at 37˚C 5% CO_2. At day 6 the cells were restimulated with PMA/ionomycin in the presence of BFA as described 7.5.2 and the T-cell phenotype was assessed by intracellular cytokine staining and flow cytometry 7.5.3 measuring the proportion of IL-17^+ or $IFNy^+$ cells.

7.4.4 T cell activation: superantigen DC: T-cell

For the culture of autologous T-cell and DC cultures in the presence different concentrations of super antigen staphylococcal enterotoxin B (SEB) day 7 immature CellGro DC were harvested as described in 7.4.2. $CD4^+$ T-cells were isolated and frozen (as described in 7.1.5 and 7.2) until the moDC were generated and the autologous T-cells were resurrected on the day that the autologous T-cell:DC co-cultures were seeded. Immature DC were cultured with autologous $CD4^+$ T-cells in a total volume of 1 mL IRS10 using $1x10^5$ DC:$1x10^6$ T-cells in the presence of pro-Th17 cell cytokines (Table 5), and in the presence or absence of SEB at a concentration of 1000 - 0.1 pg/mL (Sigma). Cells were cultured for 6 days at 37 °C, 5% CO_2. At day 6 the cells were restimulated with PMA/ionomycin in the presence of BFA as described 7.5.2 and the T-cell phenotype was assessed by intracellular cytokine staining and flow cytometry 7.5.3 measuring the proportion of IL-17^+ or $IFNy^+$ cells.

7.4.5 T cell activation: DC: T-cell density

Autologous DC populations were harvested at day 7 as described in 7.4.2. Each DC population was resuspended in IRS10 to a concentration of $2x10^5$ cells/mL. Autologous T-cells were resuspened to a concentration of $2x10^6$ cells/mL. T-cells and DC were then mixed together at 1:1 volume/volume ratio. 1 mL/well of this T-cell:DC mix achieved the high density of $1x10^6$ T-cell:$1x10^5$ DC (1 DC:10 T-cell ratio). A low T-cell:DC density was achieved by a four-fold dilution of the T-cell:DC mix resulting in a final cell concentration of $0.25x10^6$ T-cell:$0.25x10^5$DC/well. Cells were cultured in IRS10 alone (no cytokines were added) until day 6. If required, cells were either split or media refreshed with pre-warmed IRS10. At day 6 the cells were restimulated with PMA/ionomycin in the presence of BFA as described 7.5.2 and the T-cell phenotype was assessed by intracellular cytokine staining and flow cytometry 7.5.3 measuring the proportion of IL-17^+ or IFNy^+ cells.

7.5 Flow Cytometry

Flow cytometry allows multiple markers of cell phenotype to be analysed within an individual cell. Furthermore multiple cells may be analysed simultaneously allowing phenotypic analysis within a heterogeneous cell population. The size, complexity, phenotype and health of a cell may all be assessed by flow cytometry. The flow cytometer passes one cell at a time through a laser using hydrodynamic localisation, generally allowing the detection of cells 1-15 µM in diameter. The size and complexity of a cell are assessed by using the forward scatter and side scatter of light as a cell passes through the laser. Forward scatter (FSc) measures the size of a cell, by detecting the scatter of light around the cell, whereas side scatter (SSc) measures the complexity and granularity of a cell by detecting the diffraction of light from a cell as it passes though a laser beam.

Generally cells are phenotyped for cell surface or intracellular molecules by using antibodies conjugated to a variety of flurophores. The lasers of the cytometer excite the fluorophore to a higher energy level, and upon the return of the flurophore to ground state, energy is emitted as light. The energy of the light emitted is determined by the energy level required to excite the flurophore and that light has a specific

wavelength and therefore colour. The cytometer detects this light by passing it through a series of filters and mirrors so that certain wavelengths are received by the appropriate detectors. This is then translated into a voltage proportional to the amount of fluorescence emitted, which may then be represented graphically.

7.5.1 Cell surface staining of T-cells

For the detection of cell surface molecule expression cells (0.2-2x10^6) were washed and resuspended in 200 µL of Fluorescence active cell sorting (FACS) buffer (PBS (Cambrex Bioscience) + 3% FBS + 2mM EDTA + 0.01% sodium azide (Sigma)) and transferred to a 96-well v-bottom plate. The plate was centrifuged at 400 g, for 3 minutes at 4°C to pellet the cells, and cells were resuspended in 50 µL FACS buffer. To block non-specific binding to Fc receptors, human IgG was added at a final concentration of 200 µg/mL. Antibodies were added at the appropriate dilutions (Table 8) and incubated for 30 minutes, in the dark, at 4°C. Cells were then washed twice in cold FACS buffer. Cells were resuspended in 300 µL FACS buffer and directly acquired on a BD FACSCanto II, or cells were fixed overnight in 1% formaldehyde and acquired the next day. Data was analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

7.5.2 PMA/Ionomycin Stimulation for intracellular cytokine staining

Large concentrations of cytokines within a cell are required for detection by flow cytometry. To achieve this, chemical stimulation may be used to turn on active transcriptional machinery, inducing production of cytokines for which the cell has active transcription. A combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin provides sufficient stimulation to detect a large number of cytokines. Ionomycin initiates a rise in intracellular Ca^{2+} levels, which in conjunction with PMA, an activator of protein kinase C, stimulates cytokine production. To concentrate the protein within the cell Brefeldin A, a chemical capable of interfering with protein transport from the within the endoplasmic reticulum to the golgi apparatus, is employed.

Cells were stimulated with PMA (10 ng/mL, Sigma) and ionomycin (1 µg/mL, Sigma) (control wells were left untreated) and incubated for 1 hour at 37˚C 5% CO_2. Brefeldin A (10 µg/mL, Sigma) was then added to both PMA/ionomycin stimulated and untreated wells and incubated for a further 4 hours at 37˚C 5% CO_2. Cells were then harvested into 1.5 mL microcentrifuge tubes and pelleted at 400g for 7 minutes (room temperature). Cells were then phenotyped for intracellular markers as described below.

7.5.3 Intracellular staining of T-cells

For intracellular cytokine detection cells were stimulated with PMA/ionomycin as described above. Cells were resuspended in 300 µL FACS buffer and 150 µL/well (x 2 wells: an unstained and a stained well for each condition) was transferred to a 96 well v-bottom plate. All centrifugation steps were performed at 400 g for 3 minutes at 4°C. Cell surface staining was performed as described in 7.5.1 if required. Cells were washed once in 150 µL/well FACS buffer, resuspended in 150 µL of fixation/permeabilisation buffer (ebioscience) and incubated for 30 minutes at 4°C in the dark. The buffer contains formaldehyde which fixes the cells by cross-linking primary amino groups in proteins to nitrogen atoms in proteins or DNA. The buffer also contains saponin which permeabilises the cell membrane allowing antibody to access intracellular molecules. Cells were washed twice in permeabilisation buffer. This buffer contains saponin at a higher concentration allowing permeabilisation of the nuclear membrane for transcription factor detection. Cells were resuspended in permeabilisation buffer (50 µL/well) containing 1% mouse serum (for mouse antibodies) or rat serum (for rat antibodies) to block non-specific binding of the antibody. Cells were then incubated in the dark at 4°C for 15 minutes. Antibodies were added directly to the wells (for dilutions refer to Table 8 and incubated in the dark at 4°C for 30 minutes. Cells were then washed twice in permeabilisation buffer (150 µL/wash) and resuspended in 300 µL of FACS buffer and directly acquired on a BD FACSCanto II. Data was analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

7.5.4 CFSE staining of $CD4^+$ T-cells

To assess cell proliferation using flow cytometry cells are stained with carboxyfluoroscein succinimidyl ester (CFSE), a fluorescent dye. CFSE is able to enter the cell where it is retained due to covalent binding of succinimidyl groups to intracellular molecules. This process causes the CFSE to fluoresce making it detectable using flow cytometry. As a cell divides the amount of CFSE divides equally between the two daughter populations and thus the degree of proliferation may be determined by observing the decrease in fluorescence intensity in the cells.

Following $CD4^+$ T-cell isolation, the cells were transferred into a 50 mL centrifuge tube and washed twice in cold PBS at 400 g for 5 minutes at 4°C. Cells were resuspended at a final concentration of $1x10^7$ cells/mL. CFSE (Invitrogen) was added at a concentration of 4 µM/mL and cells resuspended by pipetting up and down. An aliquot of $CD4^+$ T-cells which were not treated with CFSE were retained for use as negative controls. Cells were incubated with agitation at 37°C in 5% CO_2, for 10 minutes. Following this, cells were washed twice in 15 mL of RF10 at 400 g at 4°C so that proteins present within FBS would bind any unbound CFSE. $CD4^+$ T-cells were then rested for 30 minutes at 37°C at 5% CO_2. Cells were then counted with trypan blue using a Burker counter. Cells were cultured as required and acquired on a BD FACSCanto II. Data was analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). For controls non-CFSE stained $CD4^+$ T-cells were cultured in separate wells (negative control) and CFSE labelled non-activated cells (positive control for compensation and gating of a $CFSE^{hi}$ population).

7.5.5 Annexin V/Viaprobe staining

Staining with a combination of Annexin V and Via-Probe allows detection of apoptotic and/or dead cells. During early apoptosis, the cell membrane flips to expose phosphatidylserine residues, which can be detected by Annexin V. Dual staining with Via-Probe, which binds to double-stranded DNA of membrane compromised non-viable cells, allows discrimination between viable, early apoptotic and dead cells.

Following culture of T-cells under THi/TLo culture conditions the cells were harvested, washed in FACS buffer, and resuspended in 100 µL of 1 x binding buffer (0.01 M

Hepes/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM $CaCl_2$ made in-house). Then, 5 µL of Annexin V-APC (BD Biosciences) and 5 µL of Via-Probe (BD Biosciences) were added to the cells. The cells were vortexed and incubated at room temperature for 15 minutes in the dark. Following incubation 400 µL of Annexin V 1 x binding buffer was added to each tube and the cells were acquired on a BD FACSCanto II within 1 hour of staining. Data was analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Via-Probe alone can be used to stain for dead or dying cells in order to assess cell viability. Cells were harvested and washed in 150 µL of FACS buffer. Cells were resuspended in 50 µL of FACS buffer and 5 µL of Via-Probe was added. Cells were incubated at room temperature for 10 minutes in the dark. The cells were then washed in 100 µL of FACS buffer and intracellular cytokine staining procedure was performed as described in Intracellular staining of T-cells.

7.5.6 Settings and Controls

To ensure that the binding observed was specific for the marker of interest, isotype controls were used to measure non-specific antibody binding. Concentrations of the isotype controls were adjusted to the same concentration as the antibody against the marker of interest. Isotype staining employed the same protocol as that used to stain the cells in 7.5.3. An example of isotype staining is shown in Figure 94A. To ensure that the fluorescent signal detected, was not due to leakage from another fluorophore channel, the compensation was set accordingly. An example of multi-colour compensation settings are shown in Figure 94B.

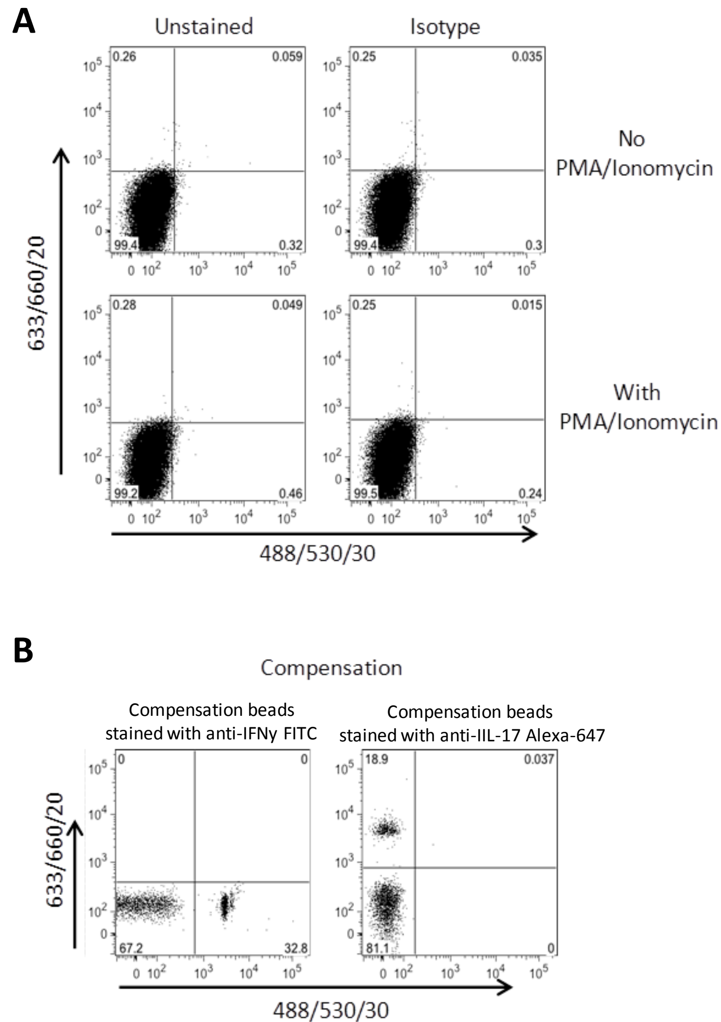


Figure 94. Isotype control (A) and compensation examples (B). (A) Cells were gated initially in forward scatter/side scatter to remove cell debris. Staining was performed according **7.5.3**. Dilutions of antibodies used are shown in **Table 9**. (B) Compensation was performed using BD anti-mouse Ig compensation beads (BD) or stained cells. Samples were acquired using a BD FACSCanto II and data was analysed using FlowJo analysis software.

7.6 Enzyme linked immunosorbant assay

An Enzyme-Linked Immunosorbant Assay (ELISA) allows the quantification of a specific protein to be determined within a sample. The protein of interest is sandwiched between two antibodies, a capture antibody bound to the surface of a well of a 96 well plate, and a biotinylated detection antibody. Addition of streptavidin (which binds to biotin with high affinity) conjugated to the enzyme horseradish peroxidise (HRP) and a substrate for the HRP enzyme results in a proportional change in fluorescence dependent on the amount of the protein of interest present within in the sample. This may then be compared to a standard curve of known protein concentrations to calculate the quantity of protein in the sample.

7.6.1 Preparation of cells for ELISA

For the detection of cytokines in culture supernatants, cells were harvested and pelleted into microcentrifuge tubes at 400g for 7 minutes (room temperature) to remove any cells/debris. The cell culture supernatant was then transferred to fresh microcentrifuge tubes and frozen at -20˚C for later analysis by ELISA.

To assess cytokine secretion in response to restimulation, cells were harvested, washed at 400 g for 7 minutes at room temperature and then counted with trypan blue using a Burker counting chamber. Cells were resuspended and plated out at $1x10^6$ cells/mL on a 24 well plate. Cells were incubated for 1 hour at 37°C, 5% CO_2 to allow the cells to settle. Following incubation one well of cells were left untreated and one well was stimulated with PMA (10 ng/mL) and ionomycin (1 µg/mL), for each condition assessed. The cells were incubated for 24 hours at 37°C, 5% CO_2. Cells were then harvested and pelleted at 400 g for 7 minutes (room temperature). The cell culture supernatant was then transferred to fresh microcentrifuge tubes and frozen at -20˚C for later analysis by ELISA.

7.6.2 ELISA

To measure the concentration of cytokines present in collected supernatants, 96 well flat bottom EIA/RIA High Bind ELISA plates (Corning Costar; Sigma) were incubated at 4°C overnight with the appropriate concentration of capture antibody (50 µl/well, Table 7). The capture antibodies were pre-diluted in coating buffer (0.06M Na_2HPO_4/0.08M $NaHPO_4.H_20$) at the appropriate dilution (Table 7). For measurement of IL-17, the IL-17 ReadySETGo! kit was used according to the manufacturer's instructions (eBioscience). The following day, the capture antibody was discarded and the plate was washed once in wash buffer (PBS + 0.1% Tween-20) before adding 100 µL/well of block buffer (PBS + 1% bovine serum albumin (BSA)) for 1-2 hours at room temperature. During the blocking incubation, samples where diluted in diluent (PBS + 1% BSA + 0.1% Tween-20). Following incubation, the plates were washed 3 times in wash buffer and the pre-diluted samples and standards were added to the appropriate wells (50 µL per well) and incubated at 4°C overnight. The following day the plate was washed 4 times in wash buffer, and the appropriate concentration of biotinylated

detection antibody (50 µL/well, (Table 7) was added to each well and incubated at room temperature for 1 hour. Following incubation, the wells were washed 4 times in wash buffer and Streptavidin-HRP (Dako; Cambridge, UK) (50 µL/well, 1/1000) was added to each well and incubated at room temperature for 30 minutes. The plates were finally washed 5 times in wash buffer, and then o-Phenylenediamine dihydrochloride (OPD) substrate was added to each well (Sigma, 50 µL/well). 1 OPD tablet was dissolved in 13 mL of citrate phosphate buffer (0.03M citric acid/ 0.05M $NaHPO_4$/0.03M $Na_2HPO_4.2H_20$) and H_2O_2 (6 µL, 30% stock) was added immediately prior to use. The plates were left for 20-40 minutes depending on the cytokine of interest **(**Table 7) for the colour to develop. The reaction was stopped by the addition of H_2SO_4 (50 µL, 3M). The absorbance was read at the appropriate wavelength (Table 7) using a Tecan sunrise plate reader (Tecan Group; Mannerdorf, Switzerland).

7.6.3 ELISA Analysis

ELISA data was analysed by plotting a standard curve of known concentrations of the cytokine of interest against the measured absorbance values. The equation of the line was calculated and this was used to calculate the unknown concentration of the cytokines present within the analysed samples. An example standard curve is demonstrated in Figure 95. The detection limit of the standard curve was determined by multiplying the background absorbance by 2.

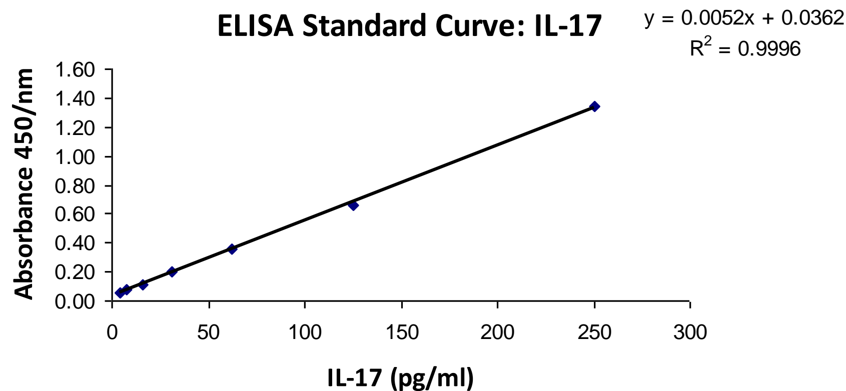


Figure 95. ELISA Standard Curve: A representative standard curve from an IL-17A ELISA plate. The absorbance was plotted against known concentrations of IL-17 using a scatter plot. The line of best fit was estimated using MS Excel. Values which were within the linear portion of the curve were selected. The equation y = mx + c and the R^2 value were calculated and used to determine the unknown concentration of IL-17 present in samples.

Cytokine	Capture antibody	Top standard pg/mL	Detection antibody	Develop (minutes)	Absorbance (nM)
Human IL-10	2 µg/mL	2000	1 µg/mL	30-40	490
Human IL-17	1/250 (kit)	500	1/250 (kit)	15-20	450
Human IFN-γ	1 µg/mL	2000	1 µg/mL	20-30	490

Table 7. Concentrations of antibodies and standards for cytokine ELISA.

7.7 Western Blotting

7.7.1 Whole Cell Lysates

Whole cell lysis buffer; 10 mL CellLytic M (Sigma) + 100 µL of 100x Halt Phosphatase Inhibitor Cocktail (Thermo Scientific; Wilmington Delaware) + 1 x Protease Inhibitor (serine and cysteine) Cocktail Tablet (Roche), was prepared and stored in 1 mL aliquots at -20˚C. Prior to use the whole cell lysis buffer was thawed on ice. Samples were normalised for cell number and transferred to a 1.5 mL microcentrifuge tube. In order to remove any residual phenol red the cells were washed once in ice cold PBS and pelleted at 13,000 g for 1 minute at 4˚C. Following careful removal of PBS to leave a dry cell pellet, 50 µL of whole cell lysis buffer was added to <$1x10^6$ cells. The pellet was then resuspended in the whole cell lysis buffer by vortexing and cell debris was removed by centrifugation at 13,000 g for 10 minutes at 4˚C. The whole cell lysate was then carefully removed from the pellet and transferred to pre-cooled microcentrifuge tubes and stored at -80˚C for use in western blotting.

7.7.2 Preparation of cells to assess NFATc1 localisation

At the required time point cells were either left untreated or re-stimulated with PMA (10 ng/mL) and ionomycin (1 µg/mL) and incubated at 37°C, 5% CO_2 for 2 hours. Following the incubation the cells were harvested and washed in cold PBS by centrifugation at 400 g for 7 minutes at 4˚C. The washed cells were used for nuclear and cytoplasmic extraction as described below.

7.7.3 Nuclear and Cytoplasmic Extraction

Nuclear and Cytoplasmic extracts were produced using the NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Scientific). The NE-PER kit allows preparation of nuclear extracts without mechanical homogenisation or multiple centrifugation or freeze/thaw cycles, thus maintaining the integrity of the protein. Reagents within the kit act in the following manner; CER 1 causes swelling of the cell, inducing cell membrane stress; CER II lyses the cell membrane allowing the collection of cytoplasmic proteins whilst leaving the nucleus intact; NER extracts nuclear proteins from the pellet.

The protocol was followed according to manufacturer's instructions. Briefly, cells were resuspended in 1 mL ice cold PBS and transferred into labelled pre-cooled 1.5 mL microcentrifuge tubes. Cells were pelleted by centrifugation at 400 g for 3 minutes at 4°C. The supernatant was carefully removed and discarded, leaving the cell pellet as dry as possible. Ice-cold CER I with 1 µL protease inhibitors per 100 µL CER 1 was added to the cell pellet at the appropriate volume, vortexed vigorously for 15 seconds to fully resuspend the pellet and then incubated on ice for 10 minutes. NER extracts nuclear proteins from the pellet. Ice-cold CER II was then added to each tube at the appropriate volume, vortexed for 5 seconds and incubated for 1 minute. The tube was then vortexed for another 5 seconds and the centrifuged for 5 minutes at 13,000 g at 4°C. The supernatant was then immediately transferred to a pre-cooled 1.5 mL microcentrifuge tube and stored at -80°C for use in western blotting. The remaining pellet containing the nuclei was resuspended in ice-cold NER + 1 µL protease inhibitors per 100 µL. Cells were then vortexed for 15 seconds and placed on ice with vortexing every 10 minutes for 15 seconds for a total of 40 minutes. The tubes were then centrifuged at 16,000 g for 10 minutes at 4°C and the supernatant immediately transferred to a pre-cooled 1.5 mL microcentrifuge tube and stored at -80°C for use in western blotting.

7.7.4 Bradford Assay

The Bradford assay is used to determine protein concentrations. Coomassie dye when bound to protein results in a colour change (red to blue) and an absorbance shift from

470 to 590 nm. The change in fluorescence is proportional to the amount of protein present.

Frozen lysates were thawed on ice, while standards were prepared using BSA diluted in water. Lysates were diluted 1:10 in the appropriate lysis buffer. Standards and lysates were then diluted 1:15 with room temperature Bradford reagent (Sigma). Standards and sample were added to a 96 well flat bottom plate (150µL/well). The absorbance was read at 595 nm using a Tecan sunrise plate reader. The protein concentration of lysates was analysed, by plotting a standard curve of known concentrations of the BSA against the measured absorbance values. The equation of the line was calculated and this was used to calculate the unknown concentration of the proteins present within the lysates. The detection limit of the standard curve was determined by multiplying the background absorbance by 2. Samples were normalised to the lowest protein concentration prior to denaturation for loading onto Sodium dodecyl sulphate (SDS)-polyacrylamide gels.

7.7.5 SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide Gel Electrophoresis (PAGE) allows proteins to be separated based upon size. Denatured polypeptides are bound by SDS molecules that impart a negative charge on the polypeptide chain proportional to molecular weight (MW). Samples are run alongside a ladder of known MW markers allowing protein size to be estimated. Polyacrylamide gels are formed when acrylamide monomer polymerises with cross-linking agent *bis*-acrylamide, which is initiated by the addition of ammonium persulfate and *N,N,N′,N′*-tetramethylenediamine (TEMED). The gel resolution is determined by the pore size of the polyacrylamide gel. The pore size is controlled by the concentration of polyacrylamide; the pore size decreases as the ratio of polyacrylamide increases. Stacking gels, with a large pore size (4% polyacrylamide) allow the protein sample to concentrate on the resolving gel surface. Proteins may then be separated by size when voltage is applied; smaller polypeptides migrate easily, where as larger polypeptides are retarded through frictional resistance.

7.7.6 Gel casting and protein loading

To cast gels, glass plates (Biorad) were set up on a gel casting stand (Biorad). Ethanol was pipetted into the plates to check for leaks and then removed. Using a Pasteur pipette plates were 2/3 filled with resolving gel (for the recipe see below). Saturated butanol was pipetted on top to remove oxygen from the gel, allowing polymerisation and formation of a level gel interface. Once set, butanol was poured off and the stacking gel (for the recipe see below) was pipetted on top of the resolving gel using a Pasteur pipette. An appropriate sized comb was then inserted into the top of the gel and the gel was left to set at room temperature.

Resolving gel (10%)

- 7.5 mL acrylimide stock solution (Sigma)
- 9 mL resolving gel buffer (0.75M Tris base, 7 mM SDS, pH 8.8 with HCL),
- 2 mL distilled H_2O,
- 180 µL ammonium persulfate stock solution (0.44M Ammonium persulfate)
- 18 µL TEMED (Sigma) added immediately prior to gel casting.

Stacking gel

- 1.8 mL acrylimide stock solution
- 5 mL stacking gel buffer (0.25M Tris base, 7 mM SDS, pH 6.8 with HCL)
- 4 mL distilled H_20
- 50 µL ammonium persulfate stock solution
- 10 µL TEMED added immediately prior to gel casting.

Once set the combs were carefully removed from the gels and the wells were rinsed with running buffer (25 mM TRIS Base, 0.2 M Glycine, 3.5 mM SDS, pH 8.3) using a Pasteur pipette. Gels were placed within the gel tank (Biorad Mini Protean II) and running buffer was placed in the inner and outer chambers.

Protein samples of known concentration were denatured into polypeptides in loading buffer (1 µL of β-mercaptoethanol per 25 µL 4x NuPage Sample buffer (Invitrogen)) at a 4:1 sample:loading buffer ratio for whole cell lysates or 5:1 for nuclear or cytoplasmic

lysates. Samples were heated to 70°C for 10 minutes to denature proteins, then returned to room temperature and pelleted. Samples were loaded onto the gel (10 µL/well). One lane of the gel was used for a PageRuler prestained protein ladder (Fermentas). The gel was run at 80 volts for ~1.5 h depending on protein size and the resolution required.

7.7.7 Protein transfer

Transfer of proteins from gel onto hydrophobic Polyvinylidene Fluoride (PVDF) membrane was achieved using a Geni Blotter (Idea Scientific; Minneapolis, USA). PVDF membrane was pre-soaked in methanol prior to use. Following careful removal of the gels from the plates the Genie Blotter was loaded according to manufacturer's instructions using transfer buffer (0.025 M Tris Base, 0.2 M Glycine diluted with 20% v/v methanol and H_2O). Blotting was performed at 4°C at 12 volts for 120 minutes.

7.7.8 Blotting

Following transfer, the membrane was washed with Tris buffered saline (0.01 M Tris base + 0.14 M SDS pH to 7.6 with HCL) + 10% w/v Tween (TBST) for 5 minutes at room temperature with agitation. After washing the membrane, non-specific binding sites were blocked with TBST + 5% w/v milk and incubated for 1 hour at room temperature with agitation. The membrane was then washed 2 times in 25 mL of TBST. The membrane was blotted with primary antibody (Table 9) diluted in TBST + 5% immunoglobulin depleted BSA (Sigma) and incubated overnight at 4°C with agitation. The following day the membrane was washed 3 times with TBST for 5 minutes at room temperature with agitation. The membrane was then incubated with an appropriate HRP-linked secondary antibody (Table 9) in 10 mL TBST + 5% w/v milk, for 1 hour at room temperature with agitation. Following this the membrane was washed 3 times for 5 minutes in TBST. Visualisation of bands was performed using either ECL or ECL+ (Amersham Biosciences; Buckinghamshire UK) for 0.5 - 2 minutes according to the manufacturer's instructions. The membrane was exposed to Kodak film for 0.5 – 1 minute, and developed using Photosol X-Ray developer and fixed using Photosol fixer (PhotonImaging Systems; Wiltshire, UK). Detection was also performed using Syngene Gel Doc (Syngene; Cambridge UK) for 0.5 to 10 minutes.

7.7.9 Stripping Blots

To re-blot membranes with different antibodies membranes were stripped using SDS and β-mercaptoethanol. Stripping buffer was made as described below, heated to 60˚C, and poured over blots in a sealed container and incubated for 30 minutes with agitation. Following stripping, blots were washed twice for 10 minutes with TBST to remove residual stripping buffer, then blocked for 1 hour with TBST + 5% milk, after which the membrane was incubated overnight with primary antibody and developed as described above.

Stripping Buffer

- 1.7 mL β-Mercaptoethanol
- 50 mL 10% SDS
- 15.63 mL 1 M Tris Base (pH 6.8)
- 182.7 mL dH_2O

7.8 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) is a powerful technique that provides a "snap-shot" of protein-DNA interactions at a chosen time point. ChIP allows the binding of transcription factors to specific regions in the genome to be assessed and offers insights into the transcriptional regulation of genes of interest. Briefly, stages of ChIP include: formaldehyde cross-linkage of DNA and proteins, cell lysis in an SDS buffer and chromatin shearing by sonication. Sonication increases the specificity of procedure allowing the presence of a protein within a region of the genome, e.g. the gene promoter, to be determined. Then immunoprecipitation is performed by incubating the sonicated lysates with antibodies which immune-select specific proteins The antibody is then pulled-down with agarose beads. The pulled down protein co-precipitates with genome fragments that were bound to the protein at the time of cross-linkage. Co-precipitated DNA is then eluted from beads, washed, and detached from proteins and purified using spin columns. The immune-selected co-precipitated DNA is then used in a real-time polymerase chain reaction (PCR) reaction with a primer set specific for a region of interest in the genome to assess transcription factor association.

I did not perform the ChIP experiments; however the details of the experiment performed by Dr J Mann are described below. Chromatin immunoprecipitation (ChIP) assay was carried out using cross-linked chromatin prepared from 6-day cultures of THi or TLo cells that were left untreated or were restimulated with PMA/ionomycin for 2 hours. The antibodies which were used for immunoprecipitation were anti-NFAT1c (clone 7A6; Santa Cruz Biotechnology; Heidelburg, Germany) or isotype-matched IgG control (Abcam; Cambridge, UK). Briefly, 5 µg of isotype-matched IgG control or anti-NFAT1c antibody were incubated overnight with 20 µg chromatin. The complexes were precipitated with Protein G–sepharose beads for 2 hours, then washed sequentially in low-salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl]), high-salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), and lithium chloride buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1). Beads were then washed twice with Tris-EDTA (TE) buffer and eluted with 500 µL of elution buffer (1% SDS, 0.1 M $NaHCO_3$). The cross-links were reversed, and DNA was obtained by phenol-chloroform extraction and ethanol purification. PCR amplification of the human *IL17A* proximal NFAT1c binding site was carried out using specific oligonucleotide primers 5'-gcagctctgctcagcttctaa-3' and 5'-ttcaggggtgacaccatttt-3'. All reactions were normalized to the isotype control and relative level of transcriptional difference calculated using the following equation: [1/(2A)] × 100. A = cycle threshold of the antibody group – the cycle threshold of the IgG Isotype control group and 2A compensates for the exponential phase of the real time PCR reaction.

7.9 PTPN22 c1858t Genotyping

7.9.1 DNA Extraction

Purification of total DNA from peripheral blood was achieved using QIAamp DNA Mini Kit (Qiagen; Crawley, UK). Briefly, samples were equilibrated to room temperature. 20 µL of QIAGEN Protease was transferred into the bottom of a 1.5 mL microcentrifuge tube. 200 µL of the whole blood sample was added to the tube. 200 µL of QIAGEN AL lysis buffer was added to the sample and mixed by vortexing for 15 seconds. The

sample was then incubated at 56°C for 10 minutes on a heat block. To remove drops from the inside the lid the sample was briefly centrifuged. 200 µL of 100% ethanol was added and the sample mixed by vortexing for 15 seconds, and again briefly centrifuged. The mixture was then carefully pipetted onto the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim, and centrifuged at 5000 g for 1 minute. The filtrate was discarded and the QIAamp spin column was placed into a clean 2 mL collection tube. 500 µL of Buffer AW1 was then carefully pipetted onto the QIAamp spin column and centrifuged at 5000 g for 1 minute. The filtrate was discarded and the QIAamp spin column was placed into a clean 2 mL collection tube. 500 µL pf Buffer AW2 was then carefully pipette onto the QIAamp spin column and centrifuged at 13000 g for 3 minutes. The QIAamp spin column was then placed into a new 2 mL collection tube and centrifuged again at 13000 g for 1 minute in order to eliminate any AW2 buffer carryover. The QIAamp Mini spin column was then placed into a clean 1.5 mL microcentrifuge tube, and the collection tube containing the filtrate was discarded. To elute DNA, 50 µL of AE Buffer was carefully pipetted onto the spin column, incubated at room temperature for 1 minute, and then centrifuged at 5000 g for 1 minute. This was then repeated with another 50 µL of AE buffer in order to maximise DNA elution. The concentration and quality of DNA was then determined using a NanoDrop (ND-1000 Spectrophotometer Thermo-Scientific) calibrated using RNAse free H_20 and AE buffer.

7.9.2 Polymerase Chain Reaction for rs2476601 - optimisation

Primers specific for rs2476601 (PTPN22 C1858T SNP) (Forward; 5' CAACTGCTCCAAGGATAGATG 3', Reverse; 5' CTCAAGGCTCACACATCAGC 3') were resuspended in RNase free H_20 (Sigma), and aliquots stored at -80°C to minimise 'freeze/thaw'. Primers were firstly optimised for annealing temperature and $MgCl_2$ concentration, a co-factor for the Taq polymerase enzyme. A PCR reaction was performed using varied concentrations of $MgCl_2$ on a temperature gradient. Briefly, the PCR mix (detailed below) containing either 1 mM, 2 mM or 3 mM $MgCl_2$ final concentrations were assessed in parallel with an annealing temperature gradient set on the Thermocycler (Peltier Thermal Cycler, MJ Research) between 55˚C-65˚C.

A 2% agarose gel (3 g of agarose (Severn Biotech; Kidderminster, UK) dissolved in 150 mL Tris-Borate-EDTA (TBE) + 1 µL ethidium bromide, which intercalates with nucleic acids) was used to analyse the PCR products. PCR products were mixed with loading dye (3 µL of loading dye was added to each 15 µL PCR reaction and 10 µL was loaded per well). A 100 base pair ladder (New England Biolabs; Ipswich, USA) was added to the gel in order to assess that the PCR product was the correct length. The gel was run at 100 V for ~1 hour. The gel was visualised under UV using the Syngene gel dock (Syngene). Figure 96 demonstrates that 1 µM $MgCl_2$ 55°C were optimal conditions for the PTPN22 rs2476601.

7.9.3 Optimised PCR reaction

- DNA (50 ng/µL)
- Forward Primer (100mM)
- Reverse Primer (100mM)
- 10 x PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3)
- $MgCl_2$ (25mM)
- dNTPs (8mM)
- AmlpiTaq Gold DNA Polymerase (Applied Biosystems)
- H_2O to make up to 15 µL

7.9.4 Optimised Thermocycling conditions

- 96°C for 14 minutes (initial denaturation)
- 96°C for 30 seconds (denaturation)
- 55°C for 30 seconds (annealing)
- 72°C for 30 seconds (extension)

x 35 cycles

- 72°C 5 minutes final extension

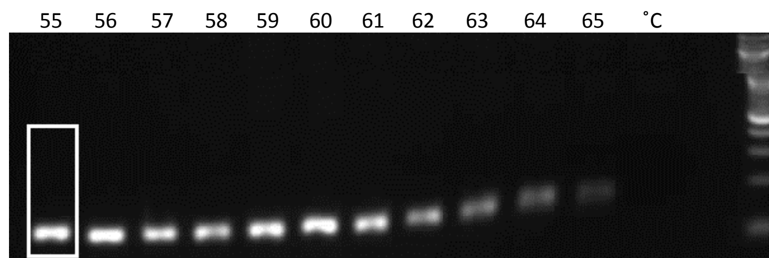


Figure 96. Optimised conditions for PTPN22 rs2476601 PCR. A single product at the correct size is seen. The box indicates optimal PCR conditions using,1 µM $MgCl_2$, and an annealing temperature of 55˚C

7.9.5 DNA Genotyping of rs2476601

Genomic DNA from 20 donors was genotyped by restriction fragment length olymorphism for a single nucleotide polymorphism (SNP) in rs2476601. Genomic DNA from each donor was isolated using the DNA extraction method described above. PCR was performed for each genomic DNA sample in a total reaction volume of 15 µl as described above using the optimised conditions. For the restriction digests 10 µl of PCR product were digested for 3 hours at 37°C with XcmI restriction enzyme (New England Biolabs) with 10x NEB2 enzyme buffer and water. Digested products were then run on a 2% agarose gel (3 µL of loading dye was added to each 15 µL PCR reaction and 10 µL was loaded per well). The gel was visualised under UV using the Syngene gel dock (Syngene). The restriction enzyme cuts at the T-allele, when visualised uncut bands were of 184 base pairs in length and cut bands were 141bp/43bp in length, for example see Figure 97.

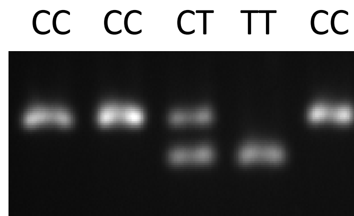


Figure 97. Example of rs2475501 restriction digested bands. The large undigested C-allele homozygote bands, T-allele homozygote digested band and the heterozgote (one undigested and one digested band for each allele)

Antibody	Fluorochome	Isotype	Clone	Cat No	Dilution	Company
Annexin V	Allophycocyanin	-	-	575504	5 µL/test	BD
Anti-human CD3	Pacific Blue	Mouse IgG1 k	UCHT1	558117	1:50	BD
Anti-human CD3	PerCP	Mouse IgG1 k	SK7	347344	1:20	BD
Anti-human CD4	FITC	Mouse IgG1 k	RPA-T4	555346	1:20	BD
Anti-human CD4	PE	Mouse IgG1 k	RPA-T4	555347	1:20	BD
Anti-human CD8	APC	Mouse IgG1k	RPA-T8	555369	1:10	BD
Anti-human CD45RA	FITC	Mouse IgG2b k	HI100	555488	1:20	BD Pharmingen
Anti-human CD45RO	FITC	Mouse IgG2a k	UCHL1	555492	1:20	BD Pharmingen
Anti-human IL-1R1	FITC	Goat IgG	AAFH02	FAB269F	1:20	R & D Systems
Anti-human IL-4	PE	Mouse IgG1 k	8D4-8	12-7049-41	1:50	eBioscience
Anti-human IL-17	Alexa 647	Mouse IgG1 k	eBio64DEC17	51-7179-42	1:20	eBioscience
Anti Human IL-21	PE	Mouse IgG1 k	eBio3A3-N2	12-7219-71	1:20	eBioscience
Anti-human IL-22	PE	Mouse IgG1	142928	IC7821P	1:20	R&D Systems
Anti-human IFN-γ	FITC	Mouse IgG1, k	4S.B3	11-7319-82	1:100	eBioscience
Anti-human IFN-γ	PE	Mouse IgG2b, k	25723.11	340452	1:10	BD
Anti-human IFN-γ	Alexa 700	Mouse IgG1, k	B27	557995	1:50	BD
Anti-human Foxp3	Pacific Blue	Mouse IgG1, k	206D	320116	1:50	Biolegend
Anti-human Foxp3	PE	Mouse IgG1, k	206D	320107	1:10	Biolegend
Anti-human RORγt	PE	Rat IgG2a	AFKJS-9	12-6988-80	1:20	eBioscience
Anti-human LAP-TGFβ	PE	Mouse IgG1	27231	FAB2463P	1:10	R&D Sytstems
Via-Probe	7-AAD			555861	10µL/test	**BD**

Table 8. Table of flow cytometry antibodies used within the study

Antibody	Isotype	Clone	Cat. Number	Dilution	Company
Anti-human AhR	Mouse IgG	RTF1	Ab2770	1:5000	Santa Cruz Biotech
Anti-GAPDH	Rabbit	D16H11	5174	1:5000	Cell Signalling
Anti-Lamin	Rabbit	Unknown	2032	1:2000	Cell Signalling
Anti-human NFATc1	Mouse IgG	Clone 7A6	SC-7294	1:5000	Santa Cruz Biotech
Anti-human STAT 3	Rabbit	Unknown	9132	1:2000	Cell Signalling
Anti-human STAT3 P Tyr 705	Rabbit	D3A7	9145	1:2000	Cell Signalling
Anti-mMouse IgG- HRP	Horse	-	7076	1:5000	Cell Signalling
Anti-rabbit IgG HRP	Goat	-	7074	1:5000	Cell Signalling

Table 9. Table of western blot antibodies used with the study

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