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The role of T cell receptor signal intensity in T helper 17 cell development

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Abstract

T-helper (Th) 17 cells are a subset of CD4+ T cells defined through the release of the cytokine interleukin-17a (IL-17a). Activation of these cells is critical for protection against some extracellular bacterial and fungal pathogens. However, a dysregulated Th17 response targeted against self is thought to play an important role in the immunopathology of a number of autoimmune conditions including Inflammatory Bowel Disease (IBD), Multiple Sclerosis (MS) or inflammatory arthritides. Further understanding of the mechanisms that influence the development of Th17 cells may aid future therapeutic targeting of these cells. Whilst the role of the cytokine milieu in Th cell polarisation is relatively well characterised, the degree of signalling through the TCR can also shape the form of the Th cell response. Both the density of antigenic peptide available and the affinity of the antigenic peptide for a particular TCR can contribute to the degree of TCR signalling.

The hypothesis of this project was that TCR signal intensity could alter the development of Th17 cells from a naive precursor population. In particular, it was of interest to determine how citrullination of a putative TCR contact amino acid in an antigenic peptide could alter the Th cell response observed. The 5/4E8 T-cell receptor transgenic (TCR Tg) mouse provides a model in which >80% of T-cells specifically recognise an immunodominant epitope derived from the G1 domain of aggrecan – peptide-84-103 (p84-103). This model allowed for the examination of these factors and the underlying mechanism *ex vivo* using a purified naive CD4+ T cell population in co-culture with LPS-matured dendritic cells (mDCs).

The data presented in this thesis show the activation, proliferation and effector responses of naive 5/4E8 TCR Tg T cells to alterations in both cognate peptide (p89-103) density and affinity through citrullination of a putative TCR contact residue (R93Cit). Interestingly, by reducing TCR signal strength the observed response shifts from one dominated by the Th2 phenotype to Th17 cells. Linking the degree of TCR activation to Th cell phenotype was the intensity of IL-2 signalling that in turn shaped the balance between phosphorylated STAT3 and STAT5. Compared to p89-103-primed T cells, T cells responding to R93Cit produced less IL-2, expressed lower levels of the IL-

2 receptor subunit CD25, and showed reduced levels of STAT5 phosphorylation, whilst STAT3 activation was unaltered. IL-2 blockade in p89-103-primed T-cells selectively reduced STAT5 but not STAT3 phosphorylation, and concomitantly enhanced Th17 development.

In summary, this work indicates the impact that changes to the intensity of TCR signalling can have on the murine Th17 response. Indeed, these data illustrate how a disease-relevant post-translational modification such as citrullination can promote Th17 development by altering the balance between STAT5 and STAT3 activation in responding T cells.

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List of Abbreviations

ACPA	Anti-cyclic citrullinated peptide antibodies
AHR	Aryl hydrocarbon receptor
AICD	Activation induced cell death
Aire	Autoimmune regulator
AP-1	Activator protein-1
APC	Antigen presenting cells or allophycocyanin (fluorophore)
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APL	Altered peptide ligand
Arp	Actin-related protein (e.g. Arp2/3)
ATP	Adenosine triphosphate
BATF	Basic leucine zipper transcription factor
BCL-6	B cell lymphoma-6
BCR	B cell antigen receptor
BFA	Brefeldin A
BLIMP-1	B lymphocyte-induced maturation protein-1
BSA	Bovine serum albumin
CarP	Carbamylated
CC(L/R)	Chemokine (C-C motif) (ligand/ receptor)
CCP	Cyclic citrullinated peptides
CD	Cluster of differentiation or Celiac disease
CDR	Complementarity determining regions

CDMS	Clinical defined multiple sclerosis
CF	Cystic fibrosis
CFA	Complete freund's adjuvant
CFSE	Carboxyfluoroscein succinimidyl ester
CFTR	Cystic fibrosis transmembrane conductance regulator
ChIP	Chromatin immunoprecipitation
ChiPseq	Chromatin immunoprecipitation sequencing
CIA	Collagen-induced arthritis
Cit	Citrullinated
CLIP	Class II-associated invariant chain peptide
CpG	—C—phosphate—G—
CPM	Counts per minute
CMA	Chaperone-mediated autophagy
c-Maf	Musculoaponeurotic fibrosarcoma oncogene homolog
CMC	Chronic mucocutaneous candidiasis
CNS	Central nervous system
CREB	Cyclic-AMP- responsive-element-binding protein
CRP	C-reactive protein
cSMAC	Central supra-molecular activation complex
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTNNB	Catenin (cadherin-associated protein), beta (e.g. CTNNB-1)

CXC(L/R)	C-X-C chemokine (ligand or receptor)
Da	Dalton
DAG	Diacylglycerol
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dSMAC	Distal supra-molecular activation complex
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephomyelitis
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbant assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
Fab	Fragment antigen binding
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
Fc	Fragement crystallisation
FcR	Fragment constant receptor
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FOXP3	Forkhead box-p3

FRC	Fibroblastic reticular cell
FSC-A	Forward scatter area
GATA-3	GATA-binding protein-3
G-CSF	Granulocyte- colony stimulating factor
GFI-1	Growth factor independent-1
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPI-6	Glucose-6- phosphate isomerase
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
HEV	High endothelial venues
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSP	Heat shock protein
HuCIV	Human collagen type IV
IBD	Inflammatory bowel disease
inDC	Inflammatory dendritic cell
ICAM	Intercellular adhesion molecule
ICOS	Inducible T-cell co-stimulator
ICS	Intracellular cytokine staining
IDO	Indolamine 2-3-dioxygenase
IFN	Interferon (e.g. Interferon-gamma)
IKK	Inhibitor of kappa B Kinase

IL	Interleukin (e.g. IL-17)
IL4I1	Interleukin-4 induced-1
Ig	Immunoglobulin
Ii	Invariant protein
IMDM	Iscoves modified dulbeccos medium
IP_3	Inositol 1,4,5-trisphosphate
IPEX	Immune dysregulation polyendocrinopathy, enteropathy, X linked
IRF4	Interferon regulatory factor 4
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motifs
Itk	Inducible T-cell kinase
iTreg	Inducible regulatory T-cell
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
JNK	c-jun kinase
L e.g.	CD62L Ligand
LAG-3	Lymphocyte activation gene product-3
LAT	Linker for activation of T cells
LCK	Lymphocyte-specific protein tyrosine kinase
LFA	Leucocyte functional antigen
LPS	Lipopolysaccharides
mAb	Monoclonal antibody

MAPK	Mitogen-associated protein kinase
Mb	Megabases
MBP	Myelin basic protein
MCC	Moth cytochrome-c
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
miRNA	microRNA
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cell
mTOR	Mammalian target of rapamycin
NCS	Non-coding sequences
NETs	Neutrophil extracellular traps
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
NMR	Nuclear magnetic resonance
NP	Nuclear protein
NO	Nitric oxide
nt	Nucleotide
nTreg	Natural regulatory T-cell

OA	Osteoarthritis
OPD	o-Phenylenediamine dihydrochloride
OVA	Ovalbumin
p	phosphorylated (e.g. pSTAT3) or pre (e.g. pre TCR β chain)
PAD	Peptidylarginine deiminases
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PD1	Programmed death-1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll protein
PGIA	Proteoglycan induced arthritis
PIP	Phosphinostil bisphosphate (e.g. PIP_2)
PKB	Protein kinase B
PKC	Protein kinase C
PLC- γ1	Phospholipase C-γ1
PGN	Peptidoglycan
PIK3	Phosphatidylinositol-4,5-bisphosphate 3 Kinase
PLC	Peptide loading complex
PMA	Phorbol 12-myristate 13-acetate
pMHC	peptide- Major histocompatibility complex
PRR	Pattern recognition receptors
PsA	Psoriatic arthritis

pSMAC	Peripheral supra-molecular activation complexes
PTK7	Protein tyrosine kinase-7
PTM	Post translational modification
PTP	Protein tyrosine phosphatase
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid arthritis
RAG	Recombination activating genes
RASF	Rheumatoid arthritis synovial fibroblast
RasGRP	Ras gaunly activating release protein
RF	Rheumatoid factor
rh	recombinant human
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROR-γT	RAR-related orphan receptor gamma T
RPMI	Roswell park memorial institute medium
RSS	Recombination signal sequences
RT	Room temperature
Runx	Runt-related transcription factor (e.g. Runx3)
SCID	Severe combined immunodeficiency
SCW	Streptococcal cell wall
SDS	Sodium dodecyl sulfate
SEM	Standard Error of the Mean

SLE	Systemic lupus erythematosus
SLP-76	SH2 binding domain leukocyte protein-76
Socs	Suppressor of cytokine signalling (e.g. Socs-3)
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 signalling
SR	Serum Replacement
SSC-A	Side Scatter Area
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
T-bet	T-box transcription factor
TCR	T cell receptor
TCR Tg	T cell receptor transgenic
T_{CM}	Central memory T cell
TdT	Terminal deoxynucleotide transferase
T_{EM}	Effector memory T cell
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
TLR	Toll like receptor
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF	Tumour necrosis factor
Tr1	Regulatory T cell type 1 (IL-10 induced)
TRAF	TNF receptor associated factor (e.g. TRAF-6)
TRECs	T cell receptor excision circles
Treg	Regulatory T cell
TSA	Tissue-specific self antigen
WT	Wild type
Y_c	Common gamma chain
ZAP-70	Zeta-chain-associated protein kinase 70

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1 Introduction

The evolution of the adaptive immune response provides the host organism with an additional form of defence beyond that evolved in the genome (1). The specialised manner through which these cells recognise pathogens renders an additional layer of complexity, meaning the immune system can target distinct families of pathogens; ranging from intracellular viruses to extracellular bacteria and multicellular helminths (1). The importance of T lymphocytes is illustrated through their loss leading to profound susceptibility to a range of otherwise harmless organisms (2). However, a dysregulated T cell response targeted against harmless antigens or self can be equally destructive leading to chronic inflammation and sustained morbidity (3-5). Thus, further understanding of how these cells can become pathogenic is important for T cell immunobiology, but also towards the development of more targeted therapies aimed at disruption of harmful responses whilst preserving overall host immunity.

1.1 T cells

Even the most evolutionary ancient organisms possess a form of innate immunity (6). First contact with a pathogen will activate cells belonging to this system. However, detection of 'danger' signals by innate cells is reliant on germline-encoded receptors (1, 7-10). By their nature these all lack a high degree of specificity and can be evaded by a particular pathogen. If the presence of the pathogen persists, cells of the innate system can escalate the immune response through the activation of the adaptive immune system. The defining characteristics of the adaptive immune system are 1) the recombination of genomic elements to form novel receptors and 2) the persistence of long-lived memory cells that serve to enhance the magnitude and kinetics of subsequent immune responses to the same pathogen.

While sharing some homology in terms of their receptor structure, the main difference between T and B cells is the manner in which they detect antigen and their developmental origin (11). In the case of B cells, the B cell receptor (BCR) can

bind to soluble antigen in the native confirmation. Indeed, because antigens are recognised in their native form, B cell epitopes can be discontinuous in terms of the protein sequence with each of the two Fragment Antigen Binding (Fab') regions binding distant sites on the target antigen. In contrast, the T cell receptor (TCR), is only capable of recognising denatured short peptides presented in the context of the Major Histocompatibily Complex (MHC) molecules; defined as MHC restriction by Zinkernagel and Docherty (12-14). Indeed, the antigenic protein must first be processed by antigen-presenting cells (APCs) in order to load the pathogen-derived peptides on MHC for interrogation by T cells through their TCR (15, 16). Since pioneering work by Davis and Tonegawa, who first sequenced the TCR loci it has become apparent that the TCR forms a heterodimer with two distinct families; either αβ or γδ which can be used to define two populations of T lymphocytes (17-23). With regards to αβ T cells, these can be further divided on the basis of other associated markers such as the MHC co-receptors CD4 and CD8 (24). CD4 T cells, also known as T-helper cells (Th), are restricted by recognition of peptides bound only to MHC class II (MHC cII)-peptide complexes. MHC cII primarily presents antigen originating from outwith the APC (25). Those expressing CD8, cytotoxic T lymphocytes (CTLs), can bind antigenic peptides bound to MHC cI (26, 27). Such peptides are typically derived from endogenously degraded proteins, although cross-presentation pathways exist for both MHC types (28). These CD4 and CD8 T cells subsets, despite expressing αβ TCRs, specialise in different aspects of the immune response. Both form effector cells with CD4 T cells mediating activation of other immune cells, mainly via cytokine driven signalling, while CD8 T cells prototypically carry out cytolytic functions to clear infected cells (26).

1.1.1 TCR Structure

The TCR was initially identified through the use of clonotypic monoclonal antibodies. It was found by immunoprecipation, in non-reducing conditions, as a 90kD protein. Treatment with Sodium Dodecyl Sulfate (SDS) to disrupt the tertiary structure revealed a slightly larger alpha chain, 48kD, linked by disulfide bonds to the beta chain of 42kD (29). The basic structure is not dissimilar from that of the

Fab' region of the BCR. Both αβ chains possess a variable region in contact with the peptide-MHC (pMHC) complex and a constant, invariant, region with a short cytoplasmic tail (see **Figure 1**) (30). Both regions are critical to the function of the TCR with the V region detecting peptide present on MHC in a restricted manner while the C region serves to interact with immediate signalling components, the CD3 complex; an interaction that conveys the information from the external environment (31).

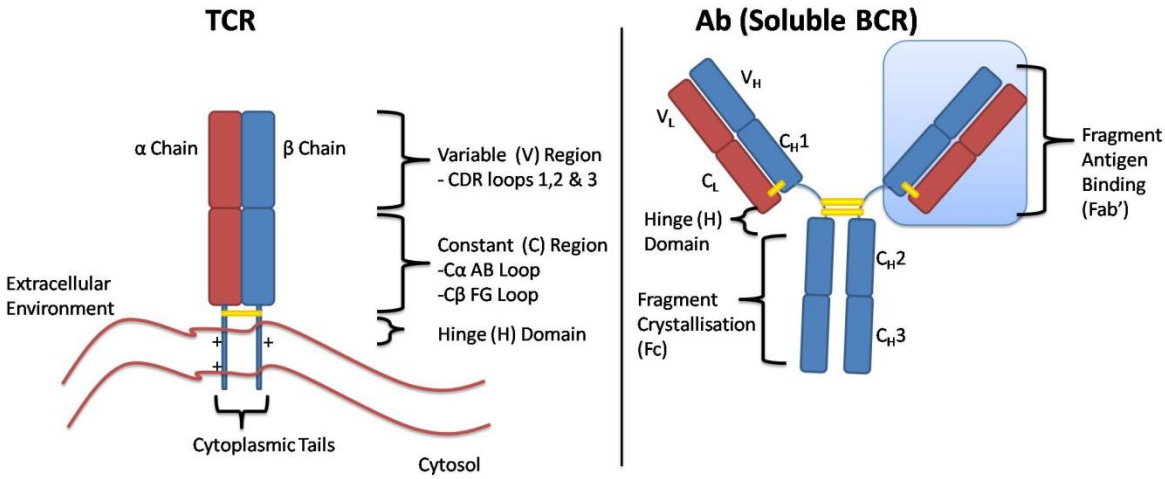

Figure 1. Comparison of TCR and BCR. Despite the high degree of homology to Fab' region of the BCR, the final TCR structure is slightly shorter and wider with the Cα domain possessing a fold that is different in form compared to other Ig-like domains. The beta-sheets adjacent to the Cβ possess a typical form yet the remaining region forms a unordered, loosely packed structures and a short α helix. Moreover, the interaction between domains is far stronger in the TCR which is further strengthened by interactions with carbohydrates moieties between the V and C regions (not shown). Unusually the TCR transmembrane domain contains positive charged residues within the transmembrane domain (indicated by + in Figure) thought to interact with reciphoral negatively charged residues in CD3 components. These polar interactions are thought to enhance the assoication between the TCR and its co-receptors.

1.1.1.1 The constant region

Unsurprisingly, the constant region shows a significant degree of conservation across all TCR structures currently obtained by X-crystallography (30). Roughly similar in size (Cα – 0.8-1.1 A^o and the Cβ -0.6-0.8 A^o), the constant domains interact through a highly polar surface. Both C regions have been found to contain sites of post-translational modifications (PTMs). The amino acid constitution of these regions is especially dominated by acidic residues in Cα interacting, through

hydrogen bonding and salt bridges, to basic residues found in Cβ (32, 33). Furthermore, the latter posses a 14 residue FG loop, that is highly conserved between humans and mice. With a hydrophobic core, this loop projects outwards into the solvent and is relatively rigid in nature. It is thought to form a section of the cavity involved in CDεγ interaction (33, 34). Loss of such a region reduces the strength of association resulting in reduced phosphorylation, causing a range of defects e.g. abrogation of proliferation, thymocyte development and cytokine production (33).

Of growing importance is another structure, the AB loop (residues 129-136), enriched with positively charged amino acids projecting into a cavity of the Cβ (34). It is thought that the lack of hydrogen bonding to other strands in the Cα may permit interactions with extracellular domains of the CD3 molecules. Thus, strengthening the obligate interaction with other components of the CD3 signalling complex.

1.1.1.2 The variable region

The antigen specificity endowed by the TCR is manifested in the V regions of both chains. It is the genetic recombination at this region that generates the diversity critical for T cell immunity (35). In terms of genetics, there are approximately 70 Vα elements and 52 Vβ elements. The complete α chain is formed by recombination with one of 61 Jα elements (1). In the case of the β chain, an additional D element (humans and mice possess 2 forms) is introduced before one of 13 Jβ elements. Each element is flanked by a recombination signal sequence (RSS) recognised by VDJ recombinase complex. Critical initiators of the process are the recombinase activation genes (RAG) of which there are two forms (RAG1 and 2) (36). Loss of either of these abrogates this process and blocks development of both T and B cells resulting in profound immunodeficiency. Additional diversity is introduced through the random nucleotides at the junction of these elements by terminal deoxynucleotide transferase (TdT) and exonucleases. Estimates suggest up to 10^{15} different TCRs in humans are possible yet this is limited by the physical numbers of

αβ T cells within an organism (approximately 10^{12} cells) (35). Thus, TCR/p-MHC interactions require a degree of cross-reactivity in order to provide at least one TCR capable of responding to a particular peptide.

Structurally, the V domains consist of a β sandwich structure with a hydrophobic core that is surrounded by a series of hydrogen bonds (35). Each chain supplies three hypervariable loops known as complementarity determining regions (CDR). It is also the domain subject to the greatest genetic diversity (37). The structure formed is relatively flat, similar to antibodies directed against proteins. The restricted number of V gene segments is beginning to allow classification of TCRs into specific subsets based on the structural features of their CDRs. Interestingly, the pairing angle between chains can directly alter the orientation of the V domains in a particular TCR. The effect is a shift in the precise specificity obtained when pairing the same VαVβ segments (35). This may contribute to the diversity of the TCR repertoire.

While the TCR locus lacks the huge number of V gene segments in comparison to the BCR, there is a significantly higher number of J segments that can be utilised (1). The region encoded by these gene segments, CDR3, shows a great degree of diversity both in terms of sequence and length (6 to 12 amino acids). Comparisons of CDR3-Loop-3s show that CDR3α is far more diverse to such an extent that it is difficult to separate these into subgroups based upon common structural features (30). Also, there is a greater addition of N and P nucleotides that further contributes to structural heterogeneity. In many structures, the CDR3 of each chain is somewhat separated and protrudes out of the TCR (38). The resulting cleft may accommodate those ligands in which the peptide projects out of the MHC. Thus, the distribution of diversity in these regions is critical to understanding the type of interaction that occurs between the TCR and its ligand. Typically CDR1 and 2 show a lack of diversity in both the α and β chains (38). This can be understood as these bind to corresponding regions of the MHC molecules. In contrast, CDR3 is thought to insert into the peptide binding- groove and contact the cognate peptide directly

in the majority of structures solved thus far (38). As the antigen-processing machinery can generate a pool of potential peptide partners far greater than the number of MHC polymorphisms found in a population, it comes as no surprise as to why this region displays such a magnitude of diversity.

1.2 Antigen processing and presentation

1.2.1 MHC cI/cII

From the moment that the α chain is successfully expressed in CD4+CD8+ thymocytes the TCR and its specificity will be critical in determining the fate of that particular cell (39). As previously mentioned the incredible diversity to be found in a T cell population is a reflection of the diversity of the ligand required to be recognised. Found on antigen presenting cells (APCs), MHC molecules allow both major classes of αβ T cells to respond to peptide antigens derived by the enzymatic processing of pathogenic components. Forming two major classes, MHC class I (cI) are expressed on th surface of most nucleated cells and are predominantly responsible for the presentation of peptide antigens originating topologically within the cell (40). Structurally, cI consists of a polymorphic α chain noncovalently bound to the conserved β2-microglobulin chain (not encoded in the MHC locus on Chromsome 15) (41). Classically, peptides degraded by the proteasome, located in the cytosol, are transferred into the Endoplasmic Reticulum (ER) by the transporter associated with antigen processing (TAP) complex prior to peptide loading (see **Figure 2A** (42)). Tapasin is associated with TAP and is involved in stabilising unbound MHC cI molecules in an open confirmation facilitating loading (43). Other chaperones such as the thiol oxidoreductase, Erp57, and calreticulin constitute a form of quality control preventing the build up of unstable p-MHC complexes (44). Following successful peptide loading, MHC cI molecules can disassociate from TAP-chaperone complex and traffic to the plasma membrane through the Golgi apparatus (40).

In contrast, MHC class II (cII) heterodimers are restricted to 'professional' APCs including dendritic cells (DCs), macrophages and B cells (1, 45, 46). It is the major

processing pathway for exogenous antigens derived via the endosomal system (see **Figure 2B** (47)). The overall structure of cII is similar to MHC cI; two independent chains, α and β, both possess transmembrane domains and are formed in the lumen of the ER (48, 49). Both MHC structures are stabilised by the presence of peptide in the binding grove (50). The major difference occurs at the peptide-binding grove. The peptides presented by MHC cI tend to be restricted to 8-10 amino acids (aa) due to the walls at the ends of the binding cleft (51). In the case of MHC cII, peptides are also anchored at key residues but bound peptides are at least 13aa and often longer (52). The increased permissiveness of binding is due to the open ends of the binding grove.

Following endocytosis of extracellular antigens, fusion of vesicles leads to increasing acidification and degradation by a range of proteolytic enzymes including members of the cathepsins. Fusion with MHC cII-containing vesicles allows loading which is catalysed in part by a nonclassical form of MHC known as MHC-DM (53). This serves a particularly important role in the exchange of cII-associate invariant peptide (CLIP) from the binding groove, allowing loading of antigenic peptide (54). However, as with MHC cI, non-classical processing pathways permit the loading of peptides from atypical topologically origins (55).

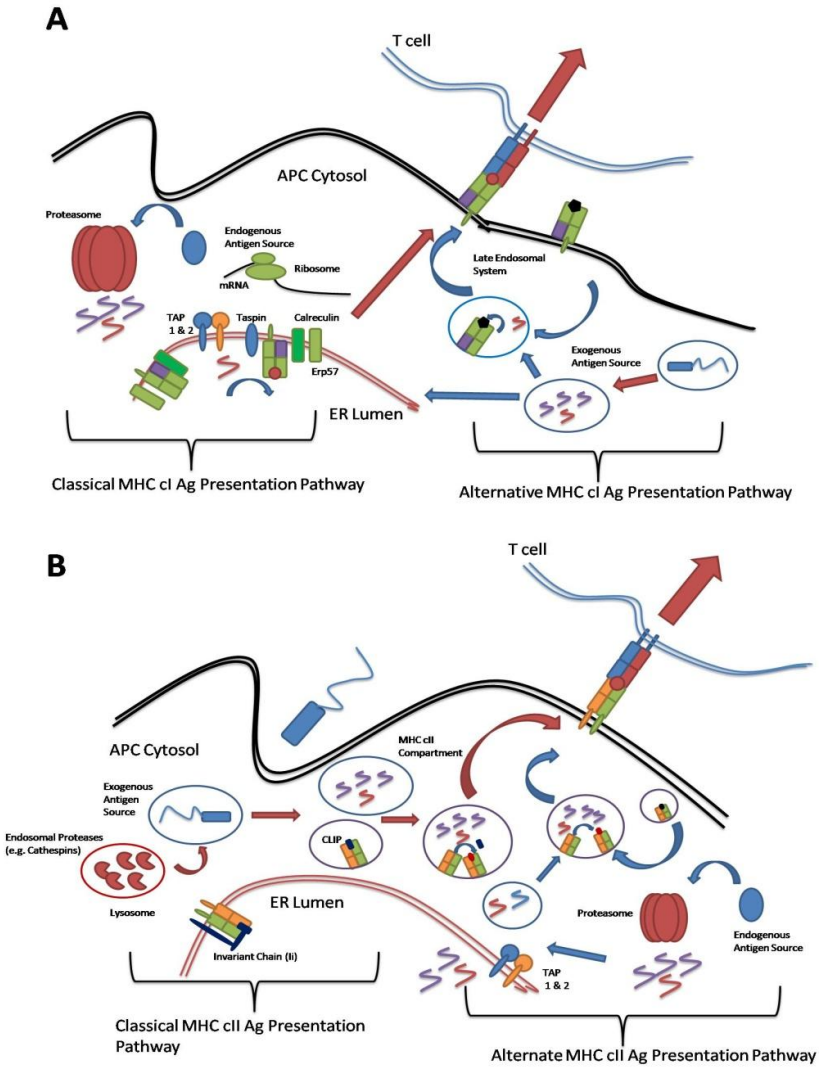

Figure 2 - Antigen processing pathways summary. (**A**) Overview of MHC cI antigen processing pathways. Endogenously synthesised antigens are degraded via the proteasome before TAP dependent transfer to the ER. Loading occurs via the Peptide Loading Complex (PLC) before MHC cI are transported to the surface. Alternatively, exogenous antigens can enter the pathway through Chaperone-mediated autophagy (CMA); Sec61 mediates retrograde transfer to the ER or re-cycling of surface cI molecules that encounter antigen in the endosomal system. The latter may be particularly important in the context of viral blockage of TAP function. (**B**) Overview of MHC cII antigen processing pathway. Guided by the Ii chain, MHC cII are targeted to the MHC cII compartment of the late endosome system. Following degradation of exogenous antigens by acidic proteases, peptides are loaded by HLA-DM catalysed mechanism. It may also permit antigen editing via repeated rounds of peptide exchanges. To some degree endogenous antigen can be presented following processing by the proteasome or calpain proteases. Delivery into the MHC cII compartment by vesicles may be driven by autophagy although the diversity in cytosplasmic antigens presented suggests that bulk autophagy alone is not the exclusive mechanism through which antigen is transferred.

1.2.2 TCR-pMHC Interactions

Both MHC molecules are presented on the surface on APCs for interrogation by T cells through their TCR. While each TCR is often described as specific for a particular pMHC complex, this interaction is of relatively low affinity (1-100μM) even for the higher affinity peptides, especially when compared to that of antibodies (37). Analysis of a number of TCR-pMHC complexes discovered thus far shows that although the alignment of the TCR over the pMHC is broadly diagonal, the precise interactions are highly variable and dependent on the precise arrangement of CDRs on the TCR and the peptide bound to the MHC (30, 37). Generally, the Vα on the TCR contacts the α2 helix and the N-terminus of the peptide, whereas the Vβ region contacts the α1 helix and C-terminus of the peptide (30, 37), thus creating an extensive surface of around 1200-2400A^{o} in area (30). However, the lack of shape complementarity results in a low affinity binding that corroborates with the data obtained from structural studies. The paradigm that CDR1 and 2 mediate contacts with the MHC while CDR3 is responsible for interactions with the peptide is now likely to be exaggerated, with numerous studies indicating exceptions. For example, Chel *et al* demonstrated a non-germline encoded CDR3 interacting extensively with the MHC while others have shown CDR1 and CDR2 contacts to antigenic peptides (56, 57). Furthermore, the precise determinate which drives the ligation of the TCR to its ligand has been shown by mutagenesis and biophysical studies to be both entropically and enthalpically driven by any of the CDRs (35). In essence, TCR recognition is highly dependent upon the context of the particular TCR and pMHC ligand.

There is growing evidence that TCRs directed against self peptides and anti-tumour complexes show atypical interactions (58). Unlike most anti-microbial structures elucidated thus far, there is a stronger interaction with the N-terminus than the C-terminus of the peptide (58), but these interactions are typically of lower affinity. Those directed against tumour antigens appear to adopt a mixed interaction sharing features typical of both autoreactive and anti-infectious TCR-pMHC complexes (59, 60). These findings reflect the need, especially for self-reactive T

cells, to preserve a sufficient degree of affinity to enable positive selection yet binding self peptide with a low affinity that permits survival of negative selection (61). Building on the apparent structural features common to these subsets of TCR, is the concept of immunodominant structures with certain antigenic peptides favouring recognition by particular TCR (38). It is thought that certain pMHC complexes represent a significant challenge for TCR recognition (37). The consequence of such is that only T cells possessing a restricted V domain or even specific CDR forms are capable of ligation and hence driving clonal expansion during an immune response (37). Furthermore, analysis of TCR-MHC cI interactions demonstrates a commonality with three specific positions, 65, 69 and 155, contacted by all TCRs so far determined (37). The suggestion is that these represent a minimal framework for binding and hence underpin MHC restriction by T cells. However, while over 400 antibody structures have been solved, construction of a paradigm based upon the modest number of ligated TCRs thus far analysed must be interpreted with a degree of caution (30).

1.2.3 Precision of the TCR-pMHC Interaction

The issue of specificity is a difficult concept to define. The notion that each TCR is capable of being selected for recognition of one peptide is naive in the implication that TCR recognition is a binary system. The cognate peptide is often the form that is capable of optimal ligation both to the particular MHC molecule and forming the strongest binding to the TCR in the context of the former. Subtle changes in peptide sequence can have dramatic effects in terms of affinity for certain TCR and the consequences of signalling triggered by such an interaction. Studies using altered peptide ligands (APLs) clearly illustrate that such micropolymorphisms can act as agonist or antagonist (62, 63). Alterations to anchor sites on the MHC also result in conformational and motility alterations to the peptide and hence detection by the TCR (64). It remains to be determined how such changes to either the peptide or MHC can be compensated for by plasticity in terms of the positioning of CDRs.

It has been established for a considerable length of time that in a polyclonal population of T cells, roughly 0.1-1% can recognise non-self pMHC and initiate a response (65). Such recognition events are critical to transplant rejection. Although some studies suggests that allogenicity is attributed mainly to the critical MHC-TCR interactions, recent evidence supports a view that it is dependent on bound peptide (66). Using peptide analogues, it has been illustrated that the CDR3 loop is vital in the distinction between allogeneic and non-allogeneic T cells. Slight alterations to the peptide sequence were able to affect which population was activated in response to two MHC polymorphisms. Thus, while some germ-line components may pre-dispose for reactivity, to yield full activation there must be a degree of peptide recognition.

1.3 T cell-APC Interactions

Following release from the thymus, T cells recirculate through the secondary lymphatic system (e.g. lymph nodes, spleen and Peyer's patches), which are located at various strategic sites in the body to collect and sample antigen (67-69). Many antigens entering at non-lymphatic sites need APC transfer to the local lymph node (70, 71). Homing to lymph nodes requires CCR7 expression (72). Upon entry to the relevant lymph node, APCs pass into the paracortical region where contact is made with T cells (73). The duration of interaction rapidly increases upon triggering of the TCR (>15h) leading to reciprocal signalling, altering the phenotype of both cells (74-76). Depending on the type of signal, T cells can either migrate to B cell areas to assist antibody production or leave the secondary lymphoid tissues and migrate to sites of inflammation. This can be visualised by imaging studies in which T cell migration in the lymph node is described as random walk on the network of fibroblast recticular cells (FRCs) (77-79). The T cells enter via high endothelial venules (HEVs) and leave by lymphatic exits. Initial contacts are described as an intermediate form making brief contact before further migration to sample several DCs. If TCRs are successfully bound, increased signalling arrests the T cell on the APC for around 16-24h before T cells resume their motility (80). These activated T cells swarm the local area and undergo several rounds of proliferation.

Interestingly, successful engagement of CD4+ T cells with antigen-loaded DCs induces production of chemokines CCL3/4 permitting the recruitment of CD8+ naive T cells, thus increasing the chance of two antigen specific T cells encountering a suitable DC in the same lymph node (81, 82). The role of stromal cells in guiding and shaping these interactions is an area of growing interest, especially given the evidence that some pathogens can alter the network to disrupt coupling of APC to antigen-specific T cells (83-85). However, this response is not limited to lymph nodes with numerous examples of priming events occurring *in situ* (86-89). Examples have been described both in the context of infection but also autoimmunity. For instance, it has been noted that T cells can undergo activation by DCs in the inflamed synovium (90, 91).

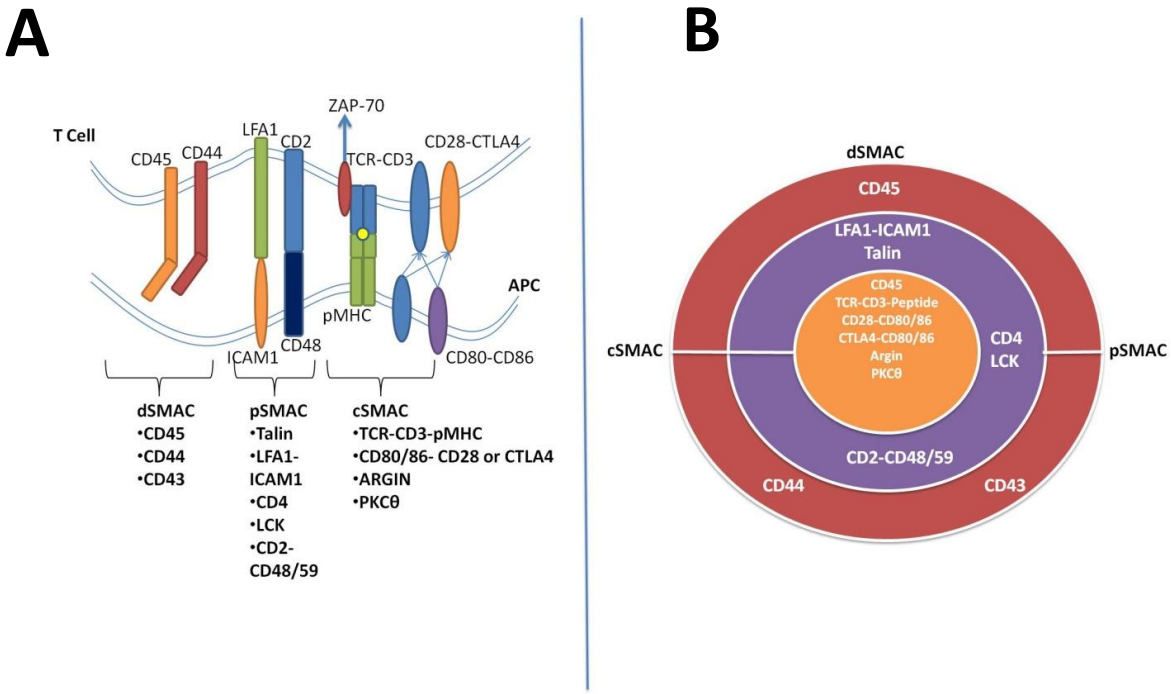

Figure 3 - Immunogical synapse. (**A**) A profile view of the IS with the activating pMHC in the centre bound to the TCR-CD3 surrounded by adhesion molecules such as LFA1 which further organise and stabilise binding. Still further out are the inhibitory molecules such as CD45 and CD43. (**B**) The bulls eye view of the IS with the cSMAC shown in yellow; the pSMAC in purple and the dSMAC in red. Assemble of this structure is dynamic depending on pMHC binding, kinase activation and Ca^{2+} mobilisation. Recruitment of negative regulators such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) is proportional to TCR signal strength. Thus, the regulation of TCR signalling is controlled by timing and activity of costimulatory receptors and ligands.

1.4 Immunological Synapse

A synapse is defined as a specialised structure formed by the apposition of plasma membranes between two cells to signal information (92, 93). Whilst lacking the persistence of neural synapses, immunological synapses (IS) are formed by T cells, B cells and NK cells (94). Classically, the structure of a mature IS is described as a 'bulls-eye' consisting of a series of supramolecular activated clusters (SMACs; see **Figure 3**) (95). The innermost area is termed the central SMAC (cSMAC) and is the location of TCR-CD3-pMHC, protein kinase-Cθ (PKCθ) and lymphocyte-specific protein tyrosine kinase (LCK). This is surrounded by the peripheral SMAC (pSMAC) where adhesion molecules such as the integrin lymphocyte associated factor-1 (LFA-1) and talin bind to the actin cytoskeleton. Larger proteins such as the phosphatase CD45 and CD43 are also located in the periphery of the pSMAC;

termed the distal SMAC (dSMAC). Although a mature IS results in the discreet arrangement of TCRs and positive promoters of signalling away from proteins thought to negatively regulate signalling, studies have shown that as little as one specific pMHC complex is sufficient to induce transient Ca^{2+} flux (95, 96). However, at least 10 pMHC complexes are needed for full activation. If co-receptors are absent then the number of specific pMHC complexes increases to around 35 to induce full signalling via the TCR. Indeed, studies by Gunzer *et al*, showed that if activated in a collagen gel matrix transient interactions that failed to yield mature IS were sufficient to induce proliferation and IL-2 production (97). Moreover, evidence shows that TCR signalling kinetics are greatest before maturation of the IS and actively signalling TCRs are located at the interface of pSMAC and cSMAC (98). The importance to the initiation of TCR signalling is doubtful given the studies that show that ZAP70 is activated 45 seconds after contact between CD4 T cells and a B cell line (95, 99). Indeed, Lee *et al* demonstrated kinase activation 15-30 minutes after initial recognition and cell-to-cell contact; too early for a mature IS to form (100). Growing evidence suggests the role of the IS is to provide a molecular framework to regulate the attenuation of TCR signalling and co-ordinate its function with downstream pathways (101, 102). This is supported by the studies using *CD2ap* knock-outs in which T cells fail to internalise activated TCRs located in the cSMAC and as a consequence proximal TCR signalling is prolonged (103).

1.5 Functional consequences of TCR interaction with pMHC

Recognition of a ligand typically induces binding of partners to the intracellular region of the bound receptor yet the TCR has no intrinsic signalling mechanism (31). Instead, it is mediated by proteins that form a larger macromolecular complex that are responsible for transmitting the extra-cellular interaction to a number of downstream signalling pathways. During the attempts to define the TCR using monoclonal antibodies, certain antibodies led to polyclonal activation of T cells and were capable of co-precipitating the TCR (104). The CD3 complex consists of γ,δ,ε and ζ subunits forming CD3ζζ homodimer, CD3εγ and CD3εδ heterodimers. Without these complexes, the TCR is not expressed and normal development of the T cell is

halted. The molecular feature that underpins this inter-dependence the three negatively charged residues found within the membrane-spanning region of the TCR (105). Clearly, these cannot exist independently within the hydrophobic lipid environment without these charges being neutralised. The reciprocal residues are found in CD3 proteins, mediating an electrostatically driven docking process that serves to orientate the TCR with the CD3 components. Each CD3 component consists of a single immunogobulin domain that is associated with the TCR.

Forming a complex with the stoichiometry of αβγεδεζζ, the TCR interacts with the CD3εγ and CD3εδ by conserved non-glycosylated regions with the TCR projecting 'above' the CD3 into the extracellular environment (31, 106, 107). Both εδ and εγ heterodimers possess adjacent immunoglobulin-like folds while the ζζ homodimer possesses an extracellular domain of only 9 amino acids. The connecting peptides, unlike those of the TCR, are relatively short at around 5-10 amino acids (31). Orientation of these relative to the TCR remains to be fully elucidated but it is thought that the CD3 εγ heterodimer is contacted by the FG loop from Cβ and also the AG loop of Cα (33, 108). Potential electrostatic interaction results from the net positive pocket formed by the AG loop which is complementary to the negatively charged surface of CD3ε, thus crosslinking the γ subunit with the TCRβ chain (108). The CD3δε is thought to bind to the opposing side interacting with TCRα chain. These link to substantial intracellular domains containing immunotyrosine activation motifs (ITAMs). CD3 ε, δ and γ retain a single ITAM per chain while CD3 ζ possesses a total of three ITAMs (31). It is these regions that are phosphorylated and bind downstream effectors following ligation. Critical is the role of CD3 in transmitting the binding event to cytosol. The diversity and size of the signalling complex likely reflects the subtle nuances and complexities that are a feature of TCR signalling.

1.6 TCR downstream signalling

After the immediate recognition of pMHC, binding leads to the activation of a number of well characterised downstream pathways (see **Figure 4**). The immediate

consequence of recognition is phosphorylation of the CD3 complex at the ITAM residues. There are 10 in a full TCR-CD3 complex. These initial phosphorylations are dependent on the Src family kinases namely Lck and Fyn, the most important in TCR signalling being LCK, found abundantly within T cells, and often in association with the CD4 co-receptor (109, 110). A key member of the 'TCR triggering signalling module' as described by Acuto *et al*, LCK serves to phosphorylate a number of ITAMs on CD3γ, CDε and CD3δ, permitting the binding of the Syk kinase ZAP-70; another LCK target (111). The combination of phosphorylation and aggregation to the TCR-CD3 complex drives structural changes to ZAP-70 that lead to kinase activity and recruitment of a key scaffold protein, linker for activation of T cells (LAT) (112-114). Following binding of SH2 binding domain leukocyte protein-76 (SLP-76), the scaffold serves as an important activator for a number of parallel signalling pathways. One of the most important is phospholipase-2γ (PLC-2γ) hydrolysing phosphatidylinositol bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The former is a diffusible second messenger binding to IP_3 receptors on the ER, and mediating the release of Ca^{2+}. Ca^{2+} flux being a common measure of the degree of TCR signalling (115). In contrast, DAG remains attached to the plasma membrane activating protein kinase Cθ (PKCθ) and Ras guanyl activating release protein (RasGRP) (116-118). The latter serves to facilitate activity of the small GTPase Ras which in turns drives MAP kinase activity.

Another key feature of TCR signalling is the rapid remodelling of the actin cytoskeleton that generates the lamellipodial sheet structure that spreads the T-cell plasma membrane against the APC. A critical stage in signalling occurs through the binding of Vav to the LAT-SLP76 complex (119). This initiates a cascade involving Cdc42 interacting with actin-related protein 2/3 (Arp2/3) that stimulates the growth of branched actin arrays (120).It is through Vav that upregulation of intergrins such as LFA-1 occurs via PLCγ and the activation of small GTPase Rap (121, 122). The enhanced polarisation of integrins is thought to play a critical role in the formation of pSMAC and the prolonged interaction between T cells and APCs (123).

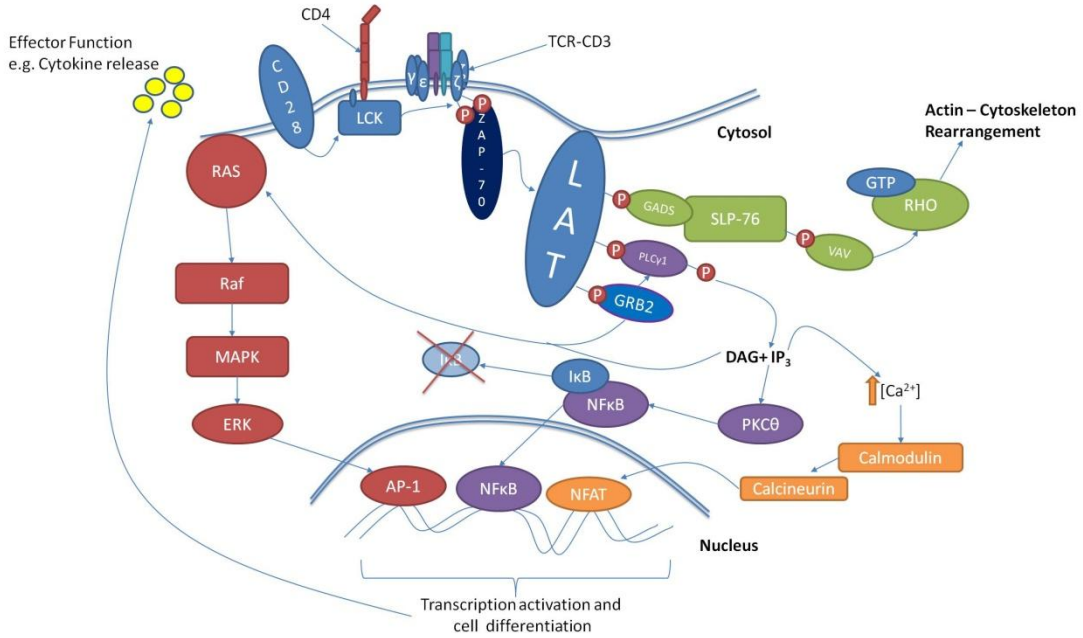

Figure 4 - TCR signalling pathway. Schematic overview of TCR signalling pathways that results in the engagement of the transcription factors Activator Protein-1 (AP-1), NFκB and NFAT. Early signalling events involve the phosphorylation of CD3 ITAMs by the Src family kinases LCK and FYN. These initiate a pathway that recruits LAT that in turn triggers PLCγ1that leads to Calcium flux via DAG and IP_3. Other key pathways include the RAS and Vav that activate the MAPK and cytoskeletal pathway respectively. The net effect is the induction of numerous genes including IL-2 and CD25.

Fundamental to TCR activation is the transduction of signals from the TCR to the nucleus and, ultimately, changes to gene expression (124). Activation of the Fos/Jun transcription factors is driven by the MAPK pathways, which involve the activation of Ras as well as Raf to induce External Regulated Kinase (ERK) and JNK/p38 signalling (125, 126). Interestingly, ERK also serves to phosphorylate LCK, offering a form of positive feedback to enhance upstream signalling. Nuclear Factor of activated T cells (NFAT) is another important mediator of transcription that is kept at low levels in the nucleus through phosphorylation by Glycogen synthase kinase-β3 (GSKβ3) (127). The influx of Ca^{2+} in the cytosol triggered by IP_3 leads to activation of the phosphatase calcineurin, activated by the Ca^{2+} binding protein calmodulin. This is further promoted by Phosphatidylinositol-4,5-bisphosphate 3 Kinase (PIK3), a target of a number of downstream signalling proteins including Ras. In addition, the PIP_3 generated serves to upregulate the survival protein Akt, which also acts on GSKβ3 to further increase NFAT translocation to the nucleus. Finally, NFκB is also

activated via Inhibitor of kappa B Kinase (IKK) targeting of the IκB. Like NFAT, NFκB is free to translocate to the nucleus (128). Together these transcription factors drive the expression of a number of genes including *IL2* and its high affinity receptor α chain *IL2RA* (129, 130). These serve to enhance cell adhesion, survival and differentiation into effector and, in some cases, memory cells.

1.7 CD28 - The influence of T cell co-stimulation

TCR triggered-signalling is often termed 'signal 1'. However, on its own it is not sufficient to induce T cell effector function. The best characterised co-receptors is CD28, a 44kD glycoprotein that forms a homodimer that is recruited to cSMAC by the presence of PYAP motifs (131, 132). Its principal ligands are CD80 (B7.1) and CD86 (B7.2) found to be expressed on all 'professional' APCs. The expression of these along with other co-stimulatory molecules, including CD40, OX40L and ICOSL, are highly dependent on stimulation of APC through Pattern Recognition Receptors (PPRs) such as Toll like receptors (TLR). Animals lacking any of these co-stimulatory molecules show a reduced immune response to an array of immunological challenges (133). The T cell response fails to induce sustained proliferation both *in vitro* and *in vivo* while germinal centres are severely reduced and class switching is also limited (134). Failure to engage these with CD28 induces a form of hyporesponsiveness (135). Upon subsequent reengagement these T cells fail to produce IL-2 and proliferate even if co-stimulation is supplied. Like the TCR, CD28 lacks any intrinsic signalling abilities and is therefore dependent on interaction with Src family of kinases (136).

Studies using microarray analysis of gene expression have shown that if CD28 co-stimulated T cells are compared to those activated by TCR alone, no *de novo* genes are induced (129, 137). However, the extent of expression (or repression) is greatly magnified. This reflects the very early upstream integration of signals from both pathways. Growing evidence suggests that the role of CD28 is not easily described as a purely qualitative receptor given that at very high pMHC-TCR occupancy responses can proceed without CD28 coupled (102). It may be that at such high

density of pMHC, CD28 is even dispensable although the maximal response of TCR alone can be increased by CD28 activity. However with a weak agonist or low pMHC density, CD28 serves a critical role in amplifying specific elements of TCR signalling (138). The latter is a situation that is arguably more likely to occur *in vivo* with limited antigenic pMHC in the lymph node. Thus, while the TCR establishes the central network of signalling pathways, CD28 co-stimulation serves to amplify certain elements.

1.8 Co-receptors; the roles of CD8 and CD4

In addition to the TCR, MHC cI and cII also bind to their co-receptors CD8 and CD4, respectively. Consisting of a heterodimer, the CD8 contacts MHC cI through the α3 domain (139-141). In contrast, CD4 binds MHC cII as a monomer, although some data suggests dimers can form to ligate two pMHC complexes. CD4 contacts MHC cII through a single domain at β2 domain although some has suggested that the β1 domain may also have an influence on CD4 ligation (142). Despite the differences in composition and binding sites, both co-receptors are thought to perform an analogous role in TCR signalling leading to around 100 fold increase in TCR signalling in response to certain pMHCs (143). Initial studies focused on the increased affinity offered by these receptors to stabilise the relatively weak pMHC-TCR interactions. Direct evidence using cell lines has demonstrated that both co-receptors can bind their respective MHC molecules even in the absence of the TCR, thus aiding to cross linking the TCR. The other critical role for the co-receptors, is the localisation of activating Src family kinase Lck to proximity with the ITAMs of the CD3 complex (102, 144, 145). Evidence suggests that in the absence of CD4, phosphorylation of the ITAMs can still occur at a high degree of TCR occupancy or high affinity pMHC ligand (146). Only with a weaker affinity peptide or at low peptide concentrations does CD4 become vital in enhancing the TCR signal (147).

1.9 Peripheral T cells

1.9.1 Naive and memory T Cells

Antigen inexperienced or naive T cells are released into the periphery following successful positive and negative selection in the thymus. Indeed, less than 5% of cells that enter the thymus leave as single positive (sp) naive T cells (148). These recent thymic emigrants can be identified through the presence of TCR excision circles (TRECs), CD31 and the protein kinase 7 (PTK7) (149, 150). These cells are also defined through the high expression of CD62L and low CD44. Typically these cells re-circulate through the vasculature and secondary lymph nodes. Entry to the latter occurs through the high endothelial venules (HEVs) driven by the receptors CD62L and CCR7 (151). However, there is increasing evidence to support the notion that some naive T cells can migrate into specific, non-lymphoid tissues with phenotypically naive CD8+ T cells being found in the liver and naive CD4+ cells in the lung, liver and the thymic medulla (152). Whilst only consisting of 1.5% of T cells compared to the numbers found in the secondary lymphatics, these could still play an important role both in responding to pathogens and perhaps tissue specific tolerance or autoimmunity.

Of those cells produced during clonal expansion, around 90% will undergo apoptosis in the 1- to 2-week contraction phase of an adaptive response (153). Those residual cells remaining form the memory compartment to that particular antigen. Memory cells are predominantly quiescent but do undergo periods of intermittent proliferation. They are a heterogeneous population of cells defined as either central memory T cells (T_{CM}) or effector memory T cells (T_{EM}) (154-156). T_{EM} cells have a diverse array of homing receptors and are recirculated to nonlymphoid tissue sites. Subsequent exposure to cognate antigen leads to rapid cytokine production (e.g. IFNγ, IL-4, IL-17a). In contrast, T_{CM} cells produce IL-2 in response to TCR stimulation with delayed production of effector cytokines in comparison to T_{EM} T cells. Like naive cells, T_{CM} express high levels of CD62L and CCR7 suggesting a very similar migration pattern, entering the secondary lymph nodes via the HEVs.

Interestingly, the subset of Th that dominates in the primary effector response tends to form T_{EM} of the same phenotype. This is by no means exclusive with the secondary response also being influenced by the factors that modulate the differentiation of naive T cells, such as cytokines.

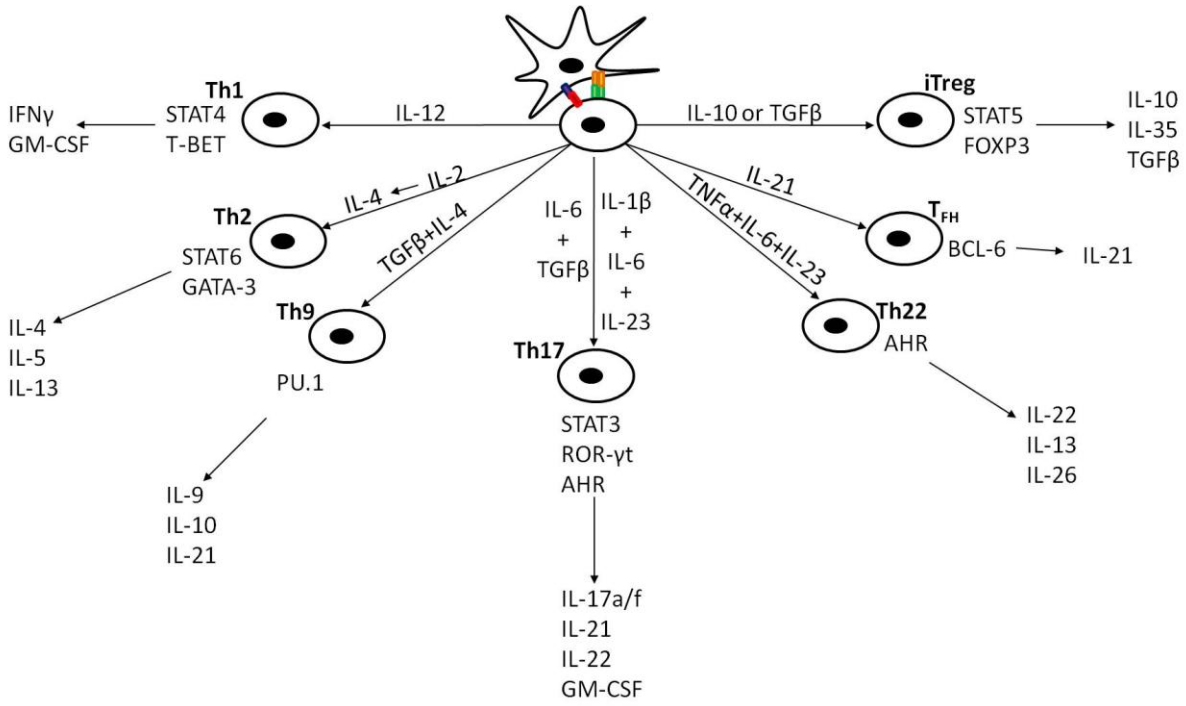

Figure 5 - CD4+ Th differentiation. Naive T cells can undergo differentiation into a number of subsets following activation. Induction can be influenced by particular cytokines that induce a range of critical transcription factors. Each subset is also defined through the production of certain cytokine profiles under the control of key transcription factors. However, some like GM-CSF or IL-22 are not exclusively released by a specific subset.

1.10 T cell effector functions

1.10.1 Th subsets

The critical role of Th cells is to direct various arms of the immune system to respond to a specific threat. Over the last 40 years this area of immunology has become increasingly well characterised (see **Figure 5**). Initial work began to characterise why some T cell clones mediated delayed type hypersensitivity (DTH) whereas a distinct subset provided help to B cells to produce antibodies (157). The pioneering studies by Mossman and Coffman began to characterise the underlying specialisation of these subsets (158). In the case of DTH, the responding subset,

termed Th type 1 (Th1), were defined through the production of IFNγ and played an important role in cell-mediated tissue damage. In contrast, type 2 Th responses (Th2) augment humoral responses dominated by IgE in allergic reactions, through the production of interleukin-4 (IL-4) (159). The underlying hypothesis has been further developed through our increasing knowledge of cytokines, transcriptional control and Th regulation leading to a further characterisation of an ever expanding number of Th subsets, such as Th9, Th17, Th22 and T_{FH}, each defined by production of particular cytokines (IL-9, IL-17 and IL-22, respectively) and associated with a particular immunological niche (see **Figure 5**). In addition, it has been widely recognised that a subset of T cells can suppress a pro-inflammatory response in an antigen-specific manner (160). These T cells can be defined as either natural or inducible regulatory Th cells (nTregs or iTregs respectively). Through both soluble (e.g. TGFβ or IL-10) and cell-contact (CTLA-4,CD39/72, LAG-3 etc) dependent mechanisms these subsets form an important arm of peripheral tolerance (161-163). However, the primary focus of this project will be on the pro-inflammatory Th1, Th2 and Th17 subsets.

1.11 Th1 - Cell mediated immunity

Since being defined, Th1 cells have been associated with cell-mediated immunity (164). The phenomena described by Mossman, Coffman and Bottomly still remains, with these cells being defined through the production of IFNγ and the expression of the transcription factor T-bet, encoded by the *TBX21* gene (158, 165, 166). In humans, these cells are vital in combating intracellular infections such as *Mycobacterium tuberulosis, Listeria monocytogenes* and *Leishmania major* (167-169). Although IFNγ is used to define these cells, they also produce IL-2, IL-3, TNFβ and are a source, although not exclusively, of granulocyte macrophage colony stimulating factor (GM-CSF; also known as CSF-2) (170). IFNγ is particularly important in driving clearance of intracellular pathogens through increased anti-microbial activities; increased lysosome fusion, production of oxygen free radicals and nitric oxide (NO). Th1 cells can also aid production of antibodies, in particular promoting the switching to IgG2a isotype (171).

The key to induction of the Th1 phenotype in naive murine T cells is the presence of IL-12 during activation derived from macrophages, monocytes or DCs (172). This cytokine acts through the IL-12R via Signal Transducers and Activators of Transcription-4 (STAT4) to upregulate T-bet and induce IFNγ (173). The latter forms a positive feedback through IFNγR that leads to STAT1-mediated T-bet expression (174). IL-18 is another cytokine that can serve as a co-factor for IL-12 to bring about Th1 induction (175). T-bet$^{-/-}$ mice lack Th1 cells (176). However, they can still produce some IFNγ due to the effects of Eomesodermin (Eomes), another Tbox protein, which is responsible for IFNγ release by CD8 T cells and is upregulated in Th1 cells (177, 178). Runx3 is also expressed in Th1 cells enhancing the phenotype whilst also impairing the Th2 transcriptional programme (179).

A dysregulated Th1 response targeted against self was thought to play a critical role in inflammatory autoimmune diseases such as RA and multiple sclerosis (MS) (180). However, this has since been complicated by the discovery of the Th17 subset (see Section **1.12.1.13**). A lack of Th1 cells results in a dysregulated Th2 response, with T-bet 'knockouts' developing asthma with a severe eosinophil infiltrate (176). Similarly, it has been shown that polymorphisms affecting the *TBX21* gene results in an analogous phenotype due to a loss of Th1 cells and a failure to constrain the Th2 development in these patients (170).

1.12 Th2 - Not just humoral immunity

Th2 cells were the other subset to be defined by Mossman and Coffman and are defined through their production of IL-4, IL-5, IL-9, IL-13 and IL-25 (181). Th2 cells are vital in the host response to helminth infections. These can also be classified by the expression of the chemokine receptors CCR3, CCR4 and CCR8 that are typically associated with mucosal surfaces (182).

The archetypal aspect of a Th2 response is the class switch of B cells to produce IgE that is capable of activating a range of innate cells such as basophils and mast cells (183). The binding of IgE to the high affinity Fc receptor, FcεR1, allows cross-linking of these receptors leading to degranulation of these cells (184), thus permitting the

release of various cytokines, chemokines, histamine, heparin, serotonin and proteases. These mediate changes in smooth muscle contraction, increased vascular permeability and inflammatory cell recruitment. Th2 cells can also promote an influx of eosinophils (through IL-5) and mast cells (through IL-9), leading to tissue eosinophilia and mast cell hyperplasia, respectively (185). Other cytokines such as IL-4, IL-9 and IL-13, can act on epithelial cells to induce mucin production at mucosal sites. Also IL-4 and IL-13 can exert effects on smooth muscle cells. Some evidence points to Th2 cells inducing production of amphiregulin that induces epithelial cell proliferation and IL-24 release; important in antitumour responses. The importance of Th2 in the clearance of helminths is demonstrated through a number of knockout animals lacking a range of Th2 cytokines including IL-4, IL-5, IL-25 and amphiregulin. However, a dysregulated Th2 response against inappropriate inert antigens is a prevalent feature of allergic disease. Goblet cell hyperplasia, mast cell degranulation and influx of eosinophils into the airways are classical features of allergic and asthmatic conditions (186).

The studies by Paul *et al* in the early 90s demonstrated that Th2 cells could be induced from naive T cells in presence of IL-4 together with TCR activation (187). The presence of IL-4 is thought to be critical in driving the induction of GATA3 via STAT6 (188). GATA3 has been shown to bind to at least two non-coding regions of the *IL4* and *IL13* loci. Moreover, Th2 cells express Growth Factor Independent-1 (GFI-1), an early IL-4 inducible gene, and antagonistic to Th1 development (189). Once established GATA3 is capable of binding to the *IL4* locus therefore sustaining a feed forward loop. However, IL-4 alone is not capable of Th2 induction with IL-2 also playing a role through signalling via STAT5. Neutralisation of IL-2 results in early IL-4 production but stable Th2 cells fail to develop. Moreover, STAT5 can bind to locations in the *IL4* gene as well as acting to upregulate the expression of the transcription factor c-Maf (190). Highlighting the role of IL-2 is the severe Th2 deficiency in STAT5a knockout mice (191). It is thought that two phases govern Th2 induction. The initial stage is IL-4-independent with TCR stimulation triggering a modest but sufficient increase in GATA3 activation (187). The parallel induction of

IL-2 leads to a signal through STAT5, which, if of sufficient magnitude, promotes enough IL-4 to initiate positive feedback through IL-4-signalling, leading to sustained Th2 development.

1.12.1.1 Th17

Alongside the emergence of Treg cells, the characterisation of Th17 cells proved that the exclusive Th1/Th2 paradigm was unable to account for all observed Th phenomena (192). Data reviewing autoimmune models showed how neutralisation of IL-12 using monoclonal antibodies targeting the p40 subunit failed to account for the fact that that this subunit was shared with IL-23 (193). Thus, much interest arose in how IL-23 could alter the immune response and the associated T cell response. In 2005, two independent reports in Nature Immunology by Park *et al* and Harrington *et al* described a population of Th cells releasing IL-17 (194, 195). Like other subsets, development of these cells was dependent on TCR signalling, co-stimulation and a specific cytokine milieu during activation. These groups showed that Th17 cells were distinct from Th1 cells and arose from naive T cell populations. These studies provided the basis for further characterisation of Th17 cells and their importance in a number of contexts in both infection and autoimmunity.

1.12.1.1.1 Th17 cells in infection

The potential for Th17 cells to induce profound inflammation makes their role in infection a careful balancing act. Capable of inducing recruitment of innate cells such as neutrophils and inflammatory DCs (inDCs), Th17 cells can also activate tissue resident fibroblasts to perpetuate inflammatory cytokine and antimicrobial peptide production (196, 197). Deficiency in Th17 cells due to a loss of STAT3, as occurs in hyper IgE syndrome (also termed Job's Syndrome), renders patients especially susceptible to fungal infections (198-200). The archetypal pathogen associated with Th17 immunity is *Candida albicans*, a commensal organism of the oral cavity and gastrointestinal tract. Pathology normally only arises in the context of immunodeficiency (201). Furthermore, patients with chronic mucocutaneous candidiasis (CMC) have defects in either the generation of Th17 cells or in Th17 effector mechanisms (202, 203). In respiratory tract infections Th17 cells provide

defence against *Pneumocystis carinii* and *Aspergillus fumigatus* through IL-17/23 functions (204). IL-23 knockout mice fail to fully clear these infections in the airways. However, excess Th17-mediated inflammation can drive excessive neutrophil influx and tissue damage. For example, in a gastric model of *C. albicans,* the stimulation of Th17 immunity drives severe intestinal inflammation and immunopathology (205).

It is not exclusively fungal immunity where Th17 cells play an important role. Defence against extracellular bacteria is another important aspect (204). Hyper IgE patients also suffer from susceptibility to *Staphylococcus aureus, Streptococcus pyogenes* and *Klebsiella pneumoniae* which is reflected in models in which IL-$17^{-/-}$ animals can suffer lethal infections (199). The addition of IL-17 to IL-$23^{-/-}$mice was capable of restoring host defence and clearance of the pathogen. IL-22, a Th17 cell-associated cytokine, appears to play a more important role in mucosal defence helping to maintain the integrity of the trans-epithelial barrier and so reduce the crossover of bacterial pathogens from the lumen (206). Moreover, Th17 cells are potent drivers of antimicrobial peptide production, such as Lipocanin-2 (LP-2), β-defensin-2 and S100 proteins, that help to supplement the mucosal defence (207). Together these data highlight the importance of Th17 cells as an activator of the innate immune system to provide protection. However, given the potency of the Th17 response to bring about polymorphonuclear cell influx and sustained inflammation, an excessive response can be damaging to the host. Whooping Cough is caused by a Gram-negative bacterium, *Bordetella pertussis* (208). Some evidence points to a biasing of the response towards a Th17 phenotype through abrogation of IL-12 and promotion of IL-23 by APCs. The release of Th17 effector cytokines can result in severe respiratory pathology including bronchiectasis and a persistent cough. Furthermore, Th17 cells are implicated in the pathology of cystic fibrosis (CF) patients. Defects in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a chloride channel, lead to thickening of the mucus and chronic biofilm formation by bacteria such as *Pseudomonas aeruginosa* (209). The persistent exposure to bacteria leads to heightened IL-17 and IL-22 levels in the

draining lymph nodes, which may lead to further damage to the lung and airways (209, 210).

1.12.1.1.2 Th17 cells in tumour immunity

Inflammation has been known for over a century to be associated with the tumour microenvironment, leading to tumour invasion, migration and metastasis (211). However, inflammatory reactions are also important for initiating and maintaining the antitumor response. Tumour immunity is typically thought to be negatively regulated by Tregs, that promote the tumour growth, as opposed to Th1 cells that are thought to be critical in the activation of CTLs. Investigation of Th17 function has revealed their presence in a number of tumours, including but not limited to ovarian and prostate tumours (212). Alongside IL-17, tumour- associated Th17 cells are a source of GM-CSF and TNFα which can help promote tumour regression (212). They also appear to aid the recruitment of Th1 cells to the tumour through production of chemokines. Numerous studies in mouse models have shown that transgenic Th17 cells are effective in tumour eradication. In contrast, ablation of IL-17 can yield greater tumour mass, accelerated growth and migration to tissues such as the lung (213). In humans, immunotherapies such as blocking of Indolamine 2-3-Dioxygenase (IDO) or vaccination with Heat Shock Protein-70 (HSP70) lead to enhanced Th17 function alongside anti- tumour immunity (214). It has been shown in prostate cancer patients that there is an inverse correlation between Th17 numbers and tumour progression (215). Treatment targeted against CTLA4 yields increased Th17 activity in melanoma patients, whilst IL-17 staining *in situ* was found to be predictive of patient survival (216, 217). All these data highlight the role of Th17 cells in protection against tumour development and the antagonistic relationship between Th17 and Treg cells.

1.12.1.1.3 Th17 cells in autoimmunity

Initial studies supported the concept that Th1 cells were the primary driver of autoimmune disease, with the Th2 phenotype being protective as described by the Th1/Th2 paradigm. Emerging data showed that antibodies targeting IL-12 were effective in suppressing a number of models of autoimmunity including Collagen

Induced Arthritis (CIA) and Experimental Autoimmune Encephalomyelitis (EAE) (218). However, the first antibodies targeted the p40 subunit of IL-12 which is shared with IL-23 (219). The growing interest in IL-23 lead to a revaluation of a number of autoimmune diseases and the role of the newly emerging Th17 subset giving the importance of IL-23 to the immunobiology of these cells. Further studies demonstrated that $p19^{-/-}$ (the specific subunit of IL-23) mice failed to develop CIA whilst $p35^{-/-}$ (specific for IL-12) mice were still susceptible (220). Similarly, despite being cloned in 1995, the pro-inflammatory role for IL-17 was not established until studies showed it was capable of inducing IL-6 production from rheumatoid synoviocytes and could synergise with TNFα to bring about joint inflammation (221). Further studies demonstrated elevated IL-17 levels in supernatants from RA patients compared to osteoarthritis (OA) controls (222). The levels of IL-17 correlated with disease progression (223). Moreover, prior to the identification of Th17 as an independent lineage, IL-17+CD3+ T cells were found to be present in the RA synovium (224). Epigenetic evidence also shows a correlation between autoimmune disease and a number of micro-RNAs (miRNAs). These short sequences of RNA, typically 21-24 bases in length, are capable of negatively regulation transcription of a number of target genes depending binding to target mRNAs (225). A number of miRNAs linked to the Th17 phenotype have been detected in the joint including miR-146a, miR-326, miR-155, miR-133b and miR-206 (226).

1.12.1.1.4 Therapeutic targeting of Th17 cells

The growing knowledge of Th17 cells and their importance to a number of autoimmune conditions has led to interest in therapeutics that can impinge of their development and function. A number of pharmaceutical companies have began to develop biological therapies against Th17 targets (227). The list of conditions that these are being trialled in ranges from RA, psoriasis, psoriatic arthritis, ankylosing spondylitis, MS, asthma, autoimmune uveitis and Crohn's disease. Trials in RA have demonstrated an ability of anti-IL-1R-antagonists to reduce Th17 numbers in patients (228). The most advanced trials to date are in Phase III for psoriasis

targeting IL-23 and IL-17, with a high degree of efficacy and a good safety profile (227). Importantly, these have comparable or better effects than the current biological therapies ustekinumab (targeting p40 subunit of IL-23 and IL-12) and etanercept (targeting TNF) and have achieved efficacy in 70-80% of patients with at least a 75% reduction in disease activity (229). Another positive development comes from patients with Crohn's disease given an anti-IL-23 mAb. Encouraging data demonstrated that in those patients with elevated C-Reactive Protein (CRP), colonic inflammation and had failed to respond to anti-TNF, showed a positive response to therapy reducing disease burden (227). However, the results from two trials of the same antibody in irritable bowel disease (IBD) showed a lack of efficacy and in some patients increased fungal infections with pathology actually increased (230). Thus, reflecting the complexity of each condition and the role of Th17 cells in each respective context. There is a need for further understanding of the role of these cells in both the maintenance of health and in immune pathology.

1.13 Th17 effector cytokines

IL-17a is the prototypical cytokine used to define the Th17 subset. The IL-17R is most highly expressed by haematopoietic cells (231). Binding of IL-17a leads to signals transduction mainly by ACT1 and TNF-associated factor (TRAF) proteins (see **Figure 6** (227) (232)). The net effect of activating these pathways is the transcription of a number of pro-inflammatory cytokines including IL-6 and Granulocyte-colony stimulating factor (G-CSF (233)). Other target genes include the chemokines CXCL1, -2 and -5, all of which serve to promote the influx of neutrophils to the tissue. Additionally, IL-17 signalling supports the production of a range of antimicrobial peptides such as the S100s, β-defensins and Lipocanins that constrain bacterial growth and are critical for defence at barrier organs (234).

Microarrays using IL-6-stimulated cells showed that IL-21 was one of the most potently induced genes in Th17 cells (235). Ligation of the IL-21R leads to STAT3 signalling inducing both IL-23R and ROR-γT expression (235). Loss of IL-21R can severely abrogate IL-17 release by naive T cells (50-70% reduction) which also lack

IL-23R (236). The precise role of IL-21 *in vivo* disease models remains to be clarified with contradictory results as to its role in EAE. In humans, Kotlarz *et al* identified patients lacking IL-21R with a primary immunodeficiency syndrome characterised by cryptosporidial infections with a lack of class switching and impaired Th17 cytokine production following *ex-vivo* re-stimulation (237).

Th17 cells are also a potent source of IL-22 and GM-CSF. IL-22 serves a vital role in maintaining the integrity of the epithelial barrier. It can also act in synergy with other cytokines such as IL-17a and TNFα to induce expression of antimicrobial peptides including S100A7-9, β-defensin-2/3 and mucins (234). In contrast, GM-CSF is vital to the recruitment, activation and maturation of myeloid cells to inflamed tissues (238). Murine studies support the role of ROR-γT as being a potent driver of GM-CSF expression, linking it to the Th17 a phenotype (239). Data from these studies suggest that it was GM-CSF alone that was capable of inducing pathology in the EAE model (240). It is also able to indirectly support Th17 development through the induction of DC to release IL-6 and IL-23 (241). Thus, whilst not limited to Th17 cells, GM-CSF is a critical link to the myeloid arm of the immune system.

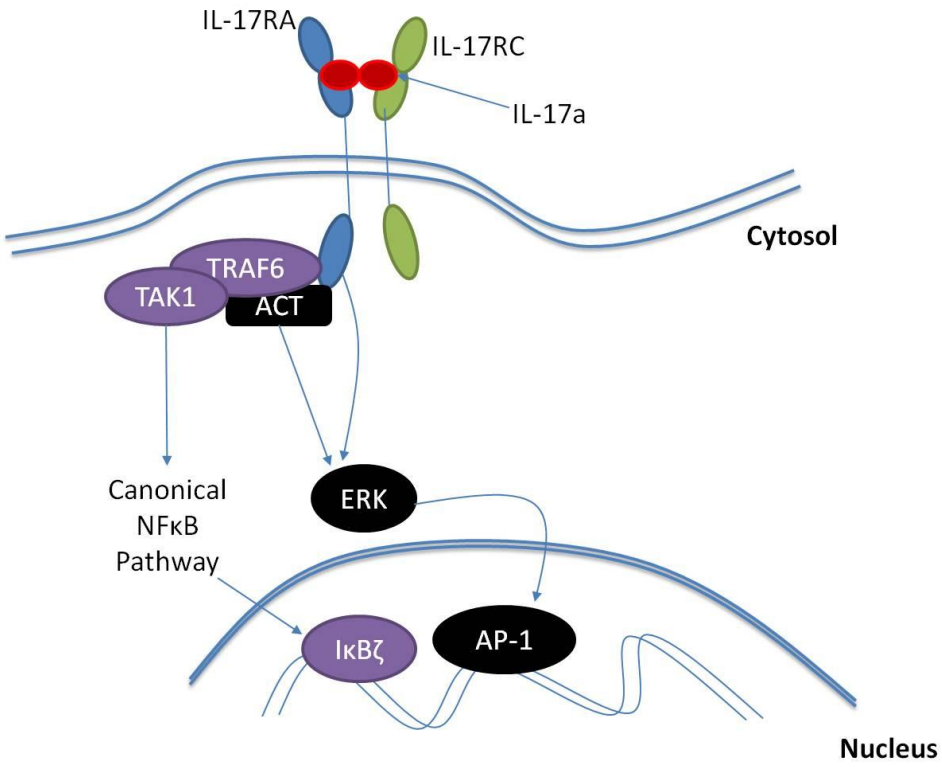

Figure 6 - IL-17a signalling pathway. The IL-17 receptor is composed of a heterodimer of IL-17RA and IL-17RC. Both of these possess the critical SEF/IL-17R and SEFEX domains that are required for signalling. ACT1 in turn activates TRAF6 via ubiquitination, which culminates in the activation of the MAPK pathway and the canonical NFκB pathway. IL-17 also activates the deubiquinase A20 that targets TRAF6 in a form of negative feedback.

1.14 Transcriptional control of the Th17 phenotype

Our current knowledge of Th17 cells is mainly derived from *in vitro*-induced populations derived from naive precursors. However, it is through these studies that we have gained some understanding of the complexity of the transcriptional networks that control the Th17 phenotype (227). One of the criteria used to define a Th subset is the presence of an exclusive 'master' transcriptional regulator. Yet these do not function alone but as part of a complex circuitry of transcription factors that serve to manage the expression of a number of genes that together define the observed phenotype (242). STATs typically function as homodimers, but can also form heterodimers with other STATs (243). Due to the potency of the cytokine environment in driving Th cell differentiation, the activity of particular STATs is intrinsically linked to a certain Th cell phenotype. For instance, STAT4 is needed for Th1 cell- and STAT6 is required for Th2 cell-development (243). In the

case of STAT4, it has been shown to positively regulate a number of Th1 genes such as IFNγ and T-bet. Moreover, it has been shown that STAT4 can also act as a transcriptional repressor for around 40 genes, with several STAT4-dependent histone repression marks in numerous Th2 genes (243). Indeed, STATs are responsible for transcription of the majority of genes within each subset**.** For example, STAT6 in Th2 cells is responsible for over 80% of genes induced (244).

In the case of Th17 cells, STAT3 is an absolute requirement for full differentiation (245). The loss of STAT3 is the cause of hyper IgE syndrome, affecting IL-17 and IL-21 expression (200). The defective Th17 response in these patients renders them susceptible to a range of pathogens, including fungi and extracellular bacteria. The converse is true in the case of a loss of the negative regulator of STAT3, Suppressor of cytokine signalling-3 (Socs3). $Soc3^{-/-}$ mice show enhanced Th17 numbers and an increased severity of EAE (246). Socs3 expression can be stimulated by IL-21 and IL-6 as a form of negative feedback, whereas TGFβ inhibits Socs3 and so can potentiate a STAT3 signal (247). By combining chromatin immunoprecipitation (CHIP) with large scale parallel DNA sequencing (seq), CHIP-seq has shown that STAT3 can bind the IL-17 promoter at multiple sites, including at conserved non-coding sequences (NCS) (248). Moreover, STAT3- binding sites have been found in other Th17 genes such as *aryl hydrocarbon receptor (Ahr), Il21, Rorγ, Batf, Irf4* and *Maf (249).* Other targets important for the stabilisation of the Th17 transcriptional program, i.e. *Il23R* and *Il6R,* are also direct targets of STAT3. It can also regulate the expression of genes not exclusive to Th17 cells, which are thought to play a role in survival and proliferation of cells (e.g. *Bcl2, Fos* and *Jun*). This correlates with the delayed kinetics and poor clonal expansion of $Stat3^{-/-}$ T cells.

Whilst not exclusive to Th17 lineage, other 'pioneer' factors have been demonstrated to be indispensible for Th17 differentiation. Basic leucine zipper transcription factor (BATF), c-Maf and Interferon Regulatory Factor-4 (IRF4) have been described to have these functions (250, 251). CHIP-seq data of IRF4 and BATF share a high number of targets in Th17 cells (252). It is thought that these serve to

permit access to these Th17-specific genes for STAT3 and ROR-γT via chromatin remodelling. Indeed, these can be induced by TCR signalling and can be found in non-polarised T cells indicating these are not aligned with a specific subset and fulfil this role of opening up genes for modifications by subset specific factors. Loss of these can severely abrogate Th17 development, although loss of IRF4 has also been shown to prevent Th2 development in the different priming settings (253).

AHR is a ligand dependent transcription factor that was first identified as the receptor for dioxin (254). Expression is found in both Th17 and Treg cells, although levels are far higher in the former. Loss of AHR was shown to lead to a reduction but not absence of Th17 cells, and attenuated EAE. Subsequent studies elucidated that Iscove's Modified Dulbecco's Media (IMDM) supported enhanced Th17 development in comparison to Roswell Park Memorial Institute-1640 (RPMI-1640) media due greater AHR activation (255). The mechanism through which AHR supports Th17 development remains to be fully characterised but it is thought to be partly dependent on AHR interfering with STAT1, a negative regulator of Th17 development (256). Others have pointed to a role of AHR in stimulating IL-22 production, of particular importance to Th22 cells (257).

Of those targets of STAT3, ROR-γT has been described as the master regulator of the Th17 phenotype (258). Overexpression of STAT3 in *Rorγ*$^{-/-}$ mice failed to produce IL-17 (259). However, the converse, with an overexpression of ROR-γT, leads to IL-17 release even on a *Stat3*$^{-/-}$ background. Encoded by a single gene, *Rorγ*, two isoforms are produced by alternative splicing (260). ROR-γT is found mainly in Th17 cells and in the thymus, whereas ROR-γ1 is more widely expressed such as muscle and kidney cells (261). Multiple binding sites for ROR-γT can be found in the *Il17a*, *Il17f* and *Il23r* genes (258).

1.15 TCR Signal Strength

The mitogenic effects of anti-CD3 antibodies have been known for over 30 years (262). Early studies in human T cells indicated that the anti-CD3 mAb OTK3 could lead to enhanced T-cell proliferation as measured by the incorporation of tritiated

thymidine (263). Follow up experiments indicated that a range of intracellular pathways could be triggered. However, the affinity of TCR-pMHC interactions is many orders of magnitude less than that of antibody-antigen binding (264). Further studies, since the advent of T cell cloning and TCR transgenic mice, have allowed assessment of both the qualitative and quantitative aspects of TCR signalling and the effects of signal 1 on Th cell differentiation using a defined TCR (see **Figure 7**) (159). It has become an area of increasing importance to our understanding of Th cell immunobiology.

1.15.1 Qualitative changes in TCR signal strength - Altered Peptide Ligands (APLs)

In order to achieve a sufficient degree of interaction leading to signalling, the TCR contacts the peptide by a relative few critical residues. These can be identified and the hierarchy of their importance determined by stepwise alterations to these points. Pioneering studies led by Paul Allen defined Altered Peptide Ligands (APLs) as analogues of immunogenic peptides in which substitutions of critical contact residues lead to alterations in signalling cascades (62). Established using a Th1 clone against a haemoglobin peptide, the APL produced shifts in the phosphorylation status of CD3ζ and a failure to activate ZAP-70 in comparison to the cognate peptide (265). Other studies have changed the affinity for MHC cII binding through single amino acid substitutions in the peptide sequence (266). As a result of either enhancing or decreasing MHC cII affinity, differential cytokine responses were induced (159).

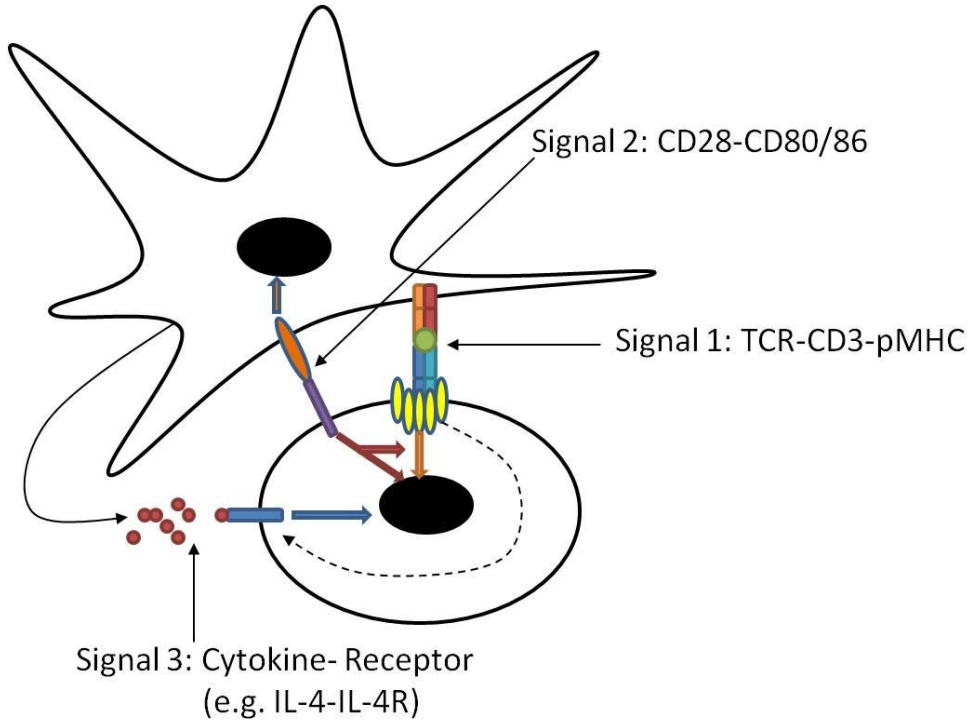

Figure 7 - Three signal model of T cell activation. In order to become fully activated naive T cells require three signals. (1) TCR-mediated recognition of a peptide ligand presented by MHC cII. Signalling induces cytokine release, cytoskeletal rearrangements and can influence the expression of cytokine receptors such as IL-12R. (2) Co-stimulation signals are provided by binding of CD80/86 to CD28. These in turn modulate TCR signalling, particularly important when TCR is weakly activated or negatively regulated by the expression of CTLA-4 on T cells. Lack of co-stimulation can result in a failure to proliferate or gain effector functions, termed anergy. (3) The cytokine milieu present during activation. The type of cytokines released by an APC are in part dependent on which PRRs are triggered. Sensitivity of the responding T cell is suggested to be in part dependent on Signal 1.

The functional outcomes of such alterations, when compared to the cognate peptide, can lead to agonism, partial-agonism or antagonism, affecting all aspects of the T cell response including proliferation and cytokine expression (267). For example, the APL to a Moth Cytochrome-c (MCC) peptide, K99R, has a lower affinity for the B10.A(5R) TCR leading to suppressed Th1 development compared to the cognate peptide. Addition of IL-2 to these cultures could not restore IFNγ production to equivalent levels as induced by the cognate peptide. In contrast, reduction of the cognate peptide to its core 9-mer caused no changes to the cytokine profile induced but did reduce proliferation. Similar effects can be found in systems using Human Collagen Type IV (HuCIV) and Myelin Basic Protein (MBP)

immunogenic peptide and their corresponding analogues (268, 269). This is not limited to the Th1/Th2 paradigm with APLs to Glucose-6-Phosphate Isomerase (GPI) $hGPI_{325-339}$ peptide reducing arthritis severity through a reduction in IL-17 release (270).

The role of affinity in governing tolerance is an area of growing interest with regards to Treg function (271). In the context of the EAE model, an agonistic APL is capable of generating IL-10- producing $FOXP3^-$ Tregs (Type 1 regulatory T cells (Tr1)) from naive precursors (272). Studies in the Allison lab have shown that a high affinity APL to the MCC peptide induces the greatest frequency of FOXP3-expressing Tregs both *in vitro* and *in vivo* (271).

1.15.2 Quantitative changes to TCR signal strength - antigen density

T cells will continuously 'scan' an APC upon interaction. Each APC can have multiple antigen-loaded pMHC complexes able to bind to the TCR and trigger signalling (273). Thus, the number of available contacts will also alter the level of downstream signalling in a cumulative manner. The duration of contact can also affect the type of signal received by a certain T cell. Studies using CD8+ T cells and peptide-pulsed DCs indicated that there is an inverse relationship between the peptide density and the time needed to form stable contacts with an APC (274). Only those peptide densities that induce stable contacts led to T cell division. The margin can be as small as two-fold between quiescence and expansion (275). Interestingly, *in vivo* studies in which the antigen is removed from the system have a significant effect on CD4+ T cell proliferation and effector function. In contrast, CD8+ T cells, if antigen is removed during the immediate phases of signalling, are much more resilient in terms of maintaining effector function.

The ability of differing amounts of antigen to shift the T cell response was initially pioneered by the work of Parish and Liew, who demonstrated that immunisation of rats with the bacterial protein, flagellin, induced variable responses depending on the antigen dose (157). If very low or very high doses were administered the animals showed strong DTH responses mediated by Th1 cells. A mid-range dose

generated a robust humoral response with Th2 cells providing help to B cells against flagellin. Studies using the protozoa *Leishmania* or the helminth *Trichuris muris* again showed distinct Th1/ Th2 responses depending on the initial pathogen burden (276, 277). Low numbers of protozoa resulted in a Th1 response that gradually shifted to a predominantly Th2 response with higher numbers of infecting organisms and, assumingly, greater densities of antigen material. Others have shown that immunisation with a form of polymerised ovalbumin (OVA) protein produced the opposite trend *in vivo* (278). Thus, while all these studies demonstrate that antigen density can shift the phenotype of the immune response *in vivo,* it was difficult to control the exact density of antigen. The studies by Hoskens *et al* and Constant *et al*, published in the Journal of Experimental Medicine back-to-back, found that using TCR transgenic naive T cells to specific synthetic peptides allowed for a more controlled *in vitro* system (279, 280). The Bottomly lab demonstrated that, generally, low doses of peptide favours a Th2-dominated response whereas the O'Garra group found strong Th2 response at either very low or very high peptide concentrations. In contrast, mid-range doses supported the generation of the Th1 phenotype. However, differences in timing, antigen, TCR affinity and APC type result in variations to the exact antigen does range that makes direct comparison between these studies challenging. *In vivo*, Milner *et al* used tetramers to remove high affinity clones to MCC peptide (I-E^k restricted) which lead to expansion of Th2 cells in an IL-4-dependent manner. It has also been suggested that sustained activation of ERK at higher antigen densities inhibits GATA3, hence blocking the endogenous release of IL-4 (281).

The role of antigen density has also been assessed in other subsets including Tregs. Emerging data has shown that changes in the intensity of TCR signalling could alter FOXP3 induction. The mechanism is dependent on reduced Akt and mammalian target of rapamycin (mTOR) activity that antagonise the development of a regulatory phenotype (271). This observation has been further clarified *in vivo* where a lower of dose of immunising peptide generated the greatest induction of

iTregs. However, others have shown that nTregs require a higher than average intensity of signalling during thymic development than other effector cells (282).

With regards to Th17 cells, studies by Purvis *et al* in our group have demonstrated that T cell stimulation strength is clearly a factor in Th17 regulation (283). Through alterations to the ratio of polyclonal activating anti-CD3/CD28 coated beads to T cells, it was found that a lower ratio favours a sustained Th17 phenotype. These results can be replicated through differential loading of APCs with higher or lower concentrations of superantigen (283). The mechanism that drives this effect is the dependency of IL-17 production on lower Ca^{2+} concentrations. This in turn favours NFAT1c binding to the *IL-17* promoter. If excessive Ca^{2+} is introduced using an ionophore, the propensity of IL-17 production is lost. Hence, there is clear data indicating a role of TCR signal strength affecting the development of all Th subsets. Work is ongoing to dissect the relative importance of particular pathways and how these interact with cytokine-induced signals to shape the phenotypic response of a particular Th cell.

1.15.3 Relative influence of TCR signal strength and the cytokine milieu in shaping T cell responses

Another area of interest is the distinct influences of cumulative TCR signal strength and exogenous cytokines in the shaping of the Th effector cell response. van Panhuys *et al* have varied these factors independently by changing either the antigen density or adjuvant used during DC maturation (284). Comparison of Th1/2 differentiation demonstrated two key stages with T cells first influenced by TCR signal strength, which is also modulated through co-stimulatory receptors such as CD28. Downstream of these are the effects of the cytokine environment in which T cell priming occurs. The link between these stages is the ability of TCR signalling to control the expression of the receptors required by naive T cells to respond to the presence of certain cytokines during their activation. For example, these studies indicated that Th1 development requires a strong TCR signal in part to induce the expression of the IL-12R β2-chain, permitting an increased response to IL-12, leading to IFNγ release and T-bet expression. Thus, modulation of the initial signals

received by the TCR can influence the response of T cells to cytokine cues, with implications not only for vaccine design but also for treatment of autoimmune disease.

1.16 Signal 3: the role of cytokines in Th differentiation

Typically the cytokine environment present during priming is thought of as the critical determinant in shaping the type of T cell response. The main factor governing the type of cytokines released by APCs are the particular Pathogen Associated Molecular Patterns (PAMPs) that bind and stimulate PRR signalling (10). In the case of IL-12, Th1 cells will dominate, whereas IL-4 induces a Th2 response. In the case of iTregs, IL-10 or TGFβ alone are sufficient.

1.16.1 The role of cytokines in Th17 development

Early work in 2003 and 2005 demonstrated that IL-23 was able to expand and promote the development of a pathogenic subset of Th cells (195, 285). IL-23 is a potent activator of STAT3 and can induce the expression of ROR-γT, IL-17 and IL-23R among other key Th17 targets (286). However, IL-23 fails to induce Th17 cells from naive T cells that lack the IL-23R. Subsequent work by the labs of Kuchroo, Stockinger and Weaver showed that a combination of IL-6 and TGFβ was required for Th17 induction (287-289). IL-6 has since been shown to be an absolute requirement for Th17 cell development (290). Loss of IL-6 renders such animals unable to generate a Th17 response (235). It functions through a receptor consisting of a specific component IL-6R and a ubiquitous subunit gp130 leading to STAT3 activation (see **Figure 8** (291)). STAT3 phosphorylation in turn leads to expression of ROR-γT transcription factor that is key for the Th17 phenotype (249). Other key target genes of STAT3, such as IL-23R and IL-17a, are also induced by IL-6.

Emerging data suggests that TGFβ functions indirectly to support the development of Th17 cells, being a potent inhibitor of both T-bet and GATA3 (see **Figure 8** (288)). Loss of either the TGFβRII chain or T cell specific TGFβ impairs *in vivo* Th17 cell responses (292). It has been shown that TGFβ leads to preferential upregulation of anti-apoptotic factors such as clusterin and BCL2 in Th17 cells (227). However, TGFβ

can also limit the pathology of Th17 in murine disease models such as EAE in an IL-10-dependent manner (293). Thus, while an important factor in the initiation of Th17 responses, TGFβ can also ameliorate the inflammatory response induced.

IL-1β also plays an important role in the initiation of the Th17 development. Like IL-$6^{-/-}$ mice, loss of IL-1β prevents induction antigen-specific Th17 cells and hence disease in models such as EAE (294). IL-1R signals via MyD88 leading to PKCθ, NFκB and MAPK activation (295). Downstream signalling is important for the promotion of the transcription factor IRF4 that in needed to co-operate with and enhance the function of ROR-γT. IL-1β has an additional role in ensuring the T cell is equipped metabolically for activation through its ability to stimulate the phosphorylation of the mTOR1 (296).

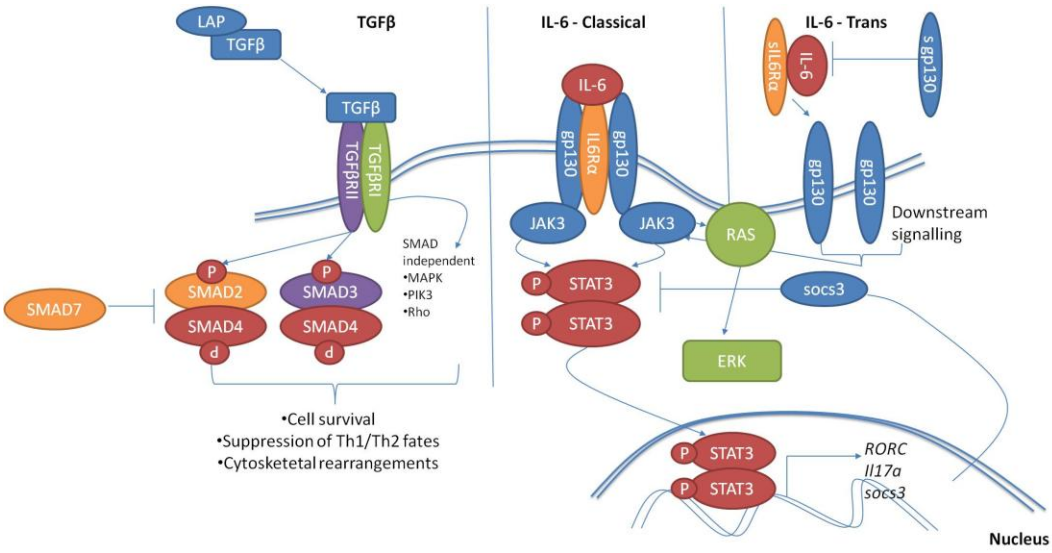

Figure 8 - Key cytokines in Th17 differentiation. TGFβ first requires activation by the cleavage of Latent Activating Peptide (LAP) that then permits its binding to the heterodimeric TGFβR. Signalling via Alk5 leads to phosphorylation of SMAD proteins that influence cell survival, suppression of other Th cell fates and cytoskeletal changes. SMAD7 acts as a negative regulator of signalling. The net effects of TGFβ signalling include reducing IL-2/ IFNγ production, Inducible tyrosine kinase (Itk) activation and reducing TCR Calcium flux. TGFβ alone also promotes FOXP3 yet IL-6 antagonises this. IL-6 signalling can function either via direct binding to IL-6R which is associated with the shared gp130 chain. In contrast the trans pathway involves IL-6 bound to a soluble form of its receptor that lacks a cytoplasmic domain. This in turns binds gp130 located on the cell membrane. A soluble form of gp130 can antagonise the trans pathway by 'mopping' up these complexes. Both lead to JAK3 activation of STAT3 that can translocate to the nucleus. STAT3 promotes a number of Th17 genes including *Rorγ, Il17a* and *Il23r.* Socs3 is a negative regulator of STAT3 activation. Ras and the MAPK pathway can

also be triggered by JAK3. Thus, the classical IL-6 signalling pathway is restricted to those cells that express IL-6R (e.g. immunocompetent cells and hepatocytes). Because of the ubiquitous expression of gp130, sIL-6R allows a wide range of cells to respond including RASFs (Rheumatoid Arthritis Synovial Fibroblasts), vascular endothelial cells and osteoclasts.

1.17 Negative regulation of Th17 cells

The potency of IL-17 and other cytokines produced by Th17 cells means that a dysregulated response is a potent threat to the host organism. As with Th1 and Th2 cells, the development of Th17 phenotype is susceptible to reciprocal counter regulation by other subsets. For instance, IFNγ and IL-12 both impair the development of Th17 in part through the action of T-bet, which prevents ROR-γT expression through binding 2kb upstream of the *Rorγ* first exon (297). Ectopic expression of T-bet in naive or committed Th17 cells was capable of inhibiting release of IL-17. Conversely, genetic ablation of T-bet in mice leads to an increased number of Th17 cells (298). Interestingly, although these mice have more Th17 cells infiltrating the CNS when used in the EAE model, the severity of disease is not drastically enhanced most likely due to the need for these cells to co-express T-bet, and hence IFNγ, required for pathogenesis (299). IFNγ can also downregulate Th17 differentiation through negative regulation of IL-23 production from APCs (300). IFNγ signalling attenuates *Il23* gene expression by blocking RelA binding, and can successfully prevent disease in the experimental colitis model (300).

Unlike IFNγ, Th2-associated cytokines have not been described as being co-expressed by Th17 cells. However, IL-4 is capable of inhibiting IL-17 release by both naive and memory Th17 cells (301). Suppression by IL-4 is dependent on STAT6 and blocks the expression of IL-23R but not IL-22. STAT6 is an absolute requirement for IL-4-mediated suppression while GATA3 is not necessary (301). However, if Th17 cells undergo repeated rounds of restimulation prior to exposure to IL-4, they become resistant to inhibition as a result of desensitisation of the IL-4R. These observations are reflected *in vivo* with a single immunisation with IL-4-transduced DCs capable of preventing CIA (302). Immunisation with IL-4 has also been shown to reduce the severity of disease in Proteoglycan Induced Arthritis (PGIA) (303).

IL-2 is a key cytokine induced upon TCR signalling and capable of supporting the expansion of naive T cells (304). The high affinity α-subunit of the receptor complex (CD25) is only expressed at high levels upon sustained TCR stimulation. Sharing the common γ chain with other family cytokines such as IL-4 and IL-15, IL-2 binding leads to Jak1/3 activation and subsequently phosphorylation of STAT5A/B, which functions as a heterodimer (305). Additionally, signalling pathways dependent on Ras and PI3K kinase are also triggered. However, whilst IL-2 boosts CTL killing and can cause T cell expansion, the simple concept as a mere proliferation factor underplays the importance of IL-2 in enhancing activation induced cell death (AICD) and affecting T cell effector functions (306). IL-2 is vital to Treg survival and function in the periphery (307). Indeed, Tregs are the only subset to constitutively express CD25 (308). Moreover, IL-2 signalling is needed for IL-4 and IL-4Rα chain expression, required for Th2 development. It has also been shown to be capable of inducing IFNγ via induction of IL-12Rβ2 chain. In contrast, it has been demonstrated that IL-2 serves to inhibit Th17 cells via increasing levels of STAT5 at the IL-17 locus (468, 469). Furthermore, IL-2 can also inhibit Th17 cell development through repression of *Il6ra* and *Il6st* expression in a STAT5-dependent manner (309), thus reducing the ability of naive T cells to respond to a critical pro-Th17 cytokine. IL-15, a related cytokine family member, has been shown to have a similar effect on Th17 differentiation (310). *Il15*$^{-/-}$ and *Il15r*$^{-/-}$ mice have increased frequencies of IL-17a+ cells. These mice also have an exacerbated form of EAE, indicating a loss of regulation and a more pathogenic Th17 population. The mechanism through which IL-15 acts is very similar to IL-2. IL-15 also promotes STAT5 activation that is able to bind and compete with STAT3 at the *Il17a* locus.

1.18 Post translational modifications (PTMs)

The genetic code specifies for only twenty amino acids (311). However, it has been apparent that a number of PTMs to the basic set of amino acids allows biological systems to function with a far expanded amino acid 'universe'. Such modifications can be the result of specific biological pathways, catalysed by enzymes, or be generated through spontaneous chemical events often as a result of ageing,

inflammation or exposure to environmental factors. Some of these PTMs play a fundamental role in basic biological processes such as phosphorylation or glycosylation, but the role they play in infection and autoimmune disease aetiology remains to be fully characterised.

One of the major effects of PTM to a protein in terms of immunology is the effect on the discrimination of 'self/ non-self', through the generation of *de novo* epitopes that can result in a breach of tolerance (312). In CIA it has been shown that glycosylation can also alter the T cell response. Immunisation with the immunodominant CII peptide 256-270 containing a O-linked glycosylated Lysine (K264) fails to induce a robust T cell response and instead induces T cell tolerance (313). However, if stripped of the sugar linked to the lysine residue, these peptides were ineffective at preventing disease onset in neonatal tolerance studies.

Another example is the conversion of amino acids, such as deamination of glutamine or asparagine that can result in conversion to glutamic or aspartic acid residues, respectively (312). The biochemical change that results can lead to different charges at that particular position. It has been shown in celiac disease (CD) that conversion of a glutamine-rich component of gluten can produce a more potent antigen in terms of its ability to stimulate DQ_2-restricted T cell specific clones originating from CD patients (312). In this case it has been shown that modification of a key glutamine residue results in increased binding to the MHC cII, and so increased presentation to T cells. The generation of iso-aspartyl residues is another example of a modification that can occur spontaneously, especially in long-lived proteins (314). This has been shown to change immunogenicity of U1/sm ribonucleoprotein (snRNP) and cytochrome c autoantigens in Systemic Lupus Erythematosus (SLE) patients. Indeed, only T cell responses against the modified autoantigen, containing an iso-aspartyl residue, can be detected *ex vivo* in patient cells. Arginine can also undergo modification by addition of a methyl group. SnRNPs can be methylated at two proteins, sm-D1 and sm-D3, and have also been shown to be immune targets in SLE (315, 316).

A third way in which PTMs can alter the T cell response is at the level of antigen processing in APCs (314). Some of the residues that are capable of modifications are critical for recognition by processing enzymes, such as the protease asparagine endopeptidase (AEP) that cleaves proteins after asparagine residues. Interest is growing given that an important epitope of human MBP (p85-99) is cleaved by AEP at asparagine 94 (312). Due to antigenic processing of the unmodified form it is destroyed in the thymus. Therefore no T cells can be negatively selected against this epitope and so can exit into the periphery. Other examples include the N-glycosylation of an influenza A nucleoprotein that blocks the generation of CD8 T cell epitopes. Hence, PTMs can both interfere with the presentation of pathogenic epitopes and preserve autoreactive epitopes, potentially breaching central tolerance.

1.18.1 Citrullination

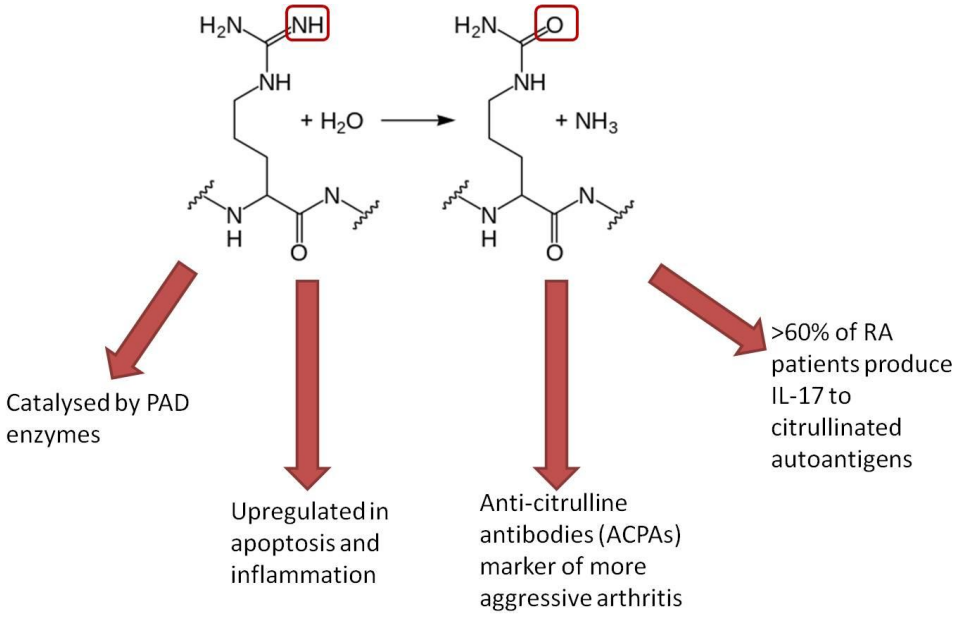

Figure 9 - Summary of citrullination and its effects on autoimmunity. Citrullination is a post translational modification involving the deimination of arginine residues to citrulline. The net effect is a loss of a net positive charge. Citrullination is catalysed by the PAD family of enzymes. This reaction is highly dependent on Ca^{2+} and prevalent during cell death and inflammation (e.g. NETosis). Anti Citrulline Protein Antibodies (ACPAs) are a set of autoantibodies capable of binding cyclic citrullinated peptides that are highly specific for RA and form part of the diagnostic criteria. Indeed, IL-17 production in RA T cells has been detected after stimulation with citrullinated peptides derived from putative autoantigens. Adapted from Wikipedia.org.

Citrullination is another modification of arginine residues through a process called deimination. In terms of biochemistry the effect is a minor increase in mass (+1 Da) and loss of a net positive charge – the arginine is replaced with a neutral citrulline that lacks the positively-charged imine group (see **Figure 9** (312)). It is thought that citrullination plays a role in a number of important physiological processes. The typical effect is a loss of ordered protein structure with studies of filaggrin suggesting that citrullination of 5% of the arginines destroys the tertiary structure. It may also play a role in the modification of cytoskeletal proteins such as vimentin during apoptosis; citrullination of vimentin results in a depolymerisation and loss of intermediate filaments. In the CNS, citrullination has a role in the regulation of protein-lipid interactions, based on binding between negatively charged ganglioside and positively-charged arginines on MBP (317). Hence, citrullinated residues reduce this interaction. The most studied influence of citrullination is in the regulation of histones and chromatin (318). Nuclear substrates include histones H2A, H3 and H4. Modification of methylated regions by citrullination can function as an antagonist for activation of transcription at certain sites within the genome.

The chemical process of citrullination is catalysed by a family of enzymes known as peptidylarginine deiminases (PADs), which consist of a family of 5 isoforms, each with a distinct pattern of tissue expression (319). Within immune cells PAD2 and 4 can be found in cells of the haematopoietic lineage, whereas PAD1/3/6 are restricted to epidermis, hair follicles and oocytes respectively (320). The catalytic activity of these enzymes is dependent on a cysteine residue that attacks the guanidino group on the target arginine (321). However, it has been shown *in vitro* that millimolar concentrations of Ca^{2+} are required to facilitate PAD activity, which may relate to the role of citrullination in pathways linked to cell stress, such as apoptosis or terminal differentiation of the epidermis. Evidence has suggested that PAD expression can be upregulated during inflammation and has been detected in the synovium of RA patients and in the CNS of Multiple Sclerosis (MS) patients (322). Release of these enzymes by dying cells can also lead to modification of matrix protein in the vicinity. PAD4 can also be regulated by p53-controlled stress

pathways (323). Neutrophils can release web-like nuclear DNA-protein complexes upon cell death known as neutrophil extracellular traps (NETs). The extracellular release of chromatin and associated proteins includes citrullinated histones that may be processed and presented by APCs (324).

In MS, citrullination has been described to play an important role in disease development and especially in the hyperacute Marburg's syndrome (325). Interestingly, the increased rate of citrullination leads to an increased ratio of citrullinated MBP to non-citrullinated MBP, similar to the level found in infants (317). It appears it mainly affects the integrity of the myelin sheath through reduced affinity for the negatively charged lipids. Moreover, citrullinated MBP is degraded by cathepsin D more readily, due to its more open structure (326). These modified proteins can be found in the CNS of animals in EAE model and citrullinated MBP has been shown to be encephalitogenic in affected rats and mice. PAD2 has been found in the CNS in oligodendrocytes, microglia and astrocytes (321). PAD4 is expressed in infiltrating myeloid cells. Furthermore, populations of citrulline-specific T cells can be activated, meaning citrullination enhances the pool of autoantigens. Using a citrullinated MBP peptide it has been shown that this APL is capable of activating a distinct T cell population that fails to cross-react with unmodified MBP, following PAD2/4 activation in the CNS (325). Thus, citrullination can exacerbate and accelerate disease through the expanded numbers or changed repertoire of autoreactive T cells that recognise self-antigens.

The role of citrullination in RA has been typically characterised by autoantibodies against citrullinated antigens are highly specific for RA and can be found in approximately 75% of RA patients (327, 328). Indeed, anti-citrulline autoantibodies can be detected in patient sera up to 14 years prior to clinic symptoms of RA (329). PAD2 and PAD4 have been found to be expressed within Rheumatoid synovium and are expressed at levels that exceed those found in healthy controls (320). ACPA titires offer an importnat diagnostic marker and are widely used clinically (330). Studies of RA patient groups show that over 60% of patients can respond to

citrullinated peptides by releasing IL-17 (331). Moreover, a strong IL-6 response was found in an Australian cohort of RA patients to a number of citrullinated autoantigens including filaggrin, vimentin and aggrecan (332). Furthermore, neutrophils of RA patients have an increased potential to undergo NETosis (324). This process exposes and possibly creates citrullinated autoantigens and can stimulate the production of anti-citrulline autoantibodies. It can be enhanced by the presence of pro-inflammatory cytokines such as IL-17 and TNFα, leading to an increased susceptibility to NETosis.

The factors that lead to an immune response against citrullinated proteins have been linked to environmental influences such as periodontal disease and smoking (333). It has been hypothesised that these factors induce greater cell death and so generate a pool of citrullinated antigens either by apoptotic release of citrullinated nuclear antigens or PAD modification of extra cellular matrix (ECM) proteins. A polymorphism of PAD4 has also been linked to an increased likelihood of RA development (334). Whilst found in Japanese and Korean cohorts, it remains to be replicated in European RA patients. In the CIA animal model, ACPAs have been described specific for collagen II and are cross-reactive with both citrullinated and non-citrullinated collagen II (335). Moreover, disease severity has been shown to correlate with the level of PAD4 expression and citrullinated collagen. Interestingly, the clinical signs of arthritis precede the development of citrullinated antigens and ACPAs suggesting that these exacerbate pathogenesis but are not necessary for induction (336).

1.19 Thesis aims

Evidence from the literature indicates a prominent role for TCR signal intensity in shaping the Th response (284). Indeed, the most recent data suggest these signals operate upstream of those derived from the local cytokine environment. While the influence of TCR signalling intensity is a recognised factor in the induction of other Th phenotypes such as Th1, Th2 and Tregs, it remains to be fully characterised in the Th17 subset (159, 271). Building on recent observation made by the group I work in that TCR signal strength plays a key role in human Th17 cell development (283), I hypothesised that a lower TCR signal intensity will promote Th17 induction from naive 5/4E8 TCR Tg T cells in response to changes in both peptide concentration and affinity.

Thus, the aims of this project were to:

- establish an *ex-vivo* co-culture system to study Th17 cell polarisation of naïve aggrecan-specific TCR Tg T cells
- investigate the influence of the concentration of cognate peptide on Th17 development from naïve Th cells
- determine the role of peptide affinity in Th17 polarisation using a disease relevant PTM (arginine to citrulline) of a putative TCR contact residue
- assess the possible role of IL-2 signalling as the mechanism linking changes in TCR signal intensity to the type of effector Th response

2 Materials and Methods

2.1 5/4E8 TCR Transgenic mice

5/4E8 TCR Tg mice were originally developed by Berlo *et al* and were kindly gifted by Prof. Willem van Eden (337). The TCR ($V_{\alpha1.1}V_{\beta4}$ (GeneBank accession number AY823583 and U19234 respectively)) of the 5/4E8 hybridoma recognises the aggrecan peptide epitope 84-103 (p84-103) (338). Linearized pre-TCR α chain (pTα) and pTβ 5/4E8 TCR DNA fragements were coinjected in equal amounts into the fertilized eggs of (CBA x C57BL/6) F1 mice (338). TCR Tg founders were then identified by PCR analysis of tail genomic DNA. The expression of Vβ4 was confirmed by flow cytometric assessment of blood lymphocytes. As the 5/4E8 TCR hybridoma responds to peptide in the context of $H\text{-}2^d$ MHC cII, the TCR Tg founder mice were backcrossed onto the PGIA susceptible background (338). A pure BALB/c genomic background was obtained after backcross 8. The purity of this backcrossing was confirmed using 244 sequence length polymorphic markers specific to the BALB/c strain as described by Glant *et al* (339). Throughout the backcrossing process $V_{\alpha1.1}V_{\beta4}$ expression was detected using genotyping and susceptiblilty to PGIA at each stage.

The TCR transgene was maintained on a heterozygous background by the breeding of TCR transgenic with wild type (WT) BALB/c partners. This ensured stable expression of the 5/4E8 TCR Tg gene between generations. Typing of Vβ regions 2-15 indicated a highly enriched frequency of Vβ4 expressing CD4+ T cells only in those which inherit the TCR Tg compared to WT littermates (340). In order to determine phenotype, 50µl of blood was collected in heparin via tail bleed from 4-8 week old mice before incubation with 1ml of Red Blood Cell Lysis Buffer (Sigma) for 5mins. Excess wash buffer (phosphate-buffered saline (PBS (Lonza)) plus 2% fetal bovine serum (FBS (PAA Laboratories)) was added prior to centrifugation at 400g for 5min before staining with 20µl of anti-CD4 PerCP-Cy5.5, anti-Vβ4 APC (BD Bioscience) and anti-TCRβ FITC (eBioscience) mAbs or wash buffer (see **Table 3** for clone type). Only those carrying the transgene show an enrichment of the Vβ4

population (>80%) compared to WT littermates, which had a far reduced proportion of Vβ4 CD4+ T cells (see **Figure 10**). Unfortunately, there is currently no commercially available monoclonal antibody (mAb) against the α chain possessed by the transgenic T cells of these mice (Vα1.1). All work was carried out in accordance with the Animals (Scientific Procedures) Act 1986 under the project licence PPL 60/3281 held by Prof. J.H. Robinson. All animals were housed under specific pathogen free conditions in Newcastle University's Comparative Biology Centre.

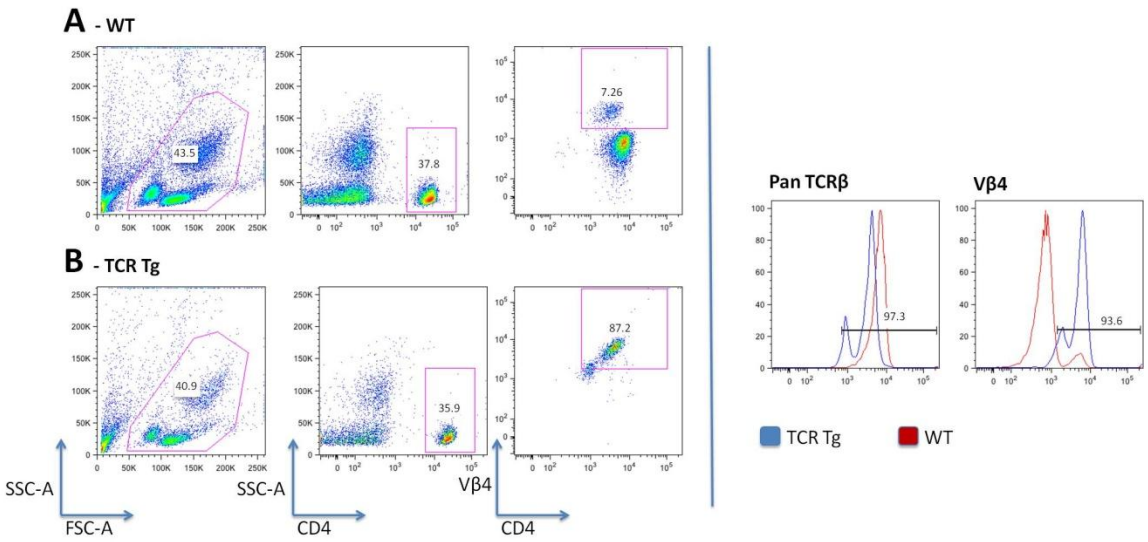

Figure 10 - Phenotype of CD4+ T cells of 5/4E8 TCR Tg mice and WT littermates. Examples of WT littermate (**A**) and a 5/4E8 TCR Tg mouse (**B**) illustrated enrichment of Vβ4+ expressing T cells in the TCR Tg mice. Phenotype was determined using peripheral blood from tail bleed of individual 4-8 week old mice followed by staining for Vβ4, CD4 and TCRβ and flow cytometry. Proportion of Vβ4+CD4+ T cells in 5/4E8 TCR Tg mice was routinely >85%. Data are representative example of five independent biological examples.

2.2 Peptides

All peptides were dissolved in sterile deionised water and stored at -20^{o}C at a concentration of 2mM. Peptides were produced by JPT Peptide Technologies GmbH, Berlin, and shown to be >85% pure assessed by mass spectrometry. Below lists the sequences of all peptides used in this study (see **Table 1**).

Peptide	Sequence	Additional Information
p84-103	VVLLVATE**GRVRVNSAY**QDK	20-mer containing core T-cell epitope
p89-103	ATE**GRVRVNSAY**QDK	15-mer containing core T-cell epitope
R93A	ATE**GAVRVNSAY**QDK	Alanine substitution of a putative TCR contact
R95A	ATE**GRVAVNSAY**QDK	Putative MHC contact
V96A	ATE**GRVRANSAY**QDK	Putative TCR contact
A99G	ATE**GRVRVNSGY**QDK	Putative TCR contact
R93Cit	ATE**GCitVRVNSAY**QDK	Citrullination of a putative TCR contact
R95Cit	ATE**GRVCitVNSAY**QDK	Citrullination of a putative MHC cII contact
R93-95Cit	ATE**GCitVCitVNSAY**QDK	Citrullination of both
F1p3	AADLTASTTATATLVEPARI	Irrelevant peptide, strong I-A^d binder (341).

Table 1- Peptides used in this study.

2.3 Extraction of Bone Marrow

6-12 week WT littermates were sacrificed and transferred to 70% ethanol. Tibias and femurs were dissected and the muscle removed before extraction of bone marrow (BM). Heavy-duty scissors were used to cut off the ends of the bones before being flushed with RPMI-1640 (Sigma); 10% FCS (PAA Laboratories); 2mM glutamine; 100units/ml penicillin; 100µg/ml of streptomycin and 50µM β-mercaptoethanol (all Sigma)) using a syringe and 25G needle. These cells were gently pipetted to disrupt clusters to obtain a single cell suspension.

2.4 Dendritic Cells (DCs)

Petri dishes (90mm triple vent polystyrene dishes, SLS) were seeded with $2x10^6$ bone marrow derived cells in 20ml of RPMI-1640 (Sigma) supplemented with 10% FBS (RF10) and 20ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems). On day 3, 6 and 8, 10ml of RF10 supplemented with GM-CSF (20 ng/ml) was added after 9ml of culture medium had been gently removed. At day 10, 14ml of the culture medium was gently removed and 5ml RF10 containing 0.1µg/ml lipopolysaccharide (LPS derived from e-coli; Sigma) and 20ng/ml GM-CSF was added to generate mature DC (mDC). After 24h, cells were harvested using a lifter following an incubation for 1 hour at 4^oC to increase cell yields. These were washed twice with 50ml of PBS before re-suspension in IMDM (Sigma) containing 10% Serum Replacement (SR; Invitrogen); 2mM glutamine; 100units/ml penicillin;

100µg/ml of streptomycin and 50µM β-mercaptoethanol (all Sigma) and counted in trypan blue (Sigma) using a haemocytometer. These cells were either stored in freeze media (90% FBS; 10% Dimethyl sulfoxide (DSMO (Sigma)) or used in functional assays.

In some examples the concentration of LPS used to induce maturation was reduced to 0.01µg/ml, 0.001µg/ml or none. Those without LPS maturation were defined as immature DCs (imDCs).

2.5 Splenocyte Extraction

6-12 week old WT or 5/4E8 TCR Tg mice were sacrificed and placed into 70% ethanol. Spleens were removed into 5ml of PBS before mechanical disruption using a 1ml syringe plug on a 100µm Cell Sieve (BD Bioscience, UK) into 50ml Falcon Tube. Aggregates were disrupted by pipetting. PBS was added to 50ml before centrifugation at 400g for 5mins. Cells were resuspended in 3ml of Red Blood Cell Lysis Buffer (Sigma) per spleen before incubation at 37^{o}C for 4mins. These were then washed twice in 50ml of PBS. Finally, cells were resuspended in 5ml of IMDM (Sigma) with 10% SR (Invitrogen) and an aliquot counted in trypan blue by haemocytometer to count dead cells.

2.6 Naive T cell Isolation

Following isolation of splenocytes, naive T cells were isolated by magnetic bead labelling using a Miltenyi MACS system. This technique allows cells to be separated from a heterogeneous population using magnetic nanoparticles bound to antibodies. Cells bound by the conjugated antibodies are passed across a column the presence of strong magnetic field. Only those labelled with conjugated antibody will be retained in the column. Thus, this technique allows for isolation of a particular target population by either positive or negative selection.

The initial step uses negative selection to isolate the CD4+ population from non-target splenocytes. Briefly, cells were resuspended in 400µl of MACS Buffer (PBS; 0.5% FBS; 2mM EDTA) per 10^{8} cells and a biotinylated cocktail of antibodies added.

These depleted a number of cell populations using antibodies against CD8a, CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b (DX5), CD105, MHC cII, Ter-119 and TCRγ/δ. After incubation to allow binding to target cells, anti-biotin microbeads were introduced and non-target cells removed by magnetic separation using a LS column and VarioMACS magnet. Further purification of naive CD4 T cells required collection and labelling of the effluent population with anti-CD62L (L-selectin) microbeads and a second magnetic isolation using positive selection by MS column with the VarioMACS magnet. The procedure was performed according to the manufacturer's protocol. Naive CD4+ T cells were collected and counted using a haemocytometer to determine cell numbers before use in either functional assay or storage by cryogenic preservation. The isolated cells were also analysed by flow cytometry to determine the degree of contamination of both non T cells and memory T cells using anti-CD4 PerCP-Cy5.5, anti-CD44 eFluro450, anti-CD62L PE and anti-I-A^d/I-E^d PE. Typically these contaminations were <10% and <6% respectively.

2.7 Functional Assays

$1.25x10^5$ isolated naive CD4+ TCR Tg T cells were co-cultured with $6.25x10^3$ mDCs (a 1:20 ratio of DCs to T cells) in 48-well plates in 500 μl culture medium: IMDM; 10% SR; 100units/ml penicillin; 100μg/ml strepomycin and 50μM β-Mercaptoethanol (ISR10). Peptide doses for primary cultures ranged from $2x10^{-3}$μM to 20μM. Cells were cultured for 5 days before washing twice with PBS and assessment of cell numbers using trypan blue (see **Figure 11**). In addition, some cultures were established in the presence of exogenous pro-Th17 cytokines: IL-1β (10ng/ml; Peprotech); IL-23 (10ng/ml; Peprotech); TGF-β (10ng/ml; R&D Systems) and IL-6 (50ng/ml; Peprotech). Recombinant human IL-2 (rhIL-2) was also added to some cultures (50IU/ml; Novartis). Cultures with irrelevant peptide (F1p3) were also established as a form of negative control. In some experiments blocking monoclonal antibody to IL-2 was used. In these cases, co-cultures were established with a cognate peptide, and anti-IL-2 (S4B6) was titrated in at varying

concentrations (1/5 to 1/80 dilution of the S4B6 hybridoma supernatant). An appropriate isotype control was also included.

Secondary cultures were established using $4x10^4$ T cells with $2x10^3$ mDCs and 2µM of cognate peptide in flat bottomed 96 well plates. Supernatants were collected after 48h and analysed for cytokine levels using ELISAs according to the manufacturers' protocols.

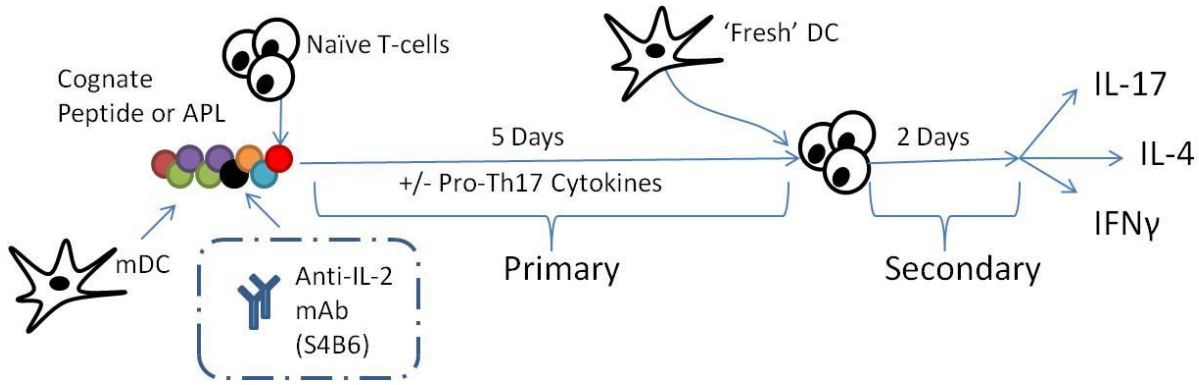

Figure 11 - Outline of secondary co-culture assay. Schematic of co-culture system used to determine how peptide density or affinity modulates Th17 dvelopment. Co-cultures of $1.25x10^5$ naive 5/4E8 TCR Tg T cells with $6.25x10^3$ LPS activated mDCs were established for an initial 5-day period with or without pro-Th17 cytokines in a 48-well plate. In some assays rhIL-2 (50IU/ml) or anti-IL-2 mAb (S4B6) was also added. After the primary culture cells were extensively washed and $4x10^4$ T cells plated with $2x10^3$ 'fresh' mDC and 2µM of p84-103 in 96 well flat bottomed plates. Supernatants were collected 48h later and analysed for cytokines by ELISA.

2.8 Proliferation Assay

Various approaches can be used to assess cellular proliferation. One of the most common uses tritiated thymidine. As cells undergo division, radioactive thymidine is incorporated into the newly synthesised DNA strands. Thus, the degree of radioactivity within a sample is proportional to amount of proliferation during exposure to the tritiated thymidine.

Unless otherwise stated $4x10^4$ naive TCR Tg T cells were co-cultured with $2x10^3$ mDCs in 200µl of ISR10 culture medium with varying doses of either cognate peptide p89-103 or indicated APL in flat bottom 96-well plates. These were then incubated at 37^oC, 5% CO_2. At the indicated time points, 120µl of supernatant was removed and 10kBq [3H] thymidine (specific activity; 74.0 GBq/mmol; Perkin Elmer)

was added for the last 12h of culture. The cells were lysed using a cell harvester (TomTech Harvester 96 Mach III M) with DNA bound to glass-fibre capture mesh (Perkin Elmer). Once dried, each mesh was placed in a plastic wallet and soaked in scintillation fluid prior to sealing (all Perkin Elmer). Radioactivity was quantified using a beta scintillation counter (Perkin Elmer Microbeta TriLux; Perkin Elmer), generating results expressed as mean counts per minute (cpm) ± SEM of triplicate wells.

2.9 Flow Cytometry

Flow cytometry is a laser-based fluidic technology that permits the assessment of multiple parameters after each individual cell is interrogated by a laser. It has the advantage that it allows analysis of multiple parameters on a single cell level within a heterogeneous population. Thus, it permits the analysis of cellular phenotype and proportion within the cohort studied. Basic understanding of the cell can be determined as each cell passes, one at a time, by the flow cell. The scatter of laser light provides two basic parameters with Forward Scatter (FSC) representing the size through the detection of the 'shadow' profile created by the cell. In contrast Side Scatter (SSC) is dependent on light diffraction, which is in turn dependent on the complexity of the cell (e.g. structures within the cytosol).

The use of fluorophores conjugated to monoclonal antibodies allows for the detection and quantification of an array of protein targets. The lasers excites a fluorophore creating an increase in energy state before returning to ground state while emitting light. Each fluorophore has a distinct wavelength at which it becomes excited and emits light at a particular set of wavelengths. Calibrated using the mirrors and filters, these specific emissions are quantified by the corresponding detectors. Finally, these detectors convert the analogue light signals into binary voltages, which are rendered graphically by software. See **Table 2** for a list of antibodies used during this project.

2.9.1 Immunofluorescent Labelling of Cell Surface Molecules

Between $1x10^5$-$1x10^6$ of the relevant cell type were transferred to a 1.5ml eppendorf and spun for 7min at 2000rpm. The supernatant was removed and cells resuspended in 150µl of FACS buffer (PBS; 2% FCS; 2mM Sodium Azide) per well. The cells were then transferred to the appropriate well of a 96 well v-bottomed plate. Following washing the cells in 150µl of FACS buffer, cells were resuspended in 50µl of anti-CD16/CD32 mAb (BD Biosciences) for 20mins at 4^oC to block Fc receptors. 5µl of staining mAb at the relevant dilution was added to the appropriate wells. These were incubated for 30min at 4^oC in the dark before two further wash steps. In the case of biotinylated antibodies, two additional washes were included prior to the addition of the streptavidin-bound fluorophore. After a further two wash steps with 150µl of FACS buffer, the cells were resuspended in 150µl of FACS buffer and transferred to FACS tubes. Each sample was acquired within 24h of fixation using FACS Canto II (BD Biosciences).

In some cases cells are fixed with 150µl of 1% of Paraformaldehyde (Sigma) for 20min at 4^oC. These were acquired the next day. FACS Diva (BD Biosciences) and FlowJo (Treestar) software was used for acquisition and analysis of data, respectively.

2.9.2 Intracellular Cytokine Detection

In order to stimulate sufficient simultaneous cytokine production at levels able to be detected using fluorophore-conjugated antibodies. Transcriptional machinery must be activated to initiate cytokine production via chemical triggering of PKC. For most cytokines, phorbol 12-myristate 13-acetate (PMA) and ionomycin are a potent trigger. Brefeldin A (BFA) is also needed to block the transport of proteins from the ER and to the Golgi apparatus, thus leading to protein accumulation within each cell. At the relevant time point, cells were stimulated with PMA (10ng/ml; SIgma, UK) and ionomycin (1µg/ml; Sigma) for 5 hours at 37^oC, 5% CO_2. After an hour BFA (10µg/ml; Sigma) was also added to each well. Cells were then collected and placed in eppendorf tubes before being spun down for 7mins at 2000rpm. Cells were resuspended in 150µl of FACS buffer before being transferred to FACS tubes. These

were topped up to 1ml before being spun at 400g for 5mins and treated with anti-mouse CD16/32 (BD Bioscience) to block FcR non-specific binding of antibodies. After a 20min incubation at 4^{o}C, mAbs to stain for surface markers were applied as previously described. In order to stain intracellular antigens, these cells were subsequently incubated in 500µl of fixation/permeabilisation buffer for 30mins at 4^{o}C (Fix/Perm; eBioscience for transcription factors or BD Bioscience for cytokines). The purpose of this buffer is to crosslink proteins and DNA using formaldehyde, and to permeabilise the membranes by saponin and so provide access to intracellular antibody targets. These cells were then washed twice in 1ml permeabilisation (Perm) buffer before resuspension in 50µl of 2% Rat Serum (Sigma) to block non-specific binding of intracellular sites. After a 15min incubation at 4^{o}C, cells were stained with the corresponding intracellular mAbs. Each sample was incubated for a further 30mins at 4^{o}C. Cells were then washed twice in 1ml of Perm buffer before resuspension in 150µl of FACS buffer. Acquisition of cells was subsequently carried out within 24h using a BD FACs Canto II with data assessed using Flowjo software (Treestar Inc.).

To ensure the binding observed was specific to a particular marker, isotype controls were used to detect non-specific binding. Isotype controls were assessed at the same concentration as the antibody of interest. An example is shown in **Figure 12D**. Gates were typically set using Fluorescence Minus One (FMO) controls, an example of which is also shown in **Figure 12E**. These help to interpret and gate cells in the context of data spread due to the presence of multiple fluorochromes within a panel.

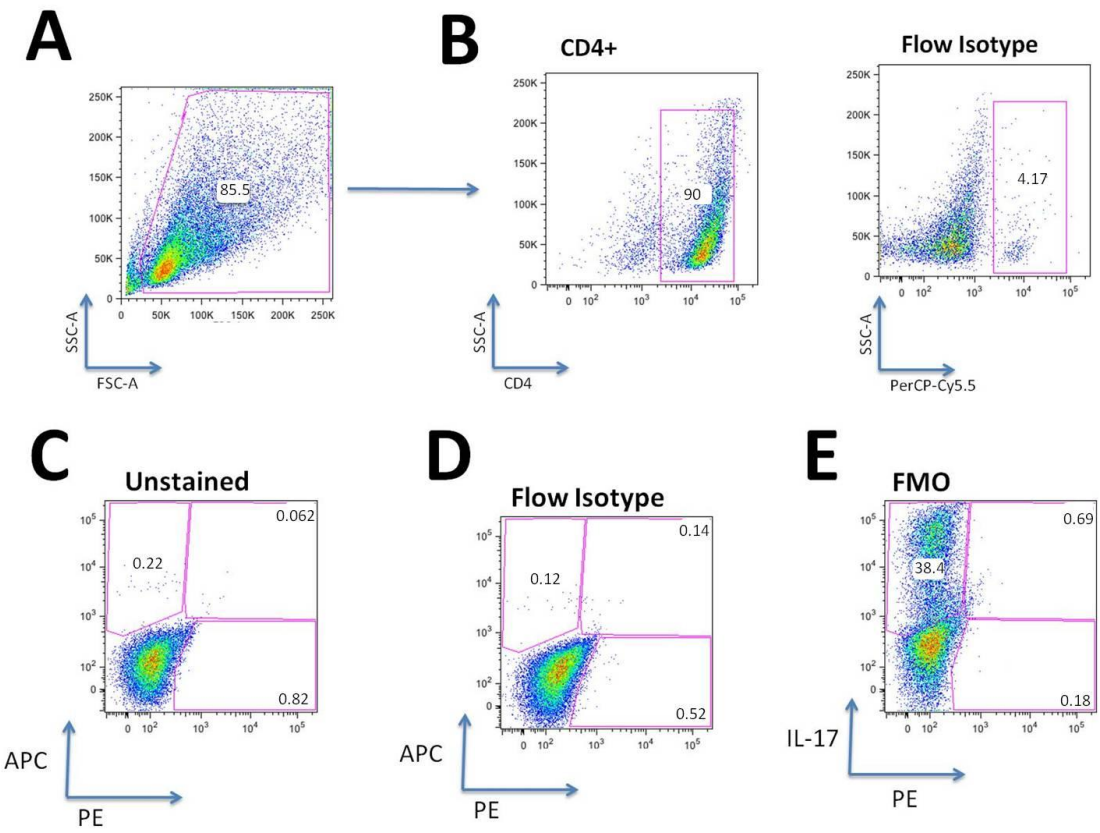

Figure 12 - Example of gating. (**A**) Cells were initially gated on the SSC-A/ FSC-A to remove cell debris before gating on CD4 expression based on an appropriate isotype control to control for non-specific binding (**B**). Gating was set using the FMO control especially in those cases where the change in fluorescence was more diffuse with no clearly distinct populations occurring (e.g. cytokines such as IL-17) (**E**). Unstained and isotypes were also determined for each assay (**C** and **D** respectively).

2.9.3 Phosflow

Protein phosphorylation can be determined either by Western blotting or by the relatively novel Phosflow method. The advantage of the latter include the reduced number of cells required, the ability to test multiple parameters for each individual cell and to assign values to particular subsets within a heterogeneous cell population. Following isolation of naive T cells as described in section 1.6, co-cultures of $1.25x10^5$ naive TCR Tg T cells were established with $6.25x10^3$ mDCs and various peptide ligands. In some cases anti-IL-2 antibodies (S4B6; 1/40 dilution of the hybridoma supernatant) or 50IU/ml of rhIL-2 (Novartis) were also added to the primary co-cultures.

At each time point, co-cultures were harvested, washed and transferred to FACS tubes. Cells were fixed by the addition of 1ml of pre-warmed Fix/ Lyse buffer ((37^{o}C) BD Bioscience) and incubated for 12mins at 37^{o}C in a waterbath. Cells were spun at 600g for 8mins before careful removal of all supernatant. After vortexing to disrupt the pellet, cells were washed in 1ml of PBS containing Ca^{2+} and Mg^{2+} (Lonza). These cells were held on ice and permeabilised by the addition of 1ml of pre-chilled Perm III (BD Bioscience). Samples were stored overnight at -80^{o}C.

Keeping the cells on ice, each sample was transferred to a FACS tube and washed with 3ml of Stain Buffer (PBS (with Ca^{2+} and Mg^{2+}), 0.1% BSA and 0.09% Sodium Azide) to each tube before spinning at 600g for 6mins. After two further washes, cells were resuspended in 50µl 4% mouse serum (2% Final Concentration given the approximately 50µl of residual volume within a FACs tube) and incubated for 15mins at room temperature (RT).

Following incubation with blocking serum, anti-CD4 Alexa Fluor 488, pSTAT3(Y705) PE and pSTAT5(Y694) PEcy7 were added to the appropriate sample and incubated for a further hour at RT. After a further wash with 3ml of Stain Buffer, cells were resuspended in 150µl of FACs buffer and immediately acquired using a BD FACS Canto II with data assessed using Flowjo software (Treestar inc.).

Antibody	Clone	Fluorophore	Catalogue Number	Dilution	Company
Anti-mouse CD4	RM4-5	PerCP/5.5	553050	1/10	BD
Anti-mouse CD4	RM4-5	Alexa Fluro 488	557667	1/10	BD
Anti-mouse CD62L	MEL-14	PE	553151	1/10	BD
Anti-mouse CD44	IM7	eFluro450	48-0441-82	1/20	eBioscience
Anti-mouse CD69	H1.2F3	PE	561932	1/10	BD
Anti-mouse CD25	PC61.5	eFluro450	48-0251-82	1/10	eBioscience
Anti-mouse CD154	MR1	BIOTIN	553657	1/10	BD
Anti-mouse TCRβ	H57-597	FITC	553170	1/20	BD
Anti-mouse Vβ4	KT4	BIOTIN	553364	1/10	BD
Anti-mouse IFNγ	XMG1.2	FITC	562019	1/5	BD
Anti-mouse IL-4	11B11	PE	554435	1/5	BD
Anti-mouse IL-17	eBio17B7	APC	17-7177-81	1/100	eBioscience
Anti-mouse GM-CSF	MP1-22E9	PE	554406	1/5	BD
Anti-mouse Foxp3	FJK-16s	APC	17-5773-82	1/10	eBioscience
Anti-mouse ROR-γt	Q31-378	PE	562607	1/10	BD
Anti-mouse GATA3	TWAJ	eFluro 660	50-9966-42	1/10	eBioscience
Anti-mouse T-bet	eBio4B10	PE	12-5825-82	1/10	eBioscience
Anti-mouse CD11c	N418	APC	17-0114-81	1/10	eBioscience
Anti-mouse MHC cII ($I\text{-}A^d/E^d$)	2G9	PE	558593	1/20	BD
Anti-mouse CD80	16-10A1	FITC	553768	1/10	BD
Anti-mouse CD86	GL1	FITC	553691	1/10	BD
Anti-mouse CD40	3/23	PE	553791	1/10	BD
Anti-human/mouse pSTAT3 (Y705)	4/P-STAT3	PE	612569	1/10	BD
Anti-human/mouse pSTAT5 (Y694)	47/Stat5	PE-Cy7	560117	1/10	BD
Streptavidin APC	N/A	APC	554067	1/10	BD
Anti CD16/32 (Fc Block)	2.4G2	N/A	553142	1/28	BD

Table 2 - Antibodies used for Flow cytometry.

2.10 Enzyme Linked Immunoabsorbant Assays (ELISA)

ELISAs provide a relative simple and high throughout assay to quantify the levels of a particular protein within a sample. Sandwich ELISAs utilised the binding of the target antigen between two specific monoclonal antibodies known as a capture and detection antibody, respectively. Typically the detection antibody is biotinylated and binds streptavidin linked to horse-radish peroxidase enzyme (HRP). This is involved in the conversion of a substrate to a coloured product that permits quantification by absorbance at a particular wavelength. Comparison to a standard curve allows values to be derived for unknown samples.

Supernatants from co-cultures were harvested at each time point and frozen at -20^{o}C to await testing. ELISAs were performed according to each manufacturer's instructions. Briefly, to each well of 96 well flat-bottom EIA/RIA High Bind ELISA plate (Corning) a 100µl of capture antibody in coating buffer (PBS without carrier protein) was added. Each plate was incubated for overnight at 4^{o}C.

The following day, each plate was washed 3 times with excess PBS-0.01% Tween-20 (Sigma) before blocking each well with 200µl of PBS-1% BSA for an hour. Thawed supernatants were diluted either 1/5, 1/10 or 1/100 with PBS-1% BSA (PBS-0.1% BSA for IFNγ Duoset ELISA or Assay Dilute for eBioscience READYSETGO kits (IL-2, IL-22, GM-CSF)), and for each plate an 8-point standard curve was also established using recombinant cytokine (top concentration as recommended by kit supplier). After discarding and washing off the block, 100µl of either sample or standard curve was added to each plate followed by a 1-2h incubation at RT. These were then washed 3 times as before and 100µl of detection antibody added to all wells. After another 2h incubation at RT and subsequent 3 washes, 100µl streptavidin-HRP (1/200 dilution) was added to each plate for 20mins at RT. Finally, after 4 washes, 100µl of o-Phenylenediamine dihydrochloride (OPD) substrate was added to each well (Sigma). Each OPD tablet was dissolved in 13ml of citrate phosphate buffer (0.03M citric acid/ 0.05M $NaHPO_4$/0.03M $Na_2HPO_4.2H_20$) and H_2O_2 (6µL, 30% stock) was added just prior to use. After a 20min incubation at RT, the reaction was

terminated by the addition of 50µl of 3M H_2SO_4 (Sigma). Each plate was read immediately using a Tecan Sunrise Plate reader (Tecan Group) at 490nm wavelength. In the case of the eBioscience READYSETGO kits OPD was substituted for 3,3',5,5'-Tetramethyl benzidine (TMB; eBioscience) and absorbance determined at 450-570nm.

The known cytokine concentrations of each standard curve were plotted against the absorbance values. Unknown values can be calculated from the equation used to generate the standard curve, as as long as the values fall within the same range (see **Figure 13**). Detection limits were calculated based on the background absorbance multiplied by 2. Duplicate or triplicate measurements were obtained for every sample and the mean determined following exploration from the standard curve. If any sample was diluted before assessment the interpolated values were then multiplied by the dilution factor.

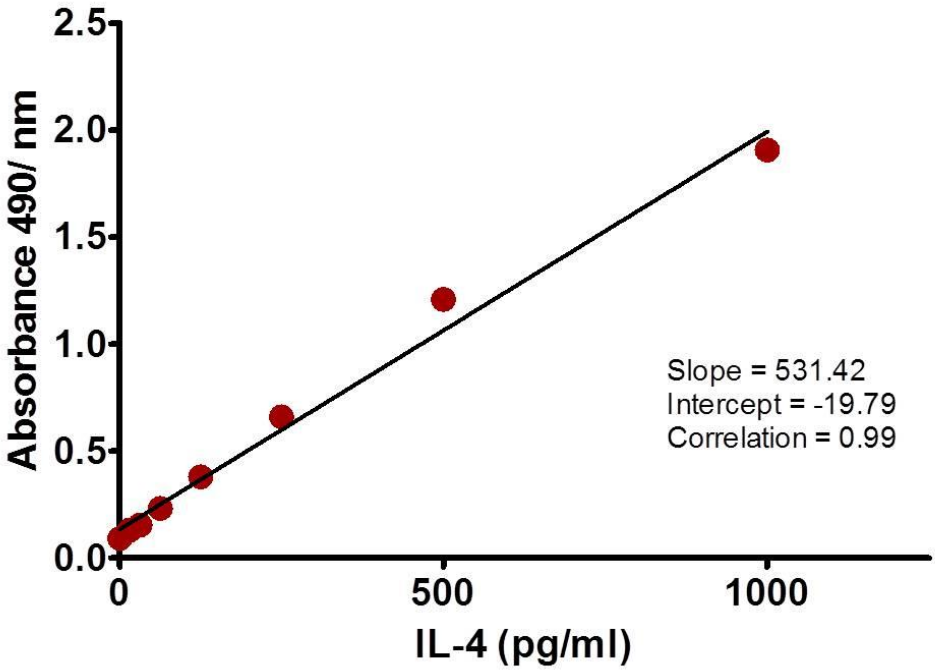

Figure 13 - Example of ELISA standard curve. Absorbance was plotted against known concentrations of the cytokine being analysed (in this example IL-4) with equation of the line determined using Microsoft Excel. Using the equation of the line values of unknown samples can be calculated and adjusted for dilution factor.

3 Characterisation of aggrecan-specific TCR transgenic T cells and the role of antigen density in Th17 development

3.1 Introduction

The influences that shape Th cell effector cell responses are of critical importance, not only for our understanding of infectious and autoimmune pathologies but also in ensuring the efficacy of therapies such as vaccines. Whilst the cytokine milieu is thought of as the primary factor in shaping the observed Th cell responses it is not, by any means, the only mechanism (273). Since the pioneering studies of the O'Garra and Bottomly labs it has been recognised that the overall density of antigen can determine the balance between Th1 and Th2 responses (159). More recently it was shown that the development of Tregs was markedly increased at higher peptide densities both *in vitro* and *in vivo* (271). Our group has shown in human T cells that Th17 responses can be influenced by the degree of TCR signal intensity following polyclonal or superantigen activation (283). Reducing the number of anti CD3/CD28 beads or DCs pulsed with superantigen to activate human CD4+ T cells profoundly increased Th17 responses in terms of both frequency and absolute Th17 cell numbers. Furthermore, the data showed that NFATc1 was capable of binding the proximal region of *Il17a* promoter only with a lower TCR signal, due to a weaker Ca^{2+} flux. It was important to expand this concept and assess the impact of peptide density in an antigen specific system using naive transgenic T cells responding to pMHC presented by APCs. The advantage of a known TCR and its cognate antigen allows for controlled changes to the peptide concentration, the primary focus of this chapter.

The 5/4E8 TCR Tg mice was developed using a TCR derived from an aggrecan-reactive hybridoma and subsequently back-crossed onto the PGIA-susceptible BALB/c background (338). A major component of the extra cellular matrix (ECM), aggrecan is composed of three globular domains of which the G1 domain is the

most characterised (342). Within the G1 domain, there are multiple T cell epitopes. Indeed, the most immunodominant arthritogenic epitope spans the positions of 84-103 (see **Figure 14** (343)). In prior studies, when activated in the context of PGIA these TCR Tg T cells tended to have a Th1 phenotype mirroring the phenotype of the 5/4E8 hybridoma from which the TCR genes used to create the TCR Tg were taken (337).

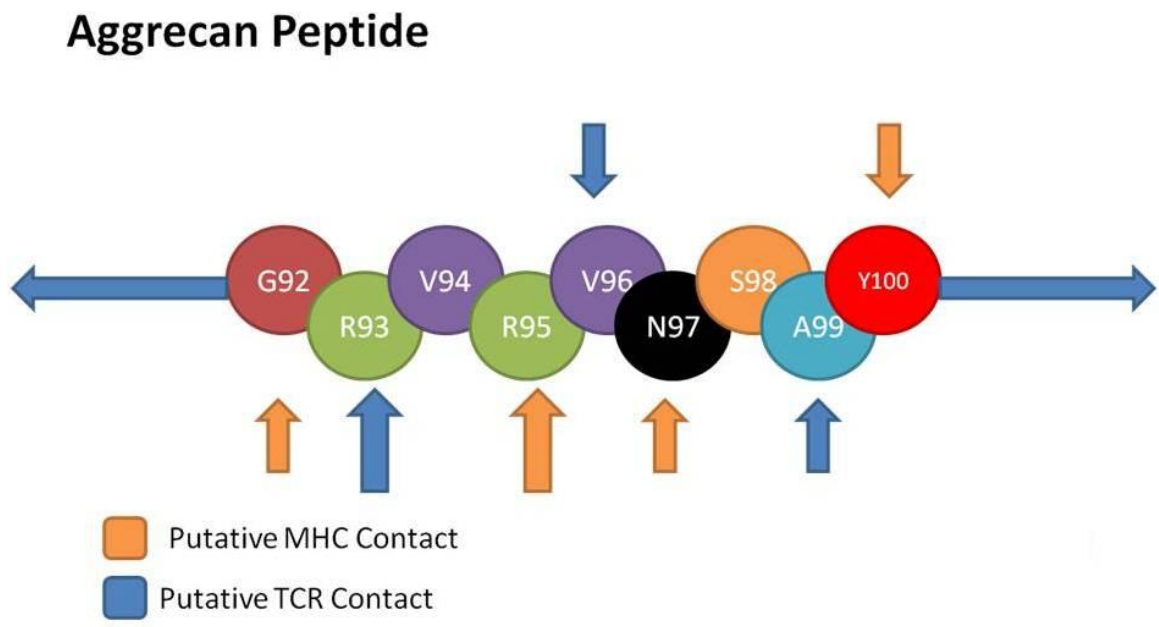

Figure 14 - Schematic representation of the core nine amino acids of the Aggrecan peptide p89-103. 5/4E8 TCR recognises this epitope derived from the G1 domain of Aggrecan in an I-A^d restricted manner. Putative TCR and MHC contact residues are illustrated using blue and orange arrows respectively (343-345).

3.2 Chapter aims

The studies using humanized mice expressing HLA DR4 and HLA DQ8 enabled the fine mapping of immune responses to aggrecan epitopes in a simpler system which lacks the polymorphic variation of the human MHC (346). These studies indicated that the G1 domain contained the most relevant epitopes (24 out of 31) that were capable of presentation by RA associated HLA alleles. Moreover, the core 9-mer of p84-103 was found to be the most potent in terms of its ability to induce disease in BALB/c and DR4 transgenic mice (347).The experiments presented in this chapter aimed to provide an *in vitro* system to evaluate Th17 differentiation of naive 5/4E8 TCR Tg T cells. This *in vitro* system was subsequently used to assess how changes in peptide concentration (current Chapter) and altered peptide ligands (Chapters 4 and 5) affected Th17 cell development.

The specific objectives are to:

- Establish an *in vitro* system to determine Th17 induction from naïve 5/4E8 TCR Tg cells
- Investigate the effect of peptide density on proliferation, surface marker expression and Th17 polarisation of naïve 5/4E8 TCR Tg cells

3.3 Phenotype of purified naive 5/4E8 T cells

The initial focus was to determine if the T cells purified *ex-vivo* were of sufficient purity both in terms of memory T-cell and APC contamination. A highly naive population was important to ensure that in subsequent experiments Th cell polarisation arose from the differentiation of naive cells, rather than the expansion of memory cells. Isolation required a two-step protocol using 1) depletion of non-CD4+ T cells followed by 2) positive selection of naive cells using anti-CD62L magnetic beads. CD62L is an adhesion molecule that acts as a 'homing' receptor allowing entry into lymph nodes from the HEVs (348). It is typically expressed at greater levels in naive T cells in comparison to those of a memory phenotype (349), thus making it a useful marker for the enrichment of these cells.

Flow cytometry was used to determine the presence of both naive cells ($CD4^{+}CD62L^{hi}CD44^{lo}$) and memory cells ($CD4^{+}CD62^{lo}CD44^{hi}$) in both the final purified population (see **Figure 15A**) and the flow-through from the second column that was used to positively select naive cells using the CD62L marker (see **Figure 15B**). The data presented shows a representative example of the two-step purification process, demonstrating that the vast majority of the eluted CD4+ T cells are of the naive phenotype. **Figure 15B** shows an enrichment of memory T cells in the flow-through of the final column. To test for the degree of APCs contamination, each population was also stained for MHC cII ($I\text{-}A^{d}/I\text{-}E^{d}$). The percentage of MHC cII-expressing cells detected were typically 10% or less. Taken together these data suggest that the overwhelming majority of the CD4+ T cells purified were of the naive phenotype with, importantly, contamination with memory cells typically <5%. This is critical to ensure that any response observed in future assays were a result of the differentiation of uncommitted naive cells rather than memory expansion.

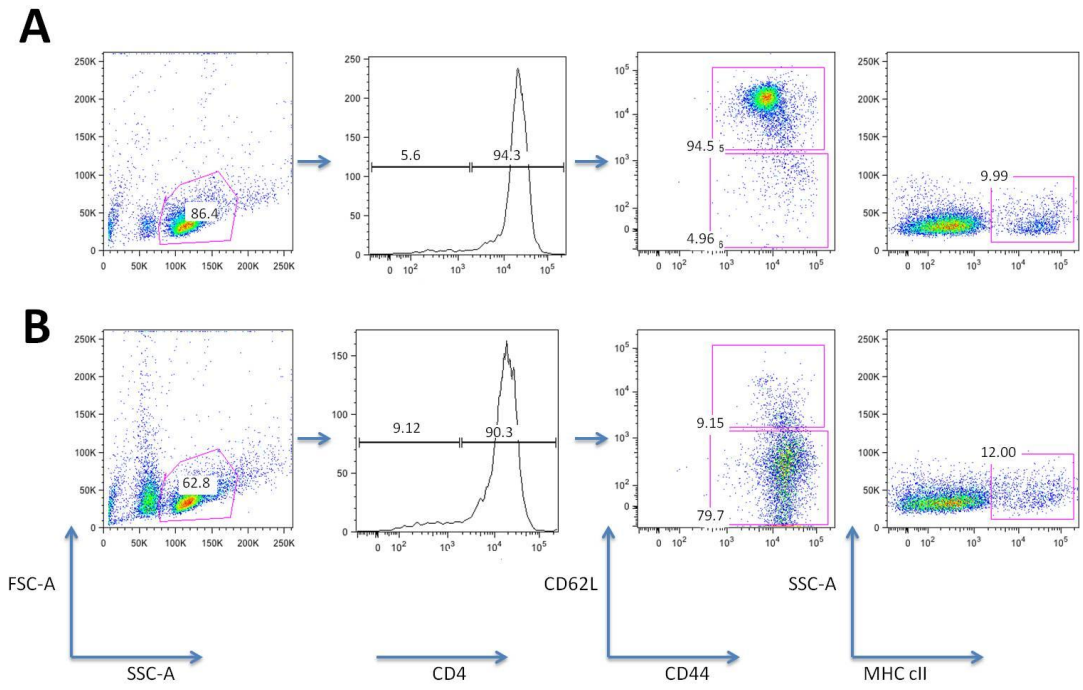

Figure 15 - Purity of starting T cell population. Naive 5/4E8 TCR Tg T cells were isolated in a two-step purification process by 1) depletion of non-CD4 cells followed by 2) positive selection on CD62L from the CD4 population. Cells from the final purified population (**A**) and the flow through (**B**) from the final column were analysed by flow cytometry both for Th purity (CD4), APC levels (MHC cII I-E^d/A^d) and naive/ memory state (CD62L and CD44 expression in the CD4 population). The data are representative of three independent biological replicates.

3.4 Characterisation of LPS-activated DCs

DCs are known to be critical in the activation of naive T cells (1). The most readily available source of these cells was through expansion of bone marrow DC precursors isolated from sex-matched non-Tg littermates, to generate bone-marrow-derived DC. Initial experiments focused on characterisation of the cells to be used as APCs in future assays. Using flow cytometry the expression of a range of markers for DCs (CD11c, MHC cII (I-E^d/A^d)) and important co-stimulatory molecules were determined (CD80, CD86, CD40) in both immature (imDCs) and 0.1µg/ml LPS-matured DCs (mDCs) following 11 days of culture in the presence of GM-CSF. In both populations over 90% of cells were CD11c+, indicating a DC phenotype (see **Figure 16A**). Exposure to LPS led to the expected rise in most co-stimulatory molecules in the vast majority of the DC population with the exception of CD40.

Further assessment focused on a range of cytokines produced following maturation. The data indicates a range of pro-inflammatory cytokines released following exposure to LPS (see **Figure 16B**). In contrast, imDCs produced minimal levels of any cytokine with only TNF-α and IL-6 being present at concentrations above the detection limit. Thus, the exposure to LPS produces a population of matured DCs that are highly homogenous with regards to cell type and expressing a range of co-stimulatory molecules necessary for Th cell activation.

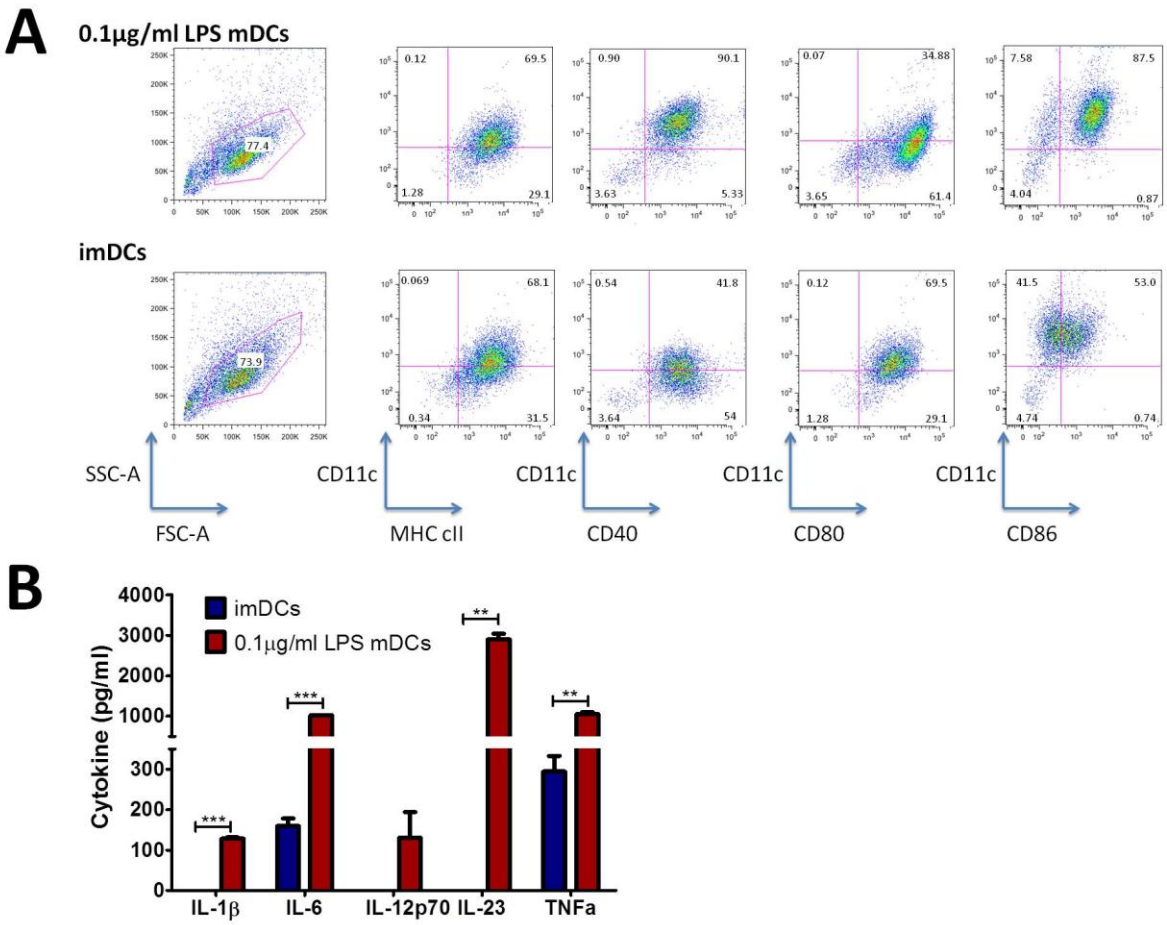

Figure 16 - Characterisation of LPS mDCs. 2x10^6 bone marrow precursors were cultured for 10 days prior to the addition of 0.1µg/ml of LPS in the case of mDCs. At day 11 cells were harvested and 1x10^6 cells stained for various markers as indicated (**A**). Flow cytometry plots show an example of three independent experiments. Cytokine production was also assessed (**B**) by ELISA 24h after exposure to LPS. Data shows mean of three independent biological experiements (two technical duplicates per biological repeat) ± SEM. Significance was determined by paired *t*-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001).

3.5 DC:T cell ratio and LPS concentration limits background IL-17 release

The initial stage was to design an *in vitro* co-culture system that would allow the evaluation of how changes to TCR signal intensity affects Th17 cell development. Therefore a number of factors were assessed to understand the optimal conditions for culture. These included the LPS concentration used to mature DCs, the number of T cells per well and the ratio of DCs relative to T cells. All of these could alter the response in terms of proliferation and cytokine production. Earlier experiments indicated a surprisingly high amount of background cytokine production in the absence of cognate peptide (data not shown). **Figure 17** demonstrates the effects of changing T cell numbers, LPS concentration and DC cell:T cell ratio on the non-specific release of both IL-17 and IFNγ. These factors were assessed by titration of T-cell density (either $2.5x10^5$ or $1.25x10^5$ naïve 5/4E8 TCR Tg cells per well in a 48-well plate) against DCs (1:10-1:40; DC: T cell ratio) matured at different LPS concentrations (1-100ng/ml). These assays were carried out in the presenece of pro-Th17 cytokines. $5x10^5$ T cells per well were also assayed although these numbers were discontinued as these produced far greater background cytokine levels. In addition, imDCs were used as a control, having not been exposed to LPS or other maturation stimuli. After 5 days of co-culture the supernatants were collected and analysed for IL-17 and IFNγ. Importantly, no cognate peptide was supplied to any group.

Of either cytokine tested, IL-17 was induced at greater levels than IFNγ, with the latter never reaching a concentration greater than 500pg/ml across any of the groups tested. Both demonstrated a proportional decline with T-cell density and ratio of DCs to T-cells. The non-specific release of IL-17 was particularly dependent on T-cell numbers and APC maturation state, as the concentration found in co-culture with either imDC or 1ng/ml LPS-matured DC was substantially lower at either density. In all groups, as the ratio of DC:T-cells was decreased, the magnitude of the IL-17 release declined. Thus, it is apparent that all these factors contribute to cytokine release in the absence of the cognate peptide p84-103. Reducing the DC:T

cell ratio to 1:20 and the total T cell number to $1.25x10^5$ cells per well reduced the background cytokine response to below 200pg/ml, independent of the LPS concentration used during DC maturation. Thus, these conditions reduced the non-specific release of cytokines to a minimal level. These ensure that the response determined in secondary cultures is influenced specifically by the peptide used in primary culture.

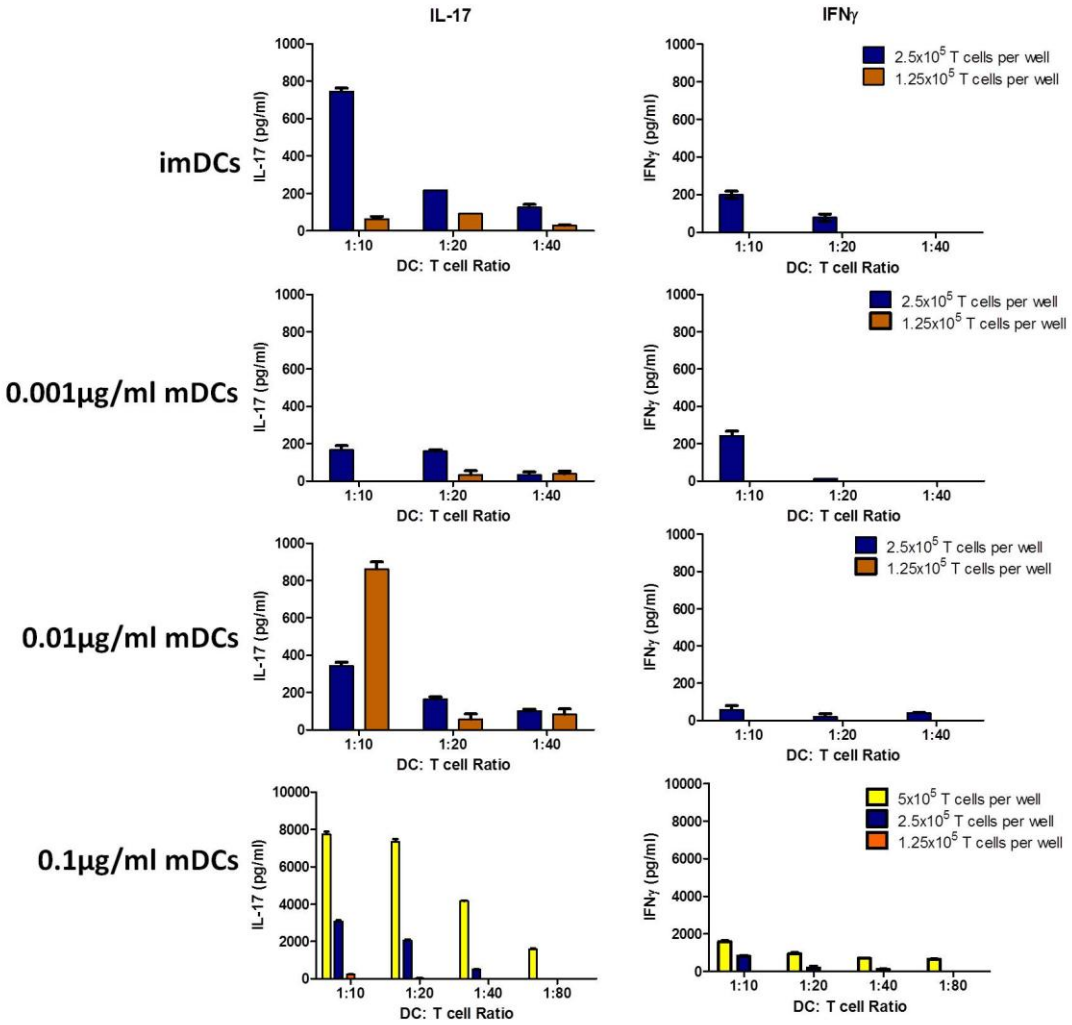

Figure 17 - Assessment of background cytokine response. Naive 5/4E8 TCR Tg T cells were co-cultured at either $5x10^5$, $2.5x10^5$ or $1.25x10^5$ cells per well in a 48-well plate with DCs at differing ratio of DC:T-cells (1:10-1:40) in the presence of pro-Th17 cytokines. DCs were matured in the presence of 1-100ng/ml of LPS for 24h prior to co-culture with T-cells. Cognate peptide was not added to any culture. Co-cultures were incubated for 5 days before collection of supernatants and cytokine concentrations determined by ELISA. Data shows mean of two independent biological experiements (three technical triplicates per biological repeat) ± SEM.

3.6 Proliferation response of 5/4E8 TCR Tg naive T cells

When varying the peptide concentration used during activation, the degree of T cell expansion was an important factor to consider. The proliferative response of naive 5/4E8 TCR Tg T cells was assessed both in terms of kinetics and in response to a titrated dose of cognate peptide. An A^d-binding peptide derived from an unrelated antigen (F1p3 - a capsular protein of *Yersinia pestis*) was also included as a negative control not expected to be recognised by the 5/4E8 TCR. 3H-thymidine incorporation was measured at either day 3, 5 or 7 of co-culture. **Figure 18A** shows a strong response to the cognate peptide even at day 3 in terms of the peak response of $1x10^5$ cpm, which declined after day 5. Given the peak response at day 3, an extended titration of p89-103 concentration was also evaluated. **Figure 18B** indicates the changing levels of 3H-thymidine incorporation as the dose of p89-103 was altered (p89-103 EC50 0.005µM). Cell counts at day 5 show a similar numbers of cells present across a range of p89-103 concentrations > 0.02µM (see **Figure 19**). Cell viability as assessed by Trypan Blue was found to be significantly greater than those 5/4E8 TCR Tg T cells stimulated with F1p3 across all concentrations of p89-103. Both cell counts and viability were similar in both the presence or absence of exogenous Pro-Th17 cytokines. Thus, it is apparent that 5/4E8 TCR Tg T cells proliferate rapidly in response to a wide range of peptide concentrations. In terms of kinetics the response peaks within 72h and was maintained until day 5.

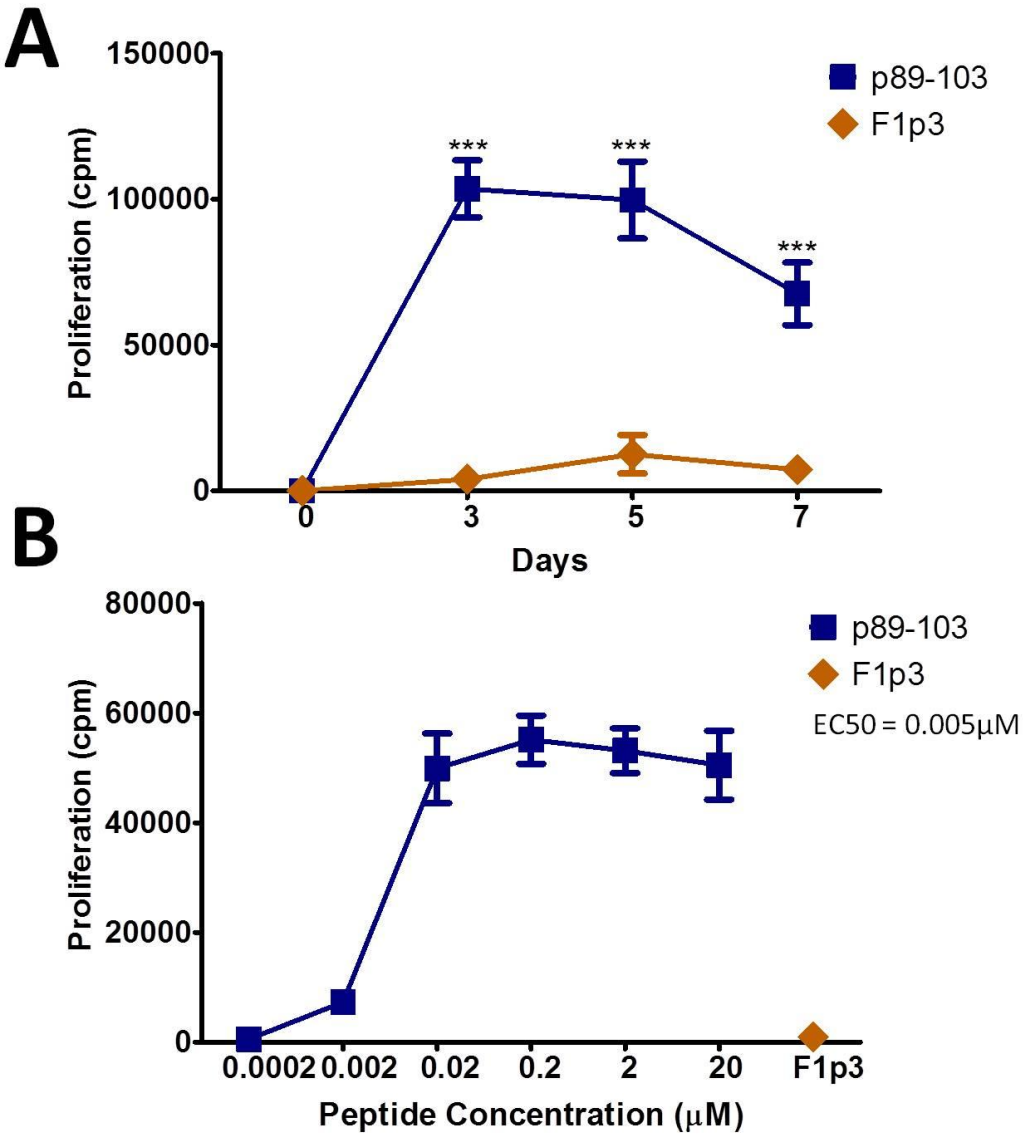

Figure 18 - Proliferative response of 5/E4 TCR Tg naive CD4+ T cells to p89-103. $4x10^3$ naive 5/4E8 TCR Tg T cells and $2x10^3$ 0.1µg/ml LPS mDCs were co-cultured with 2µM of p89-103 or F1p3 in flat bottom 96-wells plates (same ratio as **Figure 16** - scaled down to smaller surface area of 96 well plates) for either 3, 5 or 7 days (**A**). Significance between p89-103 and F1p3 was determined by two- way ANOVA - Bonferroni post t-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001). (**B**) A range of peptide concentrations were also established (0.0002-20µM) with results analysed after 72h. Co-cultures were pulsed with radioactive thymidine (3H) for the last 12h of co-culture. At the respective time points DNA was harvested onto glass fibre filters prior to counting with a scintillation counter and counts per minute (cpm) determined. Data shows mean of three independent biological experiements (two technical duplicates per biological repeat) ± SEM. EC50 was calculated using non-linear regression on a $\log_{10}$ scale.

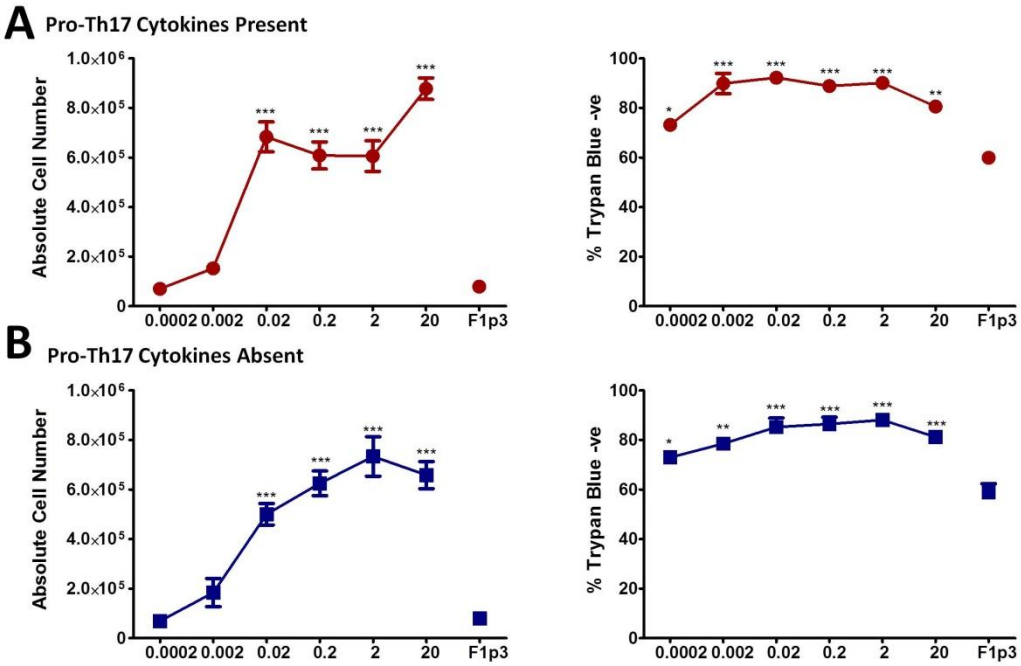

Figure 19 - Absolute cell counts and viability of 5/4E8 TCR Tg T cells activated with p89-103. Co-cultures of $1.25x10^5$ 5/E4 TCR Tg T cells and $6.25x10^3$ mDCs were established across a range of p89-103 concentrations (0.002-20µM) or F1p3 (2µM). Assays were established in the presence (**A**) and the absence (**B**) of pro-Th17 cytokines. After 5 days cells were harvested, washed and cell numbers determined using a neubauer haemocytometer. Trypan blue was used to assess cell viability with trypan positive cells being determined as dead. Data shows mean of three independent biological experiments (four counts per biological repeat) ± SEM. Significance relative to F1p3 was determined by one-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001)

3.7 Cytokine response is altered by peptide density

After determing the proliferative response to different peptide concentrations, it was important to determine if changes in peptide density would also alter the cytokine profile of the response. Previous data have shown that alterations in the density of anti-CD3/CD28 beads can affect human Th17 responses, with a low ratio of beads relative to cells promoting Th17 cells (278). It was important to build on this further using the more physiological context of naive T cells activated by cognate peptide presented by APCs.

The cytokine profile was initially determined using the two step co-culture model as optmised above. Briefly, $1.25x10^5$ naive T cells were activated with a range of peptide densities and $6.25x10^3$ mDCs. After an initial 5 days of co-culture, T cells (DC contamination was minimal as assessed by microscopy) were washed and re-plated at a fixed cell density, $4x10^4$, in order to normalise for expansion. These secondary cultures were re-stimulated with a fixed dose of p84-103 (2μM) presented by 0.1μg/ml LPS matured DC before a further 48h of culture (see **Figure 11**). Supernatants were harvested and the level of IL-17, IL-4 and IFNγ assessed by ELISA (see **Figure 20**). Given that the cell number and stimulus are equalised across all the secondary co-cultures any difference in cytokine release can be attributed to any alteration in primary culture.

Focusing on the IL-17 response, it is apparent that the presence of pro-Th17 cytokines was required for significant levels of IL-17 release, as without these IL-17 levels failed to exceed that from the negative control. Thus, there was a need for exogenous pro-Th17 cytokines despite **Figure 16** indicating DC production of IL-23 and IL-6. Even in the presence of exogenous pro-Th17 cytokines, IL-17 production varies across the range of peptide concentrations used in the primary culture. Agreeing with the hypothesis that a reduced TCR signal positively promotes Th17 cell development, release of IL-17 was found to be greater at lower peptide concentrations at the T cell priming phase. This was also evident in the negative slope value of the curve when assessed by linear regression.

In contrast, the levels of IL-4 demonstrated the inverse relationship to that of IL-17. IL-4 production did not vary greatly in the presence of pro-Th17 cytokines but increased steadly with the concentration of p89-103 used in primary cultures. Similarly, IFNγ showed a positive correlation with the peptide concentration present during the primary co-culture. Production was, again, not significantly altered by the presence or absence of pro-Th17 cytokines.

Given the length of the secondary co-cultures it was important to assess if these cells responded in the same manner, despite distinct conditions occurring during the primary stages of the assay. Therefore, after harvesting of supernatants, 3H-thymidine was added to these cells and incubated for a further 12h. The level of proliferation was determined for both a low and a high peptide concentration (see **Figure 21**). Those exposed to F1p3 during the initial culture stage were also assayed.

The results indicate that despite being exposed to peptide concentrations during T cell priming that differed a hundred-fold (2 vs 0.02μM), the degree of expansion in the secondary cultures was similar. The cells exposed to the negative control peptide F1p3 proliferated to a far less extent, perhaps reflecting a poor condition of these T cells. There was a slightly reduced response in those co-cultures not exposed to pro-Th17 cytokine although the degree of expansion remained greater than that of the F1p3 'stimulated' cells. It may be that the addition of exogenous cytokines potentiates the T cell response to a certain extent.

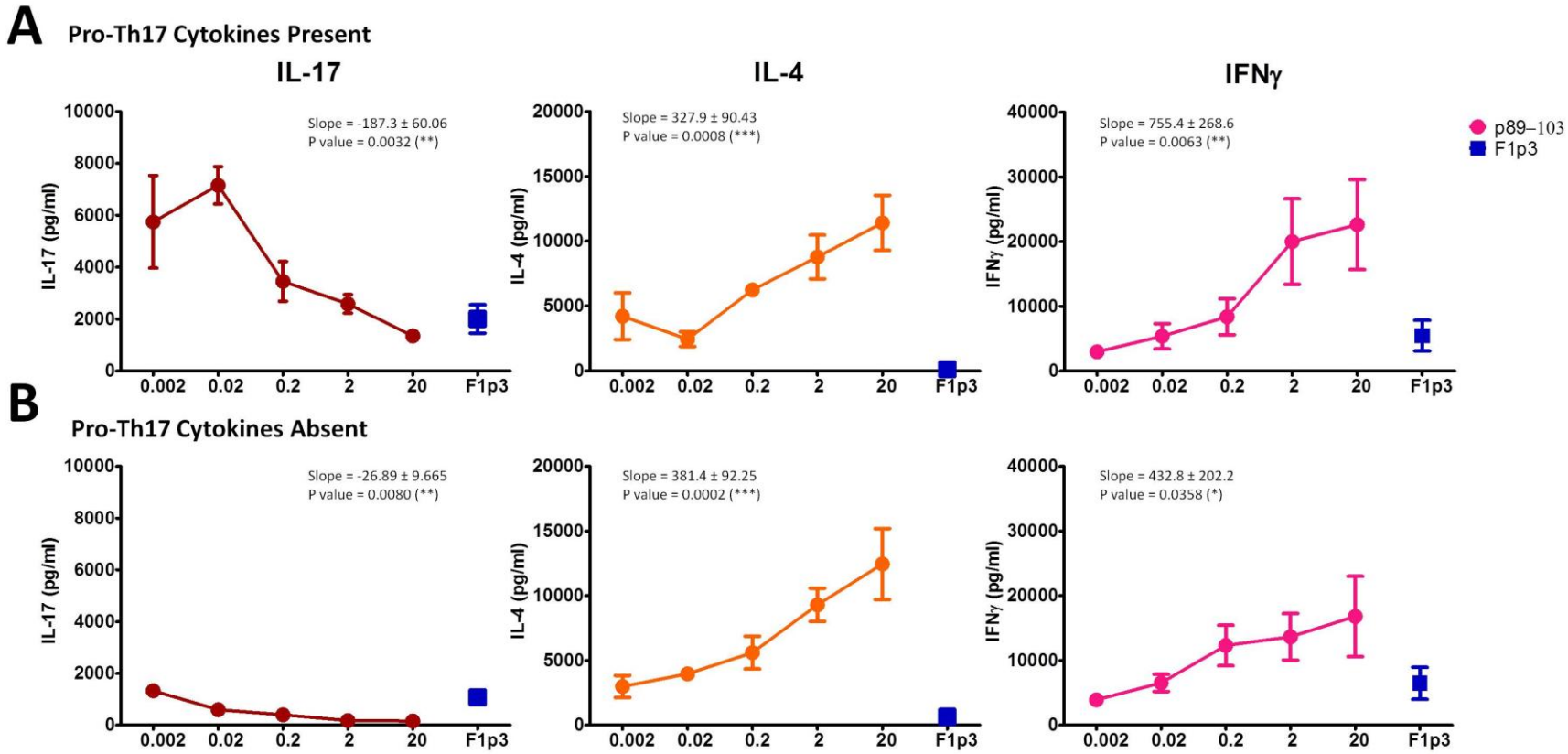

Figure 20 - Peptide concentration can shape the cytokine response. Co-cultures of 5/E4 TCR Tg T cells and mDCs were established across a range of p89-103 concentrations (0.002-20μM) or F1p3 (2μM). After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2μM of p89-103. These assays were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. After a further 48h supernatants were harvested and stored at -20°C prior to analysis. ELISAs for IL-17, IL-4 and IFNγ were performed according to the manufacturer's instructions. Data shows mean of three independent biological experiements (three technical triplicates per biological repeat) ± SEM. Linear regression was used to calculate value of the slope to determine the influence of peptide concentration.

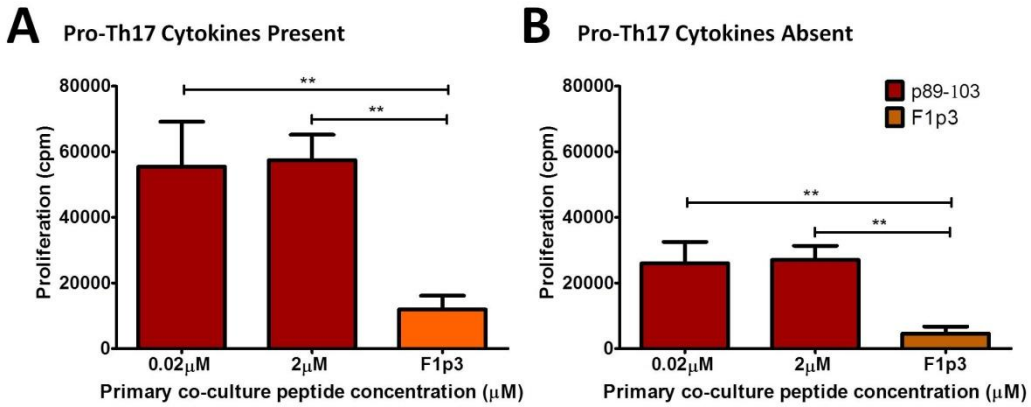

Figure 21 - Proliferation in secondary co-cultures. 4x10^4 T cells activated in primary co-cultures as described above were washed and re-plated with 2x10^3 LPS mDC and 2μM of p89-103. This was carried out from those established in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines during primary co-culture. These were incubated for a 48h prior to harvesting of supernatants and pulsed with 3H-thymidine. After a further 12h DNA was harvested onto glass fibre filters prior to counting with a scintillation counter and counts per minute (cpm) determined. Data shows mean of five independent biological experiements (three technical triplicates per biological repeat) ± SEM. Significance was determined by one- way ANOVA - Bonferroni post t-test (*p= <0.05, ** p=<0.01 *** p=<0.001).

3.8 Antigen density also alters the frequency of Th cell subsets

While determining the level of cytokine production, it was also important to assess if the changes in the type of cytokine were also reflected in the frequency of cells expressing the relevant cytokine. Co-cultures of naive 5/E4 TCR Tg T cells were established with mDCs as described above. The same dose titration of p89-103 was also used. After 5 days cells were re-stimulated with PMA/Ionomycin for 5h with Brefeldin A (BFA) added for the last 4h. The frequencies of IL-17-, IL-4- and IFNγ-producing T cells were subsequently assessed by intracellular cytokine staining (ICS).

As expected the presence of pro-Th17 cytokines was required to enhance the frequency of IL-17+ cells. **Figure 22** demonstrates that despite the presence of these cytokines across all populations, the frequency of Th17 cells varied greatly. Indeed, there was a persistent increase in the percentage of IL-17+ cells at lower peptide concentrations (see **Figure 23**) . These data were broadly consistent with the responses detected using the secondary culture system (see **Figure 20**).

By far the most frequent cell type were Th2 cells, detected on the basis of IL-4 expression. In contrast to the IL-17 response, the frequency of IL-4+ cells showed a marked increase with increasing peptide concentrations (see **Figure 23**). This correlation is confirmed by the positive value of slope when analysed by linear regression. The presence of pro-Th17 cytokines caused only a minor, non-significant, decrease in the frequency of Th2 cells (see **Figure 22A**). In comparison the frequency of IFNγ+ cells was far less than either the percentages of IL-17- or IL-4-producing T cells (see **Figure 24**). However, if pro-Th17 cytokines were removed, a positive relationship between IFNγ and peptide concentration was found. Taken together these data demonstrate a similar response across the peptide concentrations tested when compared to the amount of cytokine detected by ELISA. Furthermore, it shows how changes in peptide concentration shift the type of Th response from one with a high frequency of Th2 cells, at high peptide concentrations, to one in which Th17 cells become the most common, at low peptide concentrations.

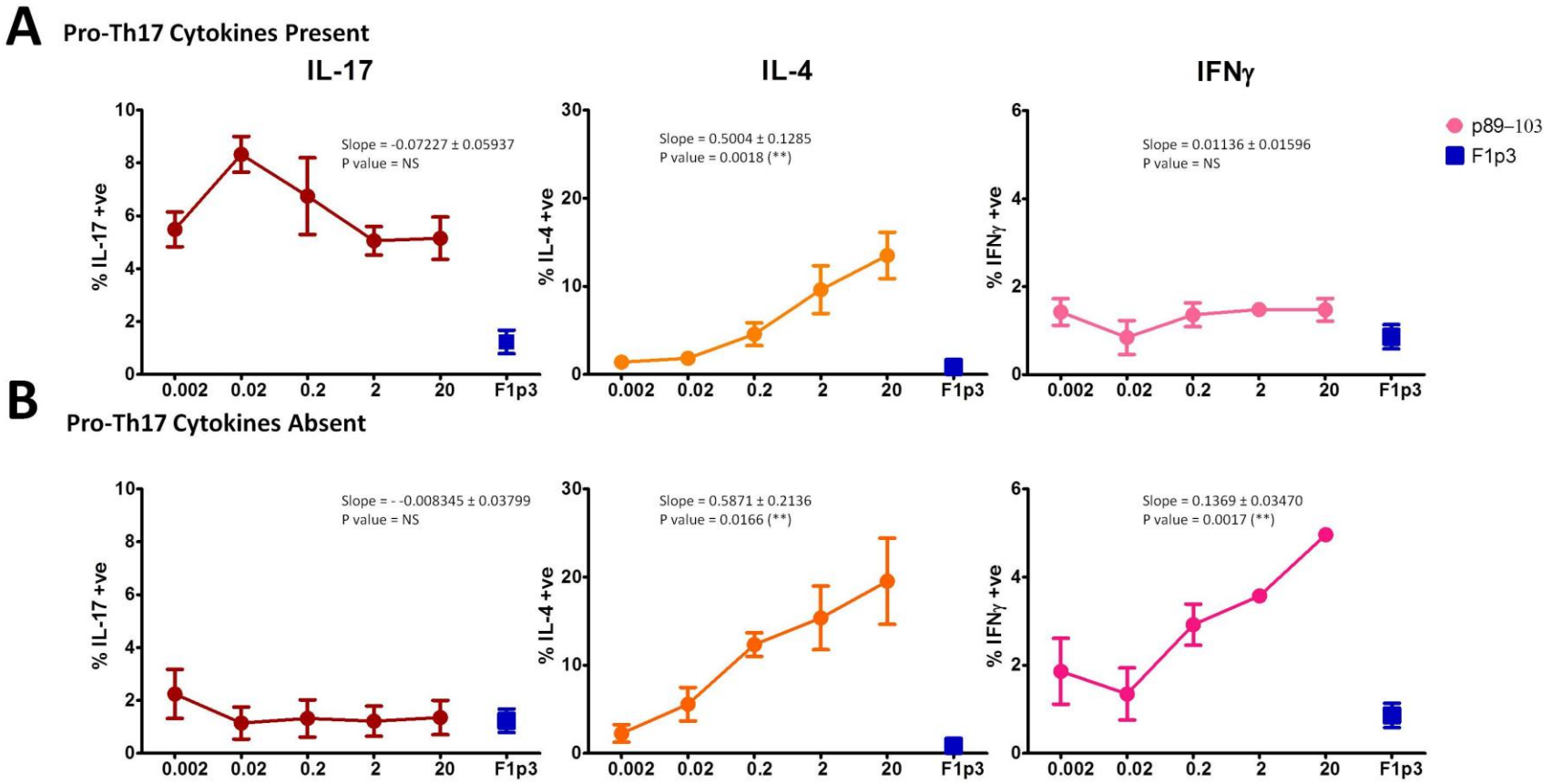

Figure 22 - Peptide density alters the frequency of cytokine-producing T cells. Co-cultures of naïve 5/E4 TCR Tg T cells and mDCs were established across a range of p89-103 concentrations (0.002-20μM) or F1p3 (2μM). After 5 days cells were re-stimulated with PMA/I for 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of IL-17-, IL-4- and IFNγ-producers within the CD4+ population. Data shows mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM. Linear regression was used to calculate value of the slope to determine the influence of peptide concentration.

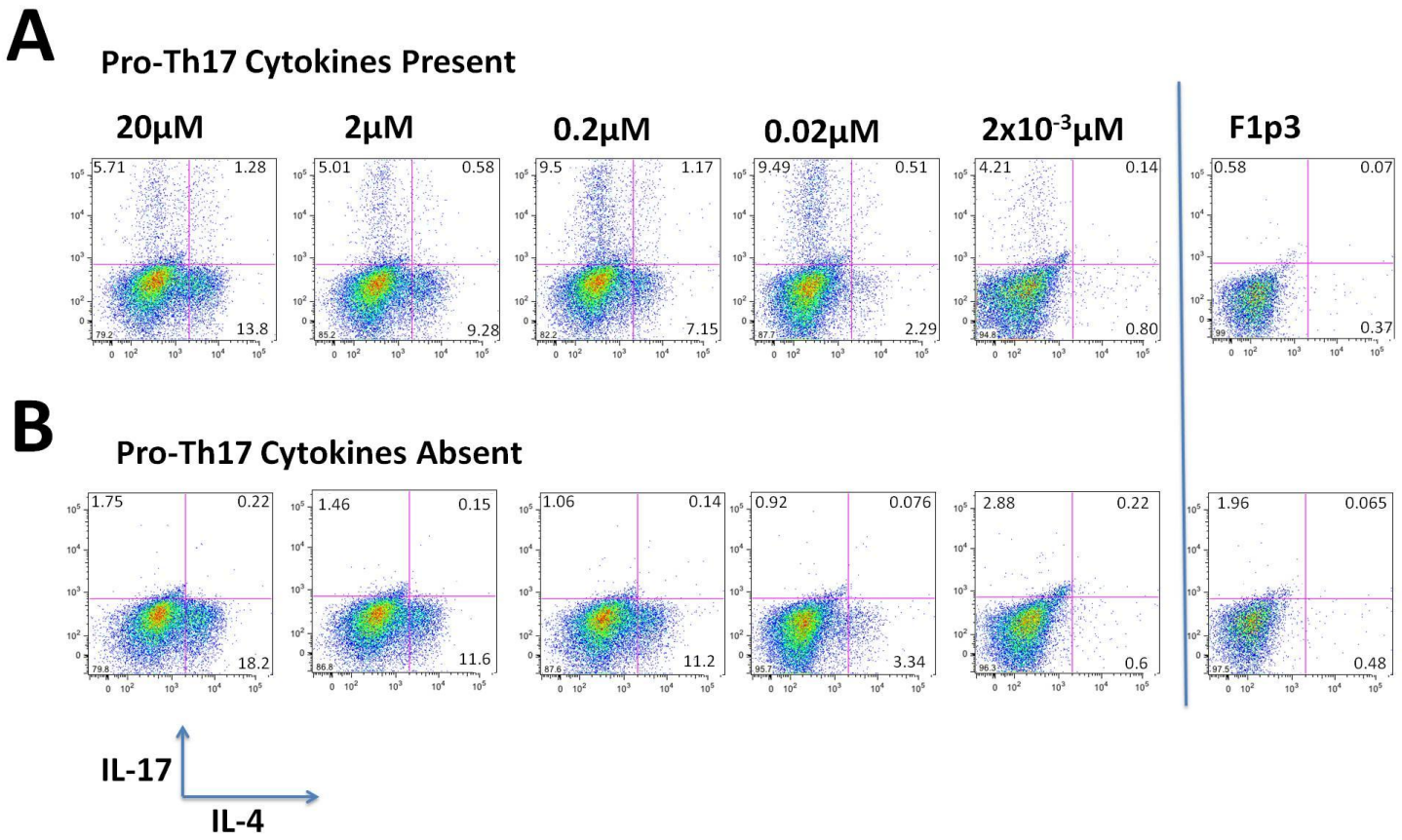

Figure 23 - Frequency of IL-17- and IL-4-expressing Th cells. Example of p89-103 activated cells stained for CD4, IL-17 and IL-4 and gated on CD4. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established over a range of concentrations of p89-103 ($2x10^{-3}$-20µM) or F1p3 (2µM). Cells were stimulated in both the presence (**A**) or absence (**B**) of pro-Th17 cytokines.After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Data are a representative example of three independent experiments.

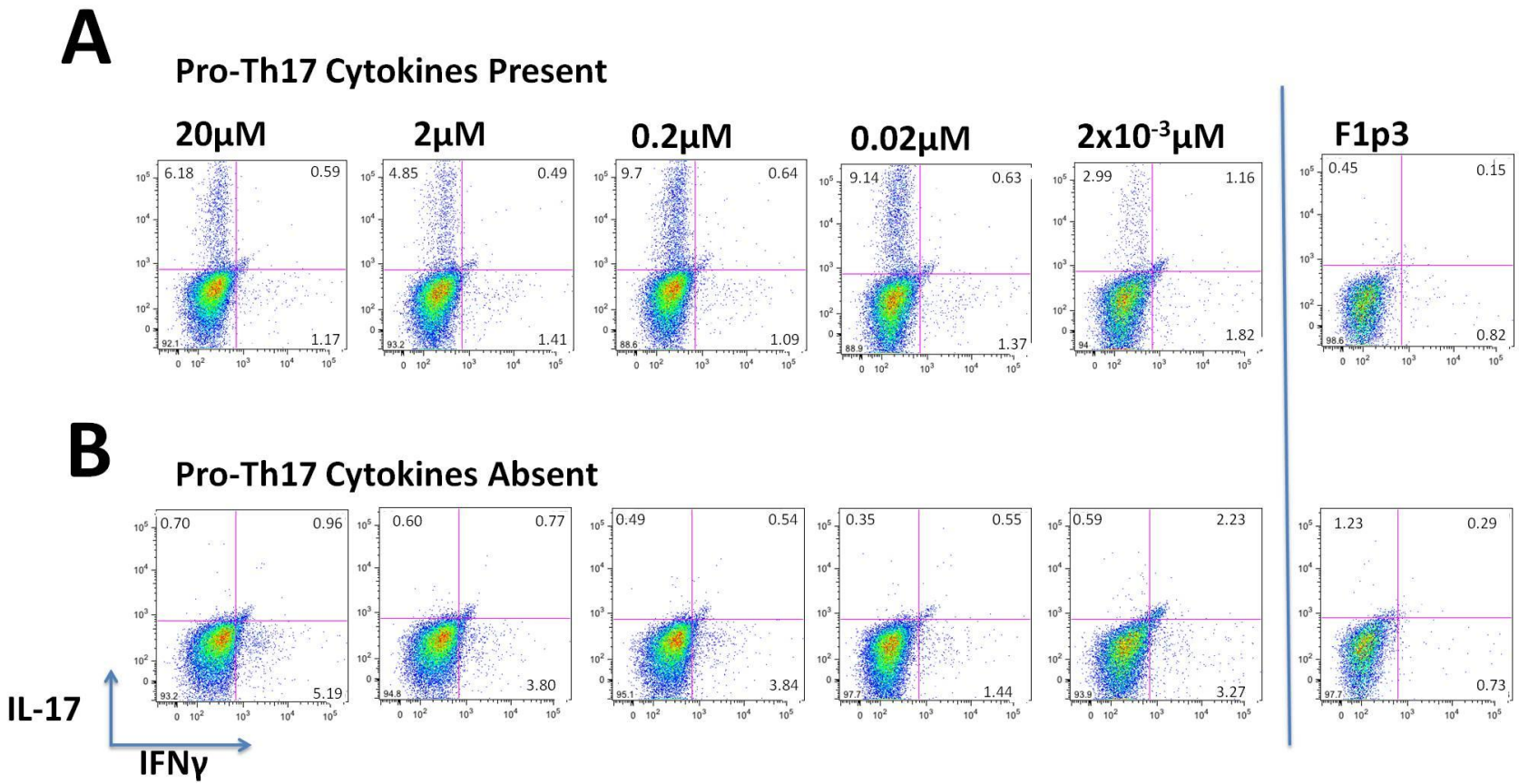

Figure 24 - Frequency of IL-17- and IFNγ-expressing Th cells. Example of p89-103 activated cells stained for CD4, IL-17 and IL-4 and gated on CD4. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established over a range of concentrations of p89-103 ($2x10^{-3}$-20µM) or F1p3 (2µM). Cells were stimulated in both the presence (**A**) or absence (**B**) of pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Data are a representative example of three independent experiments.

3.9 Transcription factor expression

Underpinning the expression of distinct cytokine profiles are the presence of a range of transcriptional regulators. These form an essential component of the classical definition of each Th cell subset, as these control multiple aspects of the cellular phenotype. Genetic ablation of these genes prevents the development of a particular subset. For example, ROR-γT is critical for Th17 development, inducing expression of both IL-17a and IL-23R (196). Similarly, GATA3 is required for IL-4 production by Th2 cells (350). Therefore, it was important to understand if changes to the cytokine profile induced by distinct peptide concentrations were reflected in changes in transcription factor expression. Naive 5/4E8 TCR Tg T cells were activated with at either a high or low peptide concentration. At day 5 cells were re-stimulated with PMA/I and the expression of ROR-γT, GATA-3, T-bet and FOXP3 assessed using flow cytometry. This allowed for simultaneous assessment of cytokine production and transcription factor expression within the same population. These assays were carried out in the presence and absence of pro-Th17 cytokines.

Figure 25 shows that peptide concentration did affect the frequency of ROR-γT+ cells. Expression was greatest in those T cells activated with at a low concentration of p89-103. Furthermore, expression of ROR-γT did depend on the presence of pro-Th17 cytokines with the frequency of ROR-γT+ cells greatly reduced in their absence. GATA-3 was found to be relatively widely expressed at both peptide concentrations used to activate naive T cells. Only in the absence of pro-Th17 cytokines did the frequency of GATA-3 cells increase significantly at a high concentration of the cognate p89-103 peptide.

FOXP3 is the key regulator of the Treg phenotype (351). Although FOXP3+ cells could be detected, the frequency of these cells did not vary at either peptide concentration tested. In those cultures activated without pro-Th17 cytokines there was no significant increase in FOXP3 expression(see **Figure 28**). With regards to the

expression of T-bet, critical to development of Th1 cells, there was no significant change when T cells were activated with a low or high peptide concentration.

GATA-3 and ROR-γT were also co-stained with IL-4 and IL-17, respectively, to allow assessment of transcription factor and effector cytokine expression simultaneously (see **Figure 26** and **Figure 27**). Unsurprisingly, the data show that all IL-4+ cells also express GATA-3. Similarly, all IL-17+ cells all co-express ROR-γT. However, the majority of cells that stain positive for either transcription factor fail to express the related cytokine. This may be related to a failure to re-stimulate all the cells sufficiently to express the cytokine. Thus, not all cells expressing a transcription factor are in an appropriate stage of differentiation to expression a particular cytokine at detectable levels. In summary, the data highlights a distinct regulation of cytokine and transcription factor expression. In the case of Th17 cells, ROR-γT levels were significantly altered in line with the cytokine profiles suggesting that the peptide concentration can influence the development of these cells at both levels. In contrast, GATA-3 was relatively resistant to changes in peptide concentration especially in the presence of pro-Th17 cytokines. The relatively high expression of this transcription factor was also unexpected.

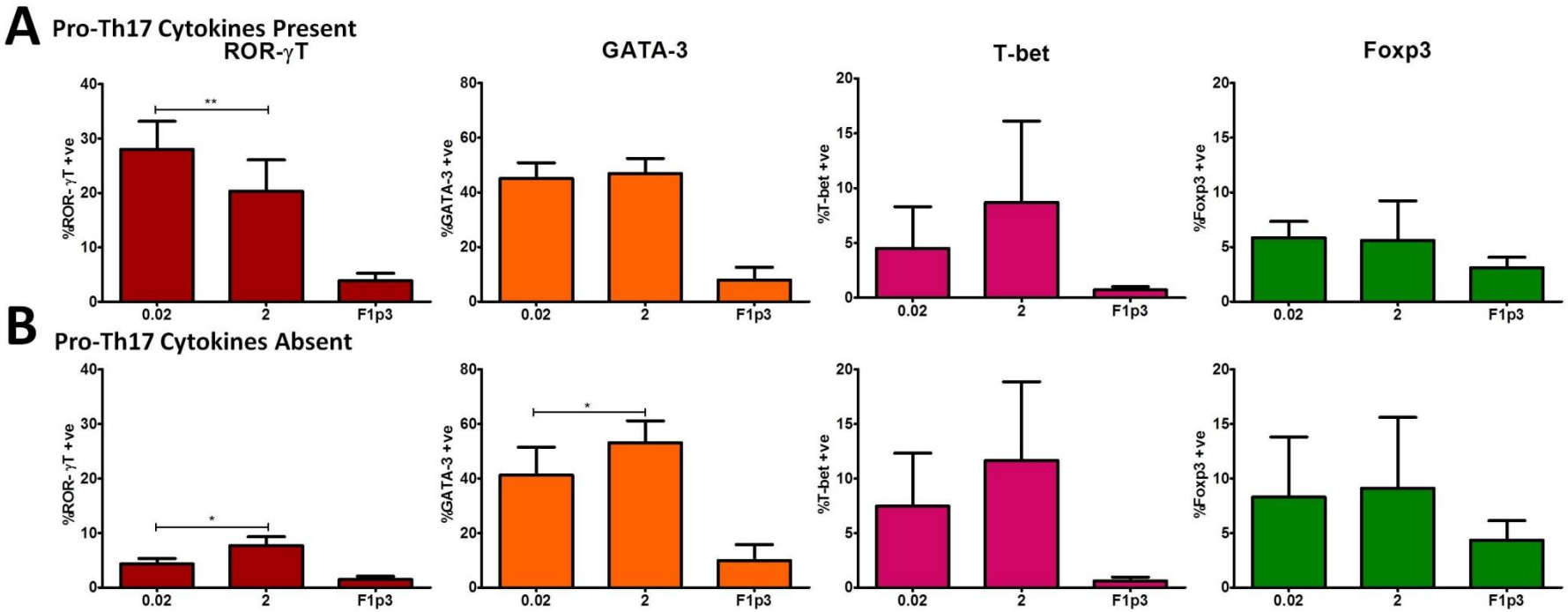

Figure 25 - Activation at a high or low peptide concentration alters the expression of transcriptional regulators. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of p89-103 or F1p3 (2µM). Cells were stimulated in both the presence (**A**) or absence (**B**) of pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of ROR-γT, GATA-3, T-bet and FOXP3 positive cells within the CD4+ population. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM. Significance was determined by *t*-test (**p= <0.05, ** p=<0.01 *** p=<0.001*).

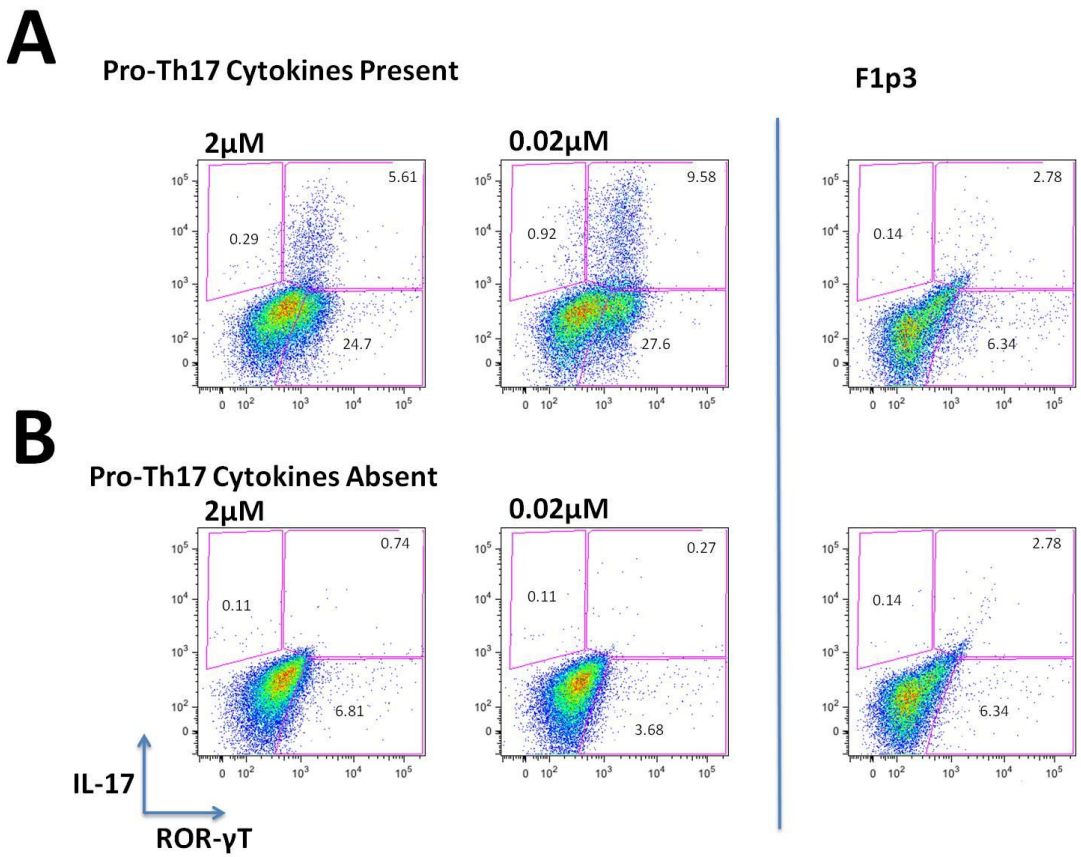

Figure 26 - Co-expression of IL-17 and ROR-γT in p89-103 activated populations. Example of p89-103 activated cells stained for CD4, IL-17 and ROR-γT. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of p89-103 or F1p3 (2µM). Cells were stimulated in both the presence (**A**) or absence (**B**) of pro-Th17 cytokines.After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Data are a representative example of three independent experiments.

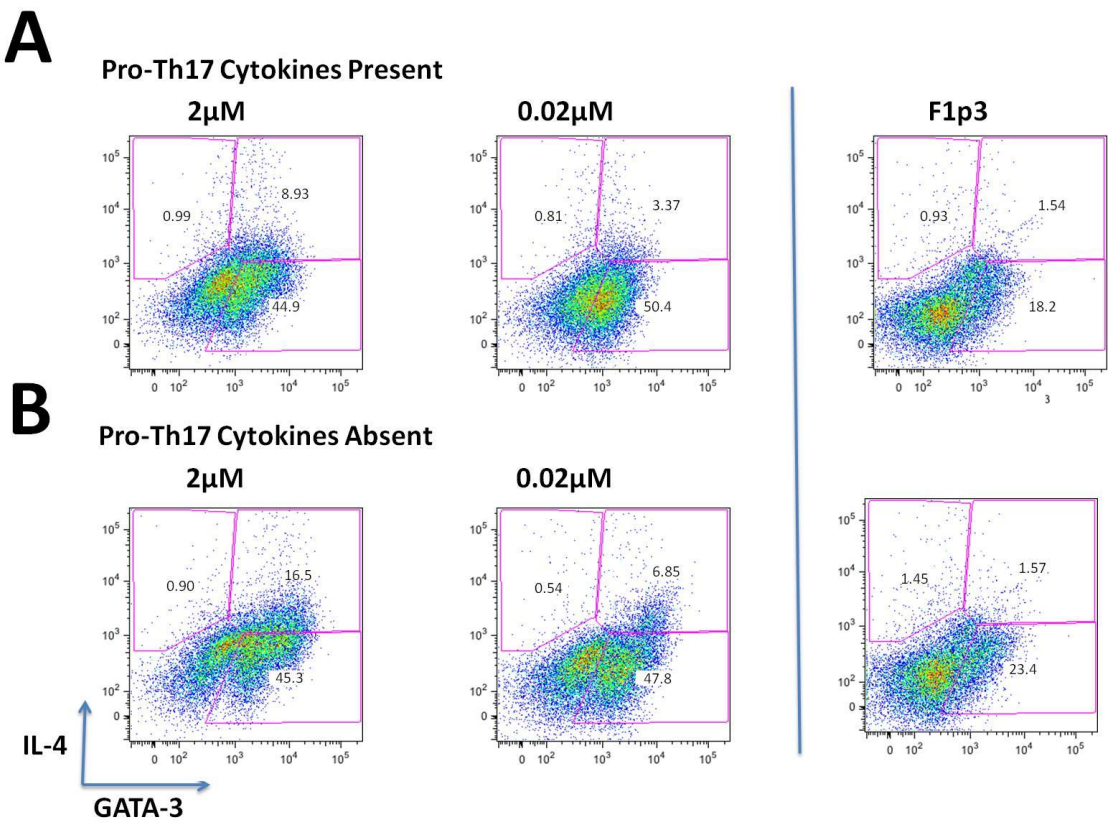

Figure 27 - Co-expression of IL-4 and GATA-3 in p89-103 activated populations. Example of p89-103 activated cells stained for CD4, IL-4 and GATA-3. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of p89-103 or F1p3 (2µM). Cells were stimulated in both the presence (**A**) or absence (**B**) of pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Data are a representative example of three independent experiments.

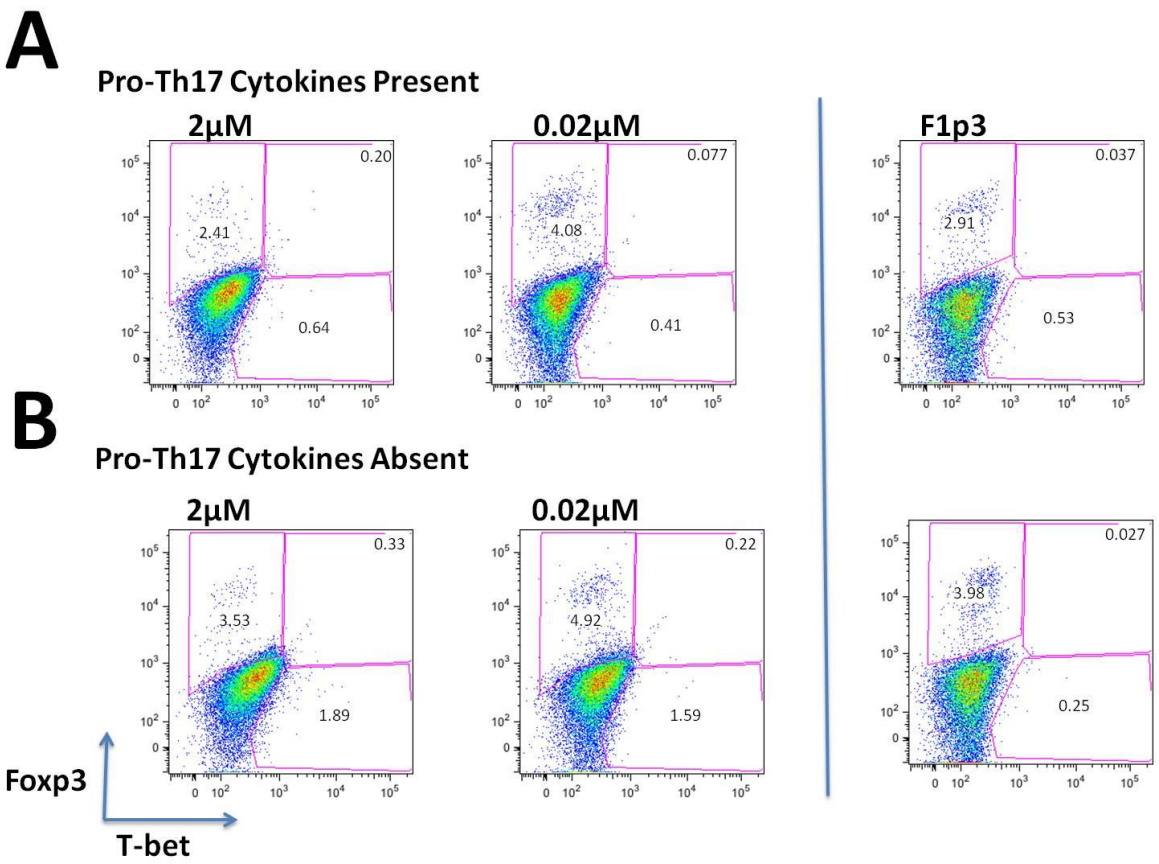

Figure 28 - FOXP3/ T-bet expression in p89-103 activated populations. Example of p89-103 activated cells stained for CD4, FOXP3 and T-bet. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of p89-103 or F1p3 (2µM). Cells were stimulated in both the presence (**A**) or absence (**B**) of pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Data are a representative example of three independent experiments.

3.10 Do changes in peptide density alter surface marker expression?

Some surface proteins can influence TCR signal strength (e.g. TCR, CD4) while the expression of others reflects the intensity of this signal (CD69, CD25, CD40L) (352-356). To understand this surface markers were assessed using flow cytometry over a number of time points. Typically T cells can down regulate the expression of TCR and co-stimulatory molecules such as CD4 in a mechanism that is dependent upon the strength of signal received through the TCR (356). Naive 5/4E8 TCR Tg T cells were activated with either a high or low concentration of p89-103 and the frequency of cells expressing a pan-TCRβ, Vβ4 (the Vβ component of the 5/4E8 TCR Tg T cells) and CD4 were established at day 0, 3, 5 and 7.

Using a generic TCRβ antibody there was a no significant variation between any of the T cell populations activated with a high or low peptide concentration (see **Figure 29A**). In contrast, expression of Vβ4, specific for the 5/4E8 Tg TCR, showed a significant decline in T cells activated with a high peptide concentration at Day 3 (see **Figure 29B**). At further time points Vβ4 expression did not vary significantly between those T cells activated at either peptide concentrations. CD4 levels are also closely linked to TCR expression. A 'strong' TCR signalling can result in the down regulation of CD4 as has been reported during the re-stimulation of cells using PMA/I (357). **Figure 29C** indicates a similar pattern of expression to that of TCRβ. Despite a slight, non-significant, decline in CD4 expression at day 3 in those T cells activated with p89-103, expression returns to day 0 levels at further time points.

Along with the expression of the TCR and CD4, the level of key activation markers was also determined at the same time points (see **Figure 30**). Both the expression of CD69, classically described as an early marker of T cell activation, and CD25, the high affinity component of the IL-2R and traditionally ascribed as a late marker of activation, were determined. Moreover, the proportion of cells expressing CD40 Ligand (CD40L; also known as CD154) was also assessed. All of these markers are significantly regulated by the degree of TCR signalling and so would be expected to be altered by changes in the TCR signal intensity.

Expression of both CD69 and CD25 followed a very similar pattern of expression. For both markers there was a marked and significant difference as a result of activation with different peptide concentrations. As expected, the higher concentration of peptide induced greater expression of CD69 and CD25 from day 3 onwards. Although the lower peptide concentration reduced frequency of positive cells, the pattern of expression remains very similar to the population activated with a higher peptide concentration. Interestingly, when activated in the absence of pro-Th17 cytokines, levels of both markers declined after Day 5.

In contrast to the relatively high percentage of CD69+ and CD25+ cells, CD40L was not expressed as widely across the time points assessed. The pattern of expression was similar to the other activation markers although the difference between high and low peptide concentrations was not significant. Thus, taken together these data highlight the distinct activation states that can be induced by alterations in the peptide concentrations. Moreover, these support the notion that these changes in peptide densities alter the intensity of the TCR signal sensed by each T cell.

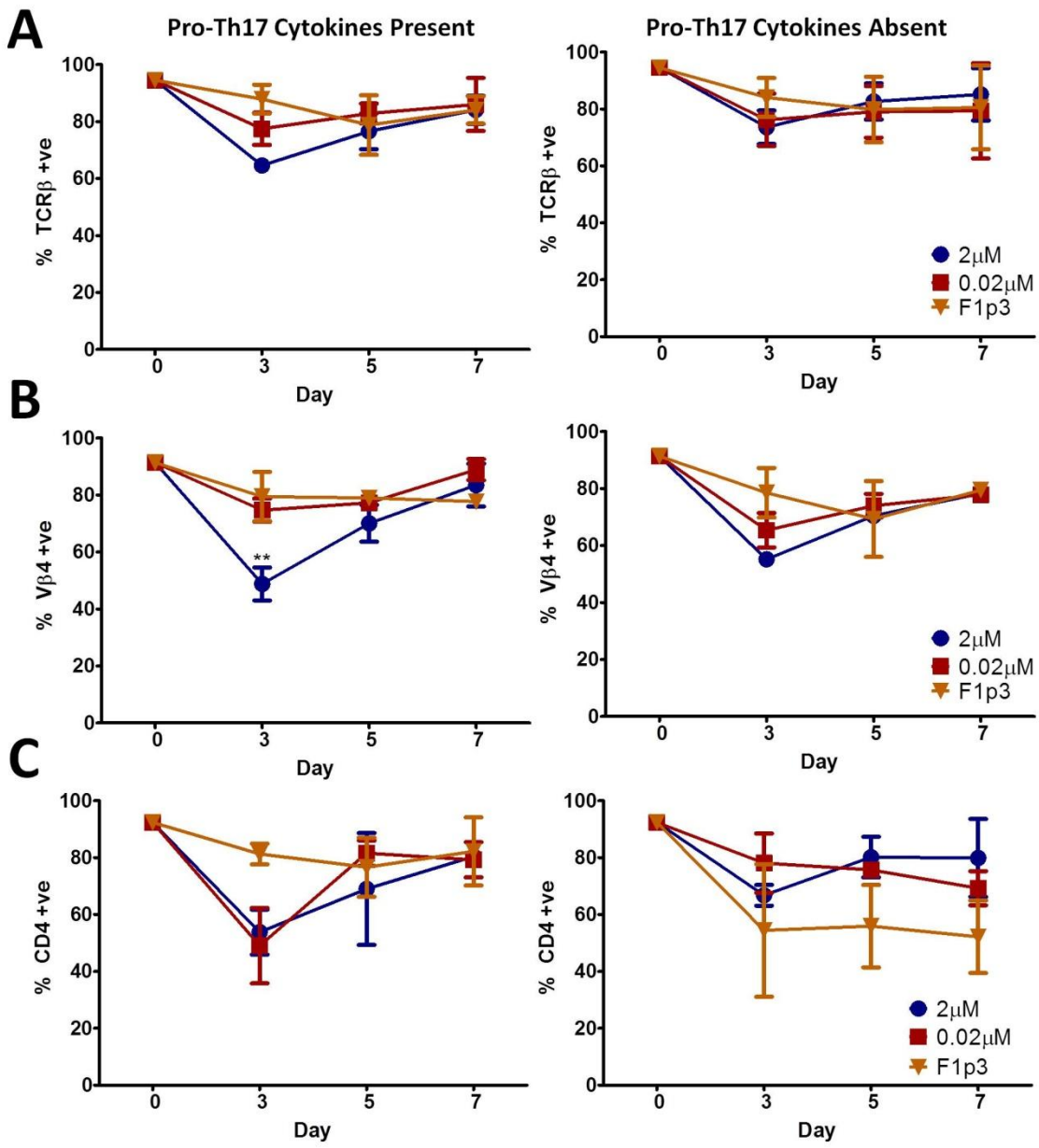

Figure 29 - The effect of peptide density on TCR and CD4 expression. Co-cultures naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2μM) or low (0.02μM) concentration of p89-103 or F1p3 (2μM). At day 0,3,5, and 7 cells flow cytometry was used to determine the proportion of TCRβ, Vβ4 and CD4 positive cells. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM. Significance was determined by two- way ANOVA - Bonferroni post t-test (*p= <0.05, ** p=<0.01 *** p=<0.001).

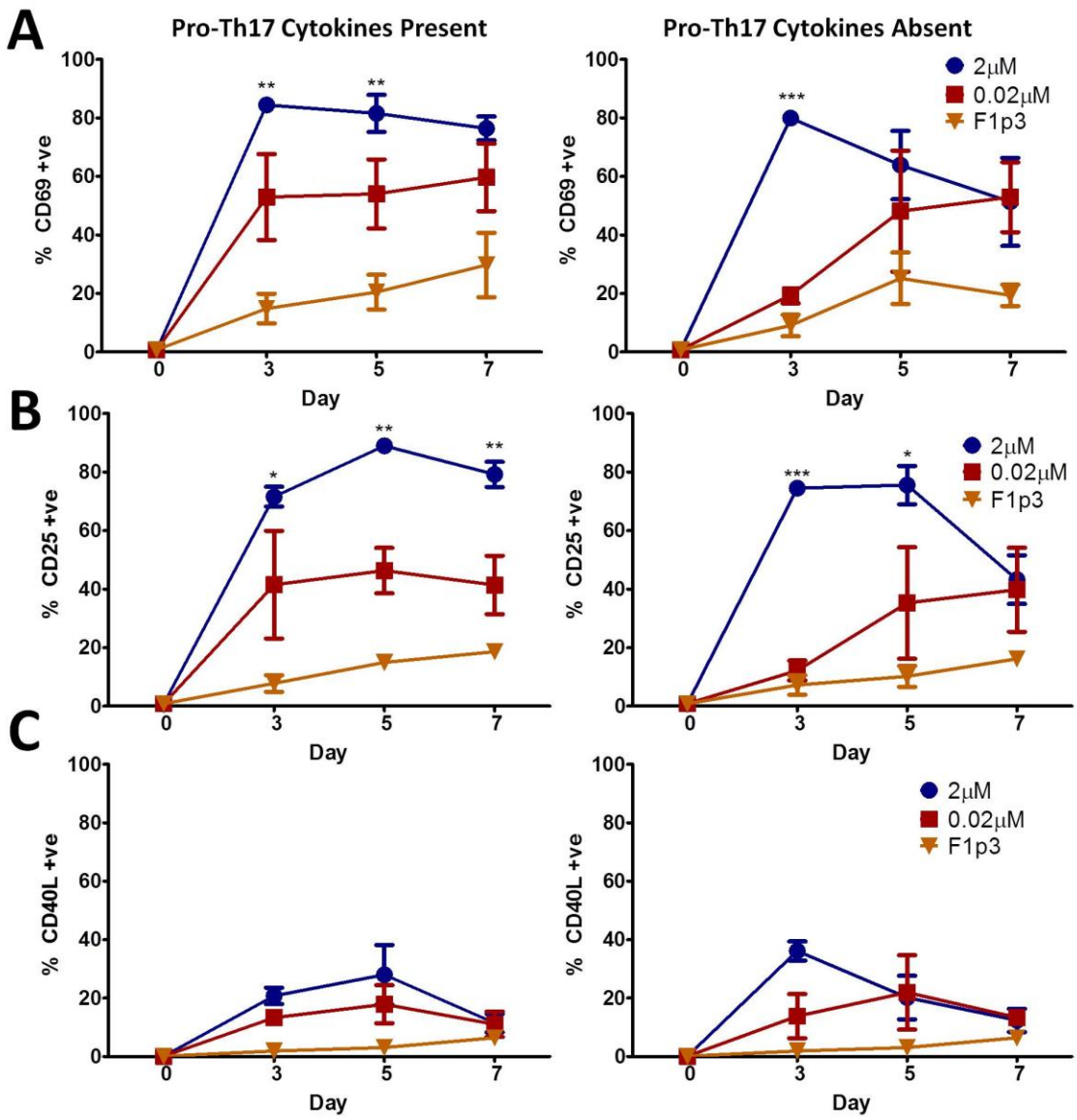

Figure 30 - The effect of peptide density on activation marker expression. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2μM) or low (0.02μM) concentration of p89-103 or F1p3 (2μM). At day 0,3,5, and 7 cells flow cytometry was used to determine the proportion of, CD69, CD25 and CD40L positive T cells. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM. Significance was determined by two- way ANOVA - Bonferroni post t-test (*p= <0.05, ** p=<0.01 *** p=<0.001).

3.11 The conversion of naive to memory cells

Exposure to antigen within the periphery promotes the conversion of naive T cells into a number of distinct states. Broadly these can be defined using the expression of the cellular adhesion molecule CD62L and its relative expression to CD44; another molecule involved in cell-to-cell contacts (358). Based on widely used definitions naive cells ($CD4^{+}CD62^{hi}CD44^{lo}$) are capable of differentiation into central memory cells (T_{CM}; $CD4^{+}CD62L^{hi}CD44^{hi}$) or effector memory cells (T_{EM}; $CD4^{+}CD62L^{lo}CD44^{hi}$). Both subsets of memory cells differ with respect to their anatomical locations, cytokine secretion and the rapidity of their responses. Using flow cytometry, the conversion of naive to memory cells was determined after 5 days of activation with a high or low peptide concentration (see **Figure 31**). As expected, exposure to peptide resulted in downregulated expression of CD62L and increased expression of CD44 on the majority of cells. While this conversion appeared greater in those activated with the higher peptide density, the difference between the high and low peptide-stimulated populations was not significant.

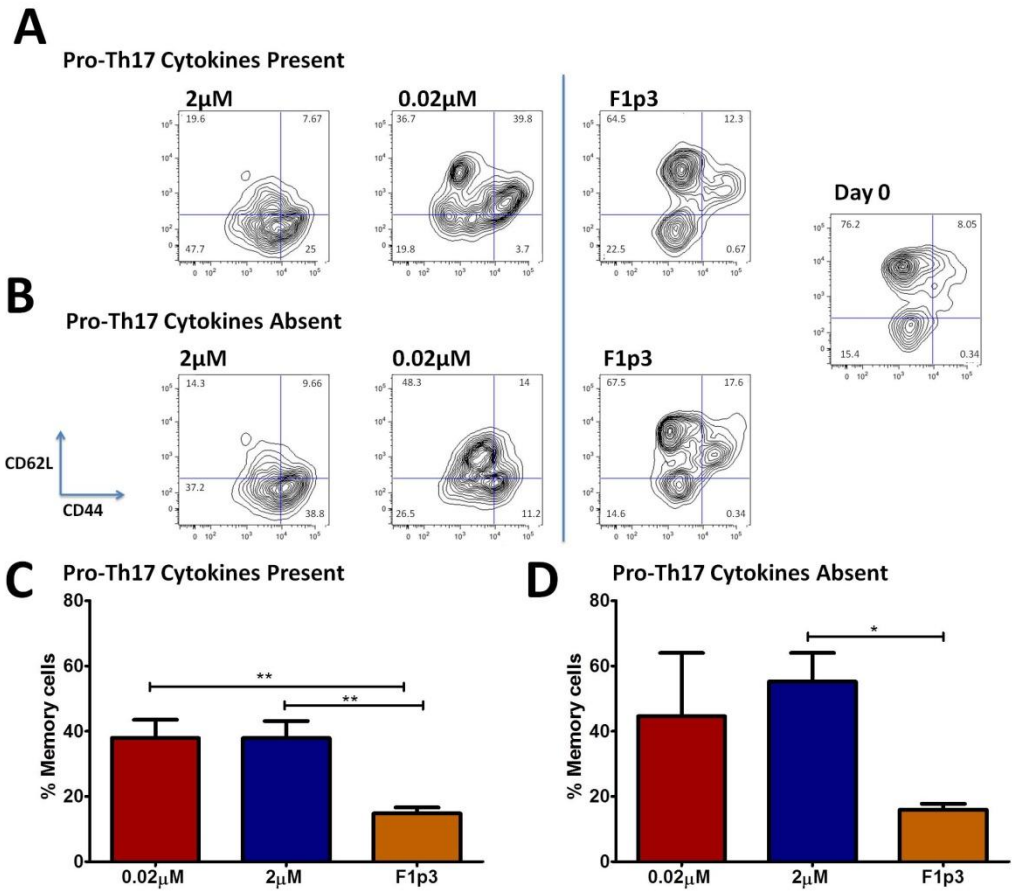

Figure 31 - Naive to memory T cell conversion. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of p89-103 or F1p3 (2µM). An example of the gating is shown in both the presence (**A**) and the absence (**B**) of pro-Th17 cytokines. After 5 days cells flow cytometry was used to assess the expression of CD44 and CD62L in the T cell population. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM in both the presence (**C**) and absence (**D**) of pro-Th17 cytokines. Significance was determined by one-way ANOVA (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001)

3.12 Production of Th17-associated cytokines is also regulated by antigen density

It is becoming increasingly apparent that IL-17 production alone is not the sole determinant of the pathogenicity of Th17 cells. Codarri *et al* demonstrated the importance of ROR-γT in the release of GM-CSF by T cells (239). Autoreactive T cells lacking GM-CSF failed to induce significant neuroinflammation in the disease model EAE (239). In contrast, IL-17$^-$IFNγ$^-$ T cells proved to be sufficient to trigger pathology. In addition, IL-22 has been described to be another Th17-associated cytokine playing an important role in the release of Matrix metalloproteinases (MMPs) and antimicrobial peptides (234). Therefore it was important to understand if GM-CSF and IL-22 were regulated in a similar manner as IL-17. Expression of these cytokines could suggest the development of a potentially pathogenic Th17 population. To understand if peptide concentration could alter the release of these cytokines, primary co-cultures were established with either a high or low peptide concentration. After 5 days cells were harvested and washed prior to restimulation with mDC and peptide as described previously.

Similarly to IL-17, both GM-CSF and IL-22 were found to be released at higher concentrations at the lower peptide concentration (see **Figure 32**). However, GM-CSF was reduced in the presence of pro-Th17 cytokines. In contrast, IL-22 increased with the addition of exogenous pro-Th17 cytokines. IL-10 is another important regulator of Th17 pathogenicity, thought to play an important role in self-regulating the T cell response (293). However, IL-10 levels were below the limits of detection when assessed by ELISA (data not shown).

Given recent data highlighting the importance of GM-CSF in the murine model of EAE it was important to determine the frequency of GM-CSF expressing cells (see **Figure 33**). Moreover, it was interesting to assess if T cells expressing GM-CSF were also co-expressing IL-17. In line with the ELISA data, the frequency of GM-CSF+ T cells was greater in those activated with a lower peptide concentration. Interestingly, the majority of GM-CSF+ cells lacked IL-17 co-expression. However, co-expression of IL-17 and GM-CSF was higher at the lower peptide density. Thus, not only does a low TCR

signal promote IL-17 expression it also increases the level of cytokines associated with the Th17 phenotype such as GM-CSF and IL-22.

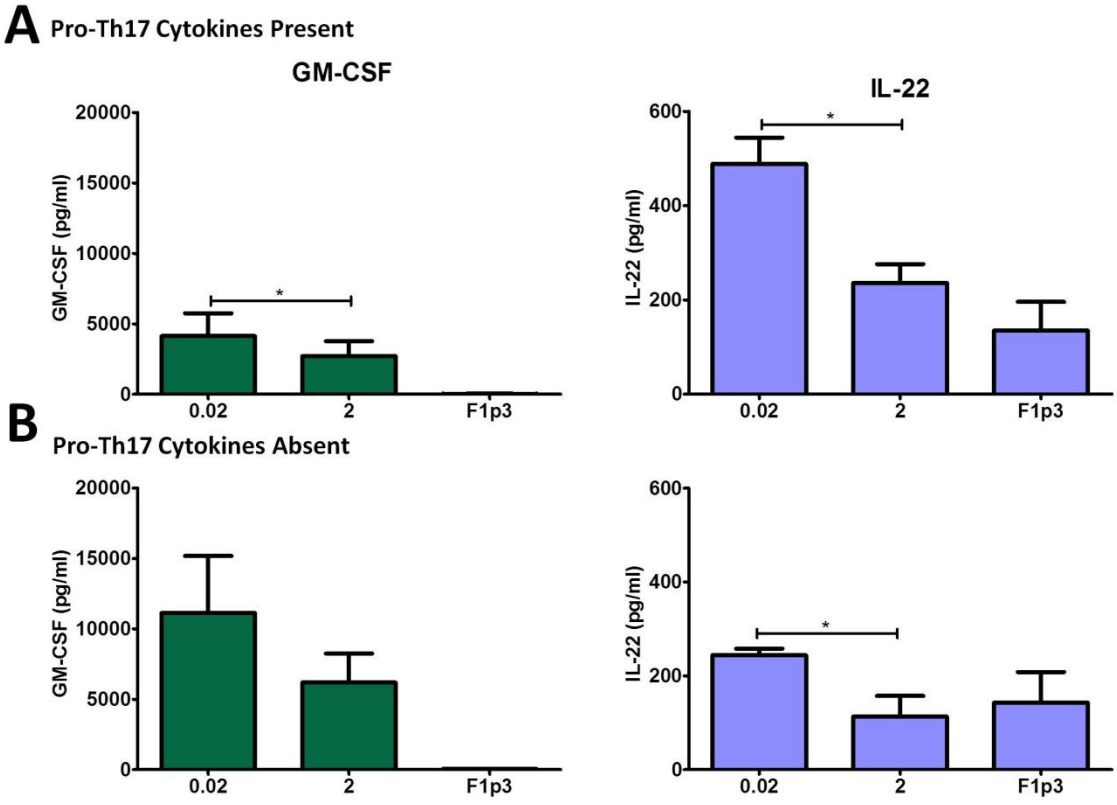

Figure 32 - Release of Th17 associated cytokine also alters with peptide density. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of p89-103 or F1p3 (2µM). These assays were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2µM of p89-103. After a further 48h supernatants were harvested and stored at -20^oC prior to analysis. ELISAs for GM-CSF and IL-22 were preformed according to the manufacturer's instructions. Data represents the mean of three independent biological experiements (three technical triplicates per biological repeat) ± SEM. Significance was determined by *t*-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001)

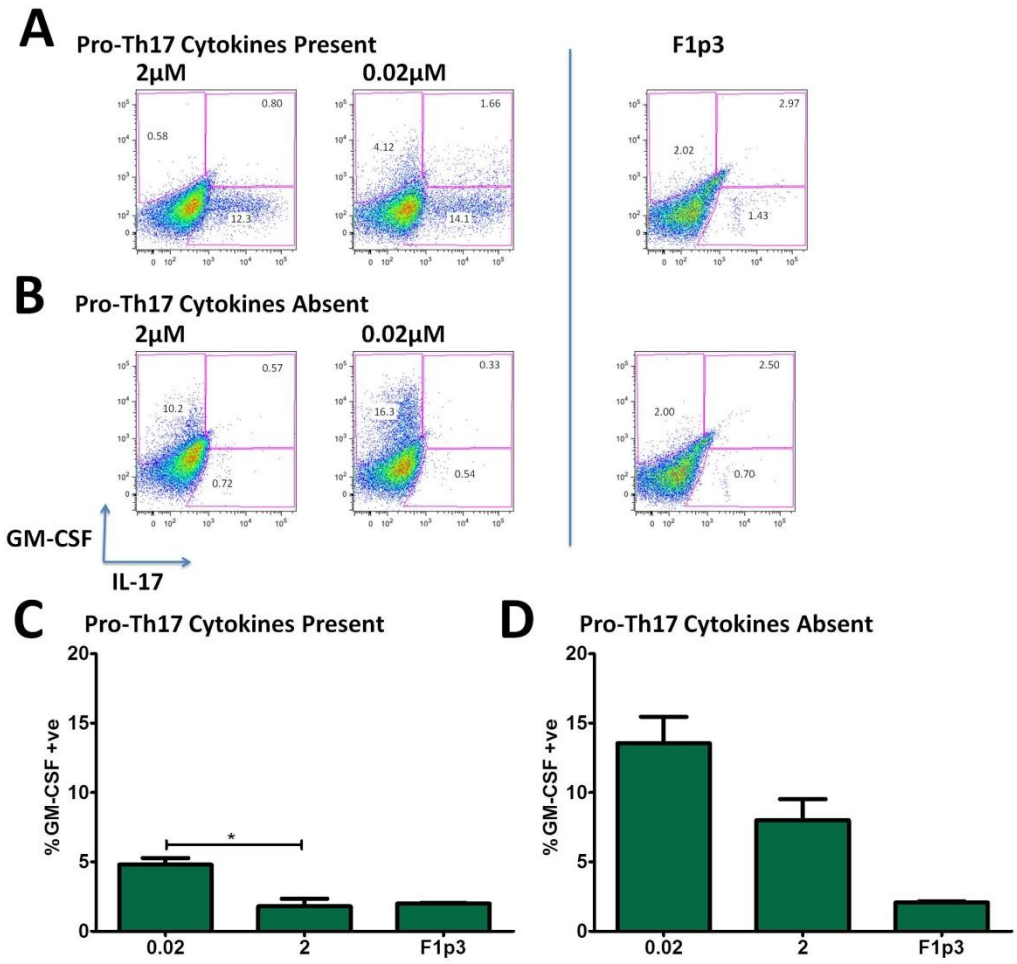

Figure 33 - Frequency of GM-CSF positive cells regulated by peptide density. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of p89-103 or F1p3 (2µM). After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of T cells expressing IL-17 and GM-CSF in the presence (**A** and **C**) and absence (**B** and **D**) of pro-Th17 cytokines. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM. Significance was determined by *t*-test (*p = <0.05, ** p=<0.01 *** p=<0.001).

3.13 Discussion

Th17 cells play an important role in the protection of a host from a range of pathogens including extracellular bacterial and fungal organisms (290). However, a dysregulated Th17 response against self antigens has implicated these cells in the development of autoimmunity (359). Given the previous data from our group and the recent studies of van Panhuys *et al,* the intensity of TCR signalling has been shown to have a vital role in regulating the development of Th17 cells as well as other subsets (283, 284). The initial focus was to optimise the experimental process to yield highly pure naive T cells. Moreover, titration of a number of factors including cell number per well, DC to T cell ratio and LPS dose used for DC maturation was required to minimise the non-specific release of IL-17. Using this system it was possible to show that activation of naive T cells with a range of cognate peptide concentrations induced distinct Th cell responses. Interestingly, Th17 cells predominated at lower peptide concentrations whereas IL-4-producing T cells were more readily induced at higher peptide concentrations. The development of a Th17 response required co-operation between the presence of an appropriate cytokine environment and a lower TCR signal intensity. Even with the addition of exogenous pro-Th17 cytokines, the fraction of Th17 cells peaked at a lower peptide concentration.

The concept of TCR signal strength involves multiple factors that can alter the degree of signalling that a particular T cell experiences (273). Thus, it can be modulated by the overall number of pMHC:TCR contacts a T cell makes, the duration of these contacts, co-stimulatory modulation and the affinity of the pMHC complex for a certain TCR (273). Most commonly anti-CD3 mAbs are used in studies to overcome the complication of competing TCRs with distinct affinities for a particular pMHC. Indeed, it is a proven tool for evaluating TCR signal strength in polyclonal T cell populations. However, the affinity of mAb for CD3 is many orders of magnitude greater than the relative weaker affinities of pMHC interacting with TCRs (273). T cell cloning provides a method of generating a monoclonal T cell population. Early studies on pMHC:TCR interactions relied on clones to define many of the general rules regarding the critical contacts in antigenic peptides. However, the process of cloning results in epigenetic modifications that render these cells unsuitable for determining effector function

(283). Thus, the knock-in of TCR transgenes allows the rapid purification of a naive Th cell population expressing a single known TCR. Such a tool allows investigation of how quantitative and qualitative changes to TCR signalling affect functional T cell differentiation. Unlike the well-studied systems using model antigens such as MCC or OVA, the use of 5/4E8 mice means that the responses detected are to a candidate autoantigen, to which T cell responses can be found in patients (332).

The data presented show an inverse relationship between IL-17 production in secondary cultures and the concentration of cognate peptide used during activation. This finding agrees with previous studies in our group by Purvis *et al,* which used varying ratios of anti-CD3/CD28 beads to memory T cells to study how overall T-cell activation strength affect Th17 cell responses (283). Thus, it appears that murine naive T cells respond to peptides in a similar manner. However, these data contradict the data from Bourgermouth *et al* and Gomez-Rodriguez *et al,* showing that an increase in anti-CD3 antibodies promotes greater induction of Th17 cells (360, 361). In the case of Bourgermouth *et al* and this project*,* the mice were both on a BALB/c background. Hence, the genetic background of the mice is not a probable cause for the differing Th17 responses observed. It may be that differences in activation using plate-bound anti-CD3 to peptide makes it difficult to compare responses.

The Bourgermouth *et al* study also showed an inhibitory role for CD28 in Th17 development. In this study mDCs numbers relative to T cells were not altered. However, the majority of DCs also expressed CD80 and CD86; the binding partners for CD28. In the study by Gomez-Rodriguez *et al,* CD28 was not found to be inhibitory for Th17 development, thus suggesting that TCR signals could overrule signals coming from CD28. Further studies would be required to define the role of co-stimulation in my project.

ROR-γT is required for the development of Th17 cells. Mice that lack ROR-γT fail to produce IL-17 and have attenuated pathology in a number of inflammatory disease models (362). In this project the expression of ROR-γT was altered by peptide density. All IL-17+ cells co-expressed ROR-γT. However, not all ROR-γT+ T cells produced IL-17. Additional factors, such as STAT3, NFAT1c and IRF4, are required for *Il17a* expression

(363). It may be that some of the ROR-γT+IL17- population fail to express one or more of these additional factors. It is also possible that negative regulators such as FOXP3 and ETS-1 could be present in ROR-γT expressing cells, preventing transcription of IL-17 in these cells. However, the expression of FOXP3 was relatively low especially in the presence of pro-Th17 cytokines.

Surprisingly, the more dominant response at the higher peptide concentrations were by Th2 cells as defined through IL-4 expression. The presence of pro-Th17 cytokines appeared to have no significant effects on the levels of IL-4 produced in secondary co-cultures. The positive correlation between IL-4 and increasing peptide concentration was also reflected in the frequency of IL-4+ cells found using ICS. Importantly, unlike other naive Th cell studies, my data were obtained without using antagonistic antibodies to IL-4 or IFNγ allowing simultaneous assessment of how multiple subsets are affected by changes in TCR signalling intensity. The IL-4 response also contradicts other studies suggesting that a lower degree of signalling was required for optimal induction of the Th2 phenotype. However, the prevailing view neglects the heterogeneity of the data from the studies of Hoskens *et al* and Constant *et al* (159, 279, 280). While in the case of the latter a clear increase was found at the lower concentrations, Hoskens *et al* show a biphasic response with Th2 cells developing at both extremes of the peptide concentrations evaluated. A comparison of peptide concentrations indicates that a contradiction exists between my study and the other examples, given that the peak Th2 responses occurred at 'low' doses of <0.02µM or 'high' doses >100µM. However, in my study IL-4 production increases steadily with increasing peptide concentrations with a peak dose at 20µM, whereas in other studies the Th1 cells dominated at this peptide dose. Direct comparisons are challenging even between TCR Tg systems due to the different antigens, different levels of peptide loading at a given concentration, the distinct sources of APCs, differences in maturation stimuli of APCs and the affinity of each TCR for their respective pMHC. It is possible that the 5/4E8 TCR has a reduced affinity for the p89-103 of aggrecan compared that of the OT-II or B10.Cg-TCR Tg, resulting in shifting thresholds in the precise concentration of antigenic peptide that triggers these distinct responses. Thus, these factors represent a limit to the use of TCR Tg and model antigen systems.

The critical cue needed for Th2 induction is the induction of IL-4 and its receptor IL-4R (187). Indeed, both of these are necessary in order to engage the expression of GATA-3. This in turns supports further IL-4 production, which through positive feedback mechanisms maintains the Th2 phenotype. In some studies IL-2 is thought to be required to establish an early IL-4 signal with STAT5 forming an important role in the remodelling of the *il4* locus to a permissive form accessible for transcription (187). Indeed, my data would agree with higher TCR signalling enhancing IL-4 production by the greater induction of IL-2 (examined further in Chapter 5). Others have suggested that a sustained ERK signal can prevent Th2 induction by preventing early GATA-3 expression and by transiently inhibiting the expression of CD25, the IL-2Rα chain, thus blocking the response to endogenous IL-2 and abrogating the 'early' IL-4 cue needed to establish the positive feedback loop (364). However, in my study CD25 was expressed at high levels by the majority of cells even at day 3, suggesting that perhaps this pathway was less active. If the 5/4E8 TCR has a weaker affinity to the cognate pMHC than those used in other studies it may be that the de-sensitisation caused by a very strong TCR signal is not occurring and so ERK inhibition of IL-2 is prevented.

An important caveat is that each of these subsets was evaluated on the basis of particular markers (e.g. IL-4 for Th2 cells compared to IL-17a for Th17 cells). These can be regulated independently from the other aspects that are attributed to that phenotype. For instance, IL-17a has been shown to be the more important mediator of Th17 effector functions than IL-17f – IL-17a binds with higher affinity to the IL-17R and is being more potent in the recruitment and activation of neutrophils than IL-17f (231). However, IL-17a and IL-17f can be regulated independently, as shown by Gomez-Rodriguez *et al;* IL-17a was regulated by TCR signal strength while IL-17f expression was not (361). This can be explained by the presence of an NFAT1c binding site in the promoter of IL-17a, meaning it is linked to Ca^{2+} and TCR signalling. In my study IL-17a alone was assessed due to its greater potency in inducing effector functions. However, it would be useful to ascertain if IL-17f was regulated in a similar manner. The study of IL-17a$^{-/-}$ mice has indicated that IL-17f can compensate functionally for the loss of the former (290). Thus, it could be that IL-17f may allow Th17 cells to functionally develop even if when activated with a high peptide density. Equally, IL-5 and IL-13 have been

shown to be important in modulating inflammation in the context of allergic asthma models (365). However, while IL-5 and IL-13 appear to mediate distinct effector functions, neither is sufficient with regards to the induction of the Th2 phenotype.

The use of naive T cells was important for my study, because it allowed for investigating the effect of peptide density on effector Th cell differentiation from uncommitted precursor cells. Memory cell contamination means the possibility that any results could be due to the preferential expansion of pre-committed memory cells. However, the high purity of naive T cells in my starting cultures suggests that differentiation of uncommitted, naïve, precursors is the most likely reason for the observed effects. Indeed, it was possible to show that following 5 days of co-culture that for both peptide doses evaluated, the majority of cells lost the naive phenotype as defined by high expression of CD62L and low levels of CD44. Moreover, this shows that despite the high frequency of responding T cells within these wells, exposure to antigen results in efficient conversion to a memory phenotype. Interestingly, although not significant, the lower peptide dose induced memory cells at a slightly reduced efficiency. Perhaps if given longer a similar level of conversion would be achieved. These observations contradict the data from Pape *et al* suggesting that the enhanced frequency of responding T cells inhibits the generation of memory cells, due to the large quantities of cytokine produced (366). Further confirmation could be obtained using flow cytometry to sort naive cells gaining purity of >99%.

Whilst the generation of memory cells did not alter significantly between the peptide concentrations evaluated, the expression of activation markers CD69, CD25 and CD40L varied significantly over the time course evaluated. CD69 has a co-stimulatory function with roles in Ca^{2+} release and there is evidence that anti-CD69 mAbs can induce IL-2 production in the presence of phorbol esters (367, 368). CD69 knockout mice have enhanced numbers of Th17 cells (369). Moreover, these mice experience a more severe form of CIA due to the dysregulated numbers of Th17 cells (370). Expression of CD69 is rapidly upregulated following TCR signal induction or PMA treatment through activation of PKC, with significant levels of expressing occurring with an hour of stimulation. Thus, it is possible that the differential expression of CD69 caused by the

altered TCR signal may also impact on the development of Th17 cells through altered IL-2 levels. Such an influence could be excluded if CD69 was ablated in the 5/4E8 model. While often ascribed as an early activation marker, the expression of CD69 persists throughout the assay possibly due to continuing presence of peptide-loaded APCs. Moreover, CD25 was found to be co-expressed with CD69 sharing a similar profile in terms of frequency of positive cells and kinetics. Similarly, CD25 expression is modulated by the intensity of signalling by the TCR via modulation of IL-2 production. Taken together these data provide a useful proxy for the degree of TCR signalling within these two populations. With regards to the implications of IL-2 on the observed effector T cell response, Chapter 5 will explore this in greater detail.

The focus of this chapter was on differentiation of naive T cells and how peptide density can shift the effector function in relatively short-term co-cultures. It would be of interest to determine the plasticity of these cells and if changing antigen density in subsequent cultures would alter the response. The plasticity of Th17 cells remains to be fully clarified. Emerging data have shown that only those Th17 cells that co-express IFNγ are pathogenic in some *in vivo* disease models (371). The ability of Th17 cells to co-express other effector cytokines has been attributed to IL-23 (372). Despite the relatively high concentration of IL-23 in my system, there was no significant development of IL-17+IFNγ+ co-expressing cells. Even IFNγ+ Th1 cells were relatively rare compared to Th2 cells. Other studies have suggested that a low level of IL-2 is required to induce co-expression of T-bet and IFNγ, despite IL-2 antagonising IL-17a expression (227). No examples as yet have identified Th17 cells expressing Th2 cytokines. No such cells arose during the cultures presented in this chapter.

Cell exhaustion could also be a possible explanation for the differences observed in T cells stimulated with either a high or low peptide concentration (275). Exhaustion is defined as a dysfunctional state that can arise due to chronic activation such as that which occurs in cancer and some forms of infection. It involves the loss of effector functions, such as cytokine production, and an inability of these cells to proliferate when exposed to further antigen. These cells express high levels of negative regulators of T cell effector function such as Programmed Death Ligand-1 (PDL-1) and

Lymphocyte Activation Gene-3 (LAG-3) (373). In the case of anergy, cells that are intrinsically functional are rendered inactive after encountering antigen in the absence of co-stimulation (374). These cells are not eliminated by cell death but persist in a hyporesponsive form. The initial study on antigen density by the Bottomly lab featured a 48h 'rest' period between primary and secondary cultures, in which T cells were withdrawn from antigen-pulsed DCs (279). However, it was not clear if this has any significant effect and was not a feature of the study by Hoskens *et al,* which also showed an antigen density effect on Th1/Th2 cell differentiation (280). In this study these factors appear to have marginal, if any, influence on the effects observed by alteration in peptide concentration. The proliferation data shows that even over an extended time course, 3H-thymidine incorporation was consistently greater than the negative control. Furthermore, those T-cells exposed to a high peptide concentration during the primary culture remained responsive to restimulation with mDC and cognate peptide. Indeed, these cells were capable of releasing effector cytokines and proliferating when re-stimulated in secondary cultures. Other studies have indicated that if a high dose of antigen persists over 10 days, the development of memory cells can be impaired. In this case, cells were stimulated for 7 days in total, five days in the primary culture and two in secondary cultures. It would be interesting to determine if exhaustion would eventually occur, if the length of culture was extended. However, given that the cultures in this chapter are relatively short term and the presence of mDCs expressing a range of co-stimulatory molecules (CD80, CD86 and CD40) it is unlikely that either of these mechanisms are responsible for the observed shift in Th phenotypes. Moreover, the proliferation and cell counts indicate a similar number of cells after 5 days of primary cultures. Assessment of cell viability using trypan blue also showed no significant difference between those activated with either a high or low peptide concentration. Thus, the lack of any significant difference in proliferation suggests that this is not responsible for the distinct cytokine responses that occurred at a high or low peptide concentration.

Although often defined by the production of IL-17a, Th17 cells can also produce IL-17f, IL-21, IL-22 and GM-CSF (375). The data presented in this study indicate that IL-22 release mirrors that of IL-17a from secondary cultures, thus suggesting these are

regulated by a similar mechanism. The induction of IL-22 was related to exposure to peptide suggesting it was T cell-derived. However, ICS in future studies would be required to absolutely ensure that it is co-expressed with IL-17a. Growing evidence suggested that Th17 cells are not a homogenous population and production of associated cytokines can be used to define sub-populations (376). Typically defined by the ability to induce pathogenicity in various disease models, the switch between these populations has been attributed to exposure to IL-23. Studies by Lee *et al* have suggest that presence of IL-23 induces the further endogenous expression of the TGFβ3 isoform (376). This study proposed a unique set of 23 genes that can be used to identify pathogenic from non-pathogenic Th17 cells (376). Both produce IL-17a but can be distinguished by the expression of either IL-10, in the case of non-pathogenic Th17s, or GM-CSF in the case of pathogenic cells. It would be interesting to investigate this in the 5/4E8 system. In the EAE model of MS, GM-CSF has been shown to have a non-redundant role. Mice lacking GM-CSF are highly resistant to disease induction and transfer of these cells to a susceptible host fails to bring about disease induction (239). Even the co-expression of T-bet and IFNγ could not induce neuroinflammation. Codarri *et al* have shown that GM-CSF expression was stimulated by ROR-γT and IL-23 (239). Given the non-redundant function of GM-CSF it was interesting to see that it showed a similar response to antigen density as IL-17a and ROR-γT. Those stimulated with the lower peptide dose had a greater frequency of IL-17a+GM-CSF+ cells. However, the majority of GM-CSF- producing cells lacked co-expression with IL-17. It would be interesting in futures studies to understand if these are unique in expressing GM-CSF alone as has been documented in some studies (377, 378). Alternatively it could be an issue of kinetics, with these cells previously expressing IL-17a or other effector cytokines. Tracking these cells by co-expression of a fluorescent marker such as YFP-IL-17 mouse developed by the Stockinger lab would be a useful approach (299). A further layer of complexity arises from the surprising increase in GM-CSF in the absence of pro-Th17 cytokines. This may be a consequence of TGFβ1 being present in the pro-Th17 cytokine cocktail, meaning further optimising of the exogenous cytokines used may be required.

Many of the genetic associations between T cells and autoimmunity affect molecules involved in TCR signalling including MHC cII, PTPN22 and CTLA-4 (379). In the context of autoimmunity, it is this characteristic of Th17 development that may explain the widespread association of these cells with such conditions as MS and inflammatory arthritis, as examination of autoreactive TCRs indicates that these have lower affinities for their cognate antigen in comparison with those responsive to infectious pathogens (60). In many examples the affinity is lower than that found in tumour-responsive T cells. These agree with the notion that these cells escape negative selection in the thymus due to their weak affinity for self peptides. Thus, these cells are more likely to develop into autoreactive Th17 cells if an appropriate cytokine environment is encountered. Additionally, the corresponding increase in IL-4 at a high peptide concentration is interesting given the negative influence Th2 cells have on autoimmune pathology. Immunisation of PGIA mice with IL-4 has been shown to ameliorate the development of disease (303).

In summary, the data presented in this chapter indicate that changes to peptide concentration can alter the type of Th response that develops from naive T cells. Co-operation between an appropriate cytokine milieu and lower TCR signal were required for optimal Th17 induction. In contrast, Th2 cells showed the opposing relationship. The Th17 cells generated at a low peptide concentration were also capable of producing both GM-CSF and IL-22, known to be critical for pathology *in vivo*. The focus on the next chapter will be explore another aspect of TCR signal strength, the affinity of a pMHC for TCR, and the effect this has on the naïve 5/4E8 TCR Tg response.

4 Citrullination of a putative TCR contact enhances Th17 responses

4.1 Introduction

Given that the genetic codes specifies for only 20 amino acids, modification of existing residues generates over a 100 distinct forms some of which are listed in **Table 3** (312). Of these Arginine residues and the resulting modifications are some of the most characterised. In the case of deimination, the result is the generation of citrulline that differs by the loss of a net positive charge as compared to Arginine. The association between citrullination and inflammatory arthritis has been understood for a considerable length of time (380-382). A prototype assay was developed over 40 years ago to detect anti-citrulline antibodies, initially known as anti-perinuclear factor (327). These assays use Cyclic Citrullinated Peptides (CCPs) that act as a surrogate target for a range of citrullinated antigens (330). Testing for ACPAs has become part of the *ACR/EULAR Rheumatoid Arthritis Classification Criteria* since July 2010 (312). The advantage with regards to CCPs is the increased specificity for RA of >97%. Growing data has indicated that Rheumatoid Factor (RF) is not exclusive to RA being present in other autoimmune diseases (383). Even in 5% of 'healthy' individuals, RF can be detected with speculation that it could represent merely a sign of polyclonal B cell activation. The value of ACPAs is not only purely diagnostic, it has been shown that ACPA levels correlate with more aggressive form of RA with faster bone degradation and joint erosion (384).

While numerous studies have evaluated the antibody response to citrullinated antigens, how these modifications impact on autoimmune T cells remains less well understood. In the murine model EAE, Carrillo-Vico *et al* demonstrated that a citrullinated form of the Myelin Oligodendrocyte Glycoprotein-35-55 (MOG35-55) peptide induced two distinct populations of T cells that could either cross-react, or not, with the native form of the peptide (325). While the citrulline-reactive T cell population was not required for disease induction, these cells could exacerbate pathology when adoptively transferred to an animal with pre-existing EAE. In terms of inflammatory arthritis, it is known that citrullination of the shared epitope increases

the magnitude of the CD4+ T cell response through the increased peptide binding to HLA-DRB1*0401 in a humanised murine model (385). Preferential binding of citrullinated synovial antigens has been demonstrated for the HLA-DRB1*0101 and HLA-DRB1*0404 alleles (312, 386, 387). The mechanism is thought to be dependent on the neutralisation of the positive charge on the citrullinated antigenic peptide. Thus, the clash with the positive charge held at position 4 (P4) of the MHC binding grove is eliminated. It has also been demonstrated that a substantial proportion of RA patients have T cells that recognise citrullinated proteins including vimentin, fibrinogen, collagen type II and aggrecan in the context of HLA-DRB1*0401, with some cross-reacting with microbial antigens (388). In the case of the vimentin, citrullination has been shown to alter antigen processing by proteases. Moreover, von Delwig *et al* have shown that 60% of 28 RA patients assessed had T cells that could proliferate in response to citrullinated aggrecan-84-103 (331). The cytokine profile of these citrulline-responsive T cells was mainly Th17-like. Not only do these studies indicate the presence of citrulline-specific T cells in RA patients, they implicate an IL-17 response induced by these antigens. These responses are not limited to RA with T cell responses to citrullinated collagen type II epitopes in juvenile idiopathic arthritis (JIA) and psoriatic arthritis (PsA) (389).

Amino Acid	Modification
Arginine	Methylation Deimination
Lysine	Hydroxylation Methylation Nitrosylation
Asparagine	N-linked glycoslation Deamidation
Aspartic acid	Isomeration
Glutamic acid	Methylation
Glutamine	Deamidation
Proline Serine Threonine	Hydroxylation Phosphorylation O-linked glycosylation

Table 3 - Amino acids and known chemical modifications (312).

4.2 Chapter aims

Given the specificity of the 5/4E8 TCR Tg mice, the focus of this chapter will be how modifications to the aggrecan peptide p84-103 can alter the T cell effector response. As mentioned previously in Chapter 3, within the core 9-mer (residues 92-100) it has been possible to putatively determine which are TCR contacts and MHC contact points (see **Figure 14**) (390, 391). Moreover, the sequence contains two Arginine residues with a putative TCR contact point at R93 whilst the second residue has been described as an MHC contact R95. Scally *et al* have recently shown by x-ray crystallography that p84-103 R93 is solvent-exposed while R95 is in contact with the MHC cII molecule (392). Studies by Misják *et al* indicated that, as expected, citrullination of the MHC contact point weakened affinity for MHC cII (A^d) whilst no significant difference in MHC binding occurred with the citrullination of the TCR contact (391). Thus, these data support the description of R93 as a TCR contact while the R95 is involved in MHC cII. These also provide an opportunity to determine how a disease-relevant peptide modification alters the type of T cell response.

Using these tools the aims of this chapter were to:

- Screen and characterise the T cell response to Altered Peptide Ligands (APLs) based on substitutions at putative TCR contacts within the p89-103 sequence
- Investigate the impact of citrullination on proliferation, surface marker expression and Th17 polarisation of naïve 5/4E8 TCR Tg cells

4.3 Screening of APLs to p89-103

Given that a lower density of p89-103 peptide enhanced the numbers of Th17 cells with a corresponding loss of the Th2 responses, it was important to determine if changes in peptide sequence could induce a similar alteration in the naïve 5/4E8 TCR Tg T cell response. APLs with Alanine substitution at the TCR contact points were synthesised, except that the Alanine at position 99 was substituted with Glycine (A99G). The Th response to an APL altered at the R95 residue, a MHC cII contact point, was also assessed as, together with R93, this residue can undergo citrullination. An initial screen was made comparing the proliferative responses of naive 5/4E8 TCR Tg T cells to cognate peptide and the APLs with Alanine substitutions at putative TCR contact points. All peptides were used at an equivalent concentration. This approach provides an insight as to the effect of modification at a particular putative TCR and MHC cII contact point. After 3 days of co-culture of DC and T cells in the presence of cognate peptide or APLs, each population was pulsed with 3H Thymidine and DNA harvested after a further 12h of incubation. **Figure 34A** indicates that the degree of proliferation varied significantly depending upon which residue in the p89-103 peptide was altered. Indeed, switching of either Arginine with Alanine severely abrogated T cell proliferation when compared to the cognate peptide. In contrast, switching at the other TCR contact points (V96 or A99) failed to alter the rate of Thymidine incorporation significantly.

With the numerous links between inflammatory RA and citrullination it was also of interest to determine the effect of such a modification on the 5/4E8 TCR Tg T cell response (see **Figure 34B**). As such the same assay was performed using the three possible forms of citrullinated p89-103, namely a single citrulline at position 93 (R93Cit) or 95 (R95Cit) or an APL with both Arginines modified (R93-95Cit). Interestingly, citrullination of the TCR contact, R93Cit, induced a slight reduction in the T cell response, which was not as severe as that found when substituted with an Alanine as above. However citrullination of the second Arginine (R95Cit) had a strong negative affect on the rate of T cell proliferation. The citrullination of both Arginines

induced proliferation at a level that was equivalent to the F1p3 peptide control, which was shown not to be recognised by the 5/4E8 TCR.

While proliferation determines the magnitude of a Th response, it was also important to determine the impact of APLs on effector function as defined by cytokine production. As such primary co-cultures were established with either cognate peptide or APLs at the same concentration in either the presence or absence of pro-Th17 cytokines. After 5 days cell numbers was normalised and secondary co-cultures were setup and re-stimulated with 2μM p89-103. Cytokine production was determined by ELISA after a further 48h of secondary co-culture. Focusing initially on the Alanine substitutes, despite being present at equivalent concentrations, IL-17 was enhanced in those that induced the poorer proliferative responses (see **Figure 35**). IL-4 release was severely abrogated by alteration to either Arginine residue. In contrast, IFNγ production was not significantly altered in any of the APL-stimulated cultures.

When assessing the impact of citrullination it became apparent that levels of IL-17 were significantly greater in response to the citrullinated peptides than in response to cognate peptide (see **Figure 36**). Although modification of the TCR contact point, R93Cit, induced the greatest release of IL-17 of any TCR site modified, it was changes to the second Arginine that induced by far the largest response. The presence of two citrullines, R93-95Cit, also significantly enhanced IL-17 release although not to same degree as R95Cit. In the case of IL-4 none of the citrullinated peptides induced levels similar to the cognate peptide. Citrullination of any residue had no significant effect on IFNγ release relative to p89-103.

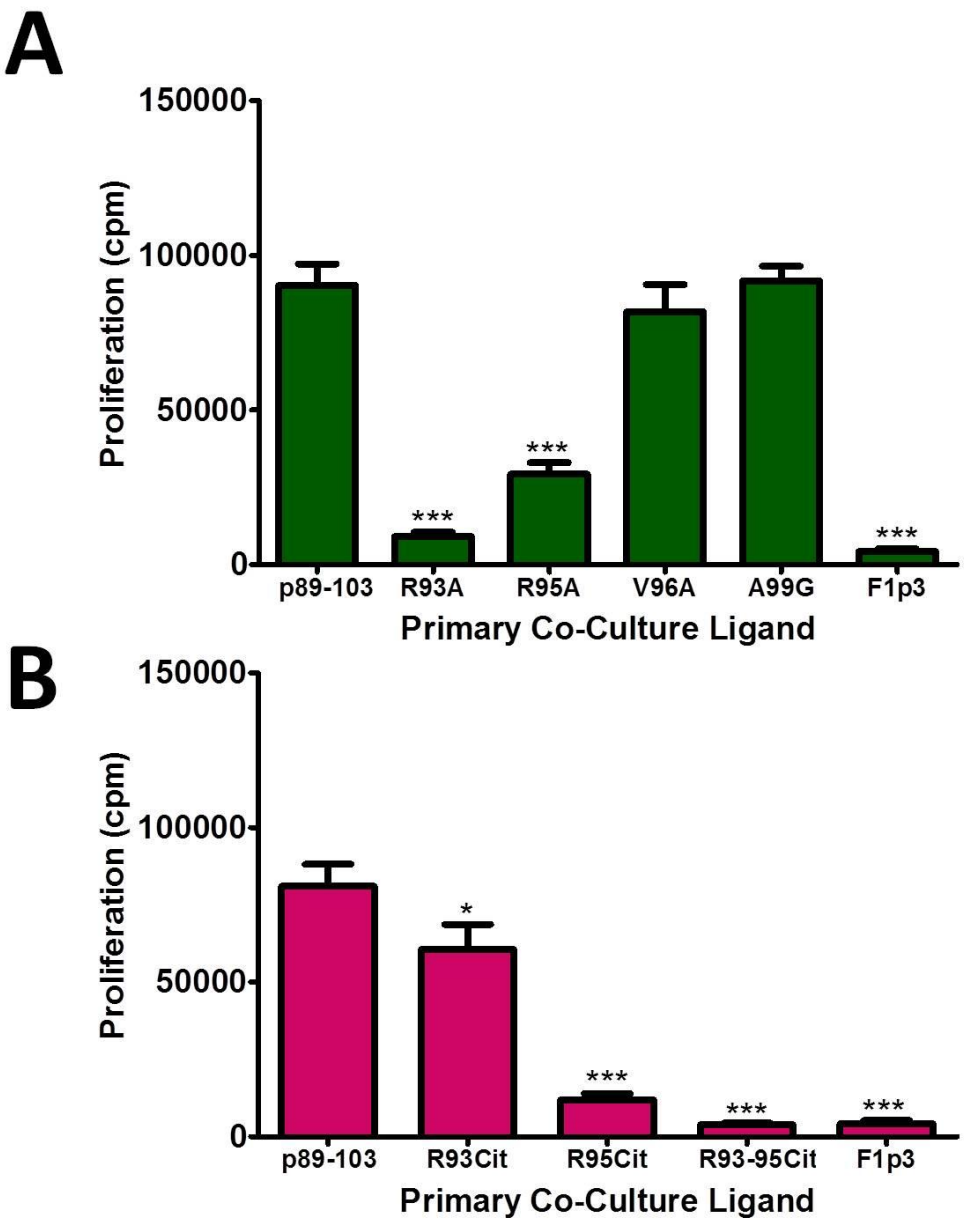

Figure 34 - APLs can alter proliferation of naïve 5/4E8 TCR Tg T cells. $4x10^4$ naïve 5/4E8 TCR Tg T cells and $2x10^3$ mDCs were co-cultured with 2µM of p89-103, APL or F1p3 in flat bottom 96-well plates. Alanine substitutes are shown in (**A**) and citrullinated APLs in (**B**). After 3 days, co-cultures were pulsed with radioactive thymidine (3H) for the last 12h of co-culture. DNA was harvested onto glass fibre filters prior to counting with a scintillation counter and counts per minute (cpm) determined. Data represents the mean of three independent biological experiements (three technical samples per biological repeat) ± SEM. Significance relative to p89-103 was determined by one-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001).

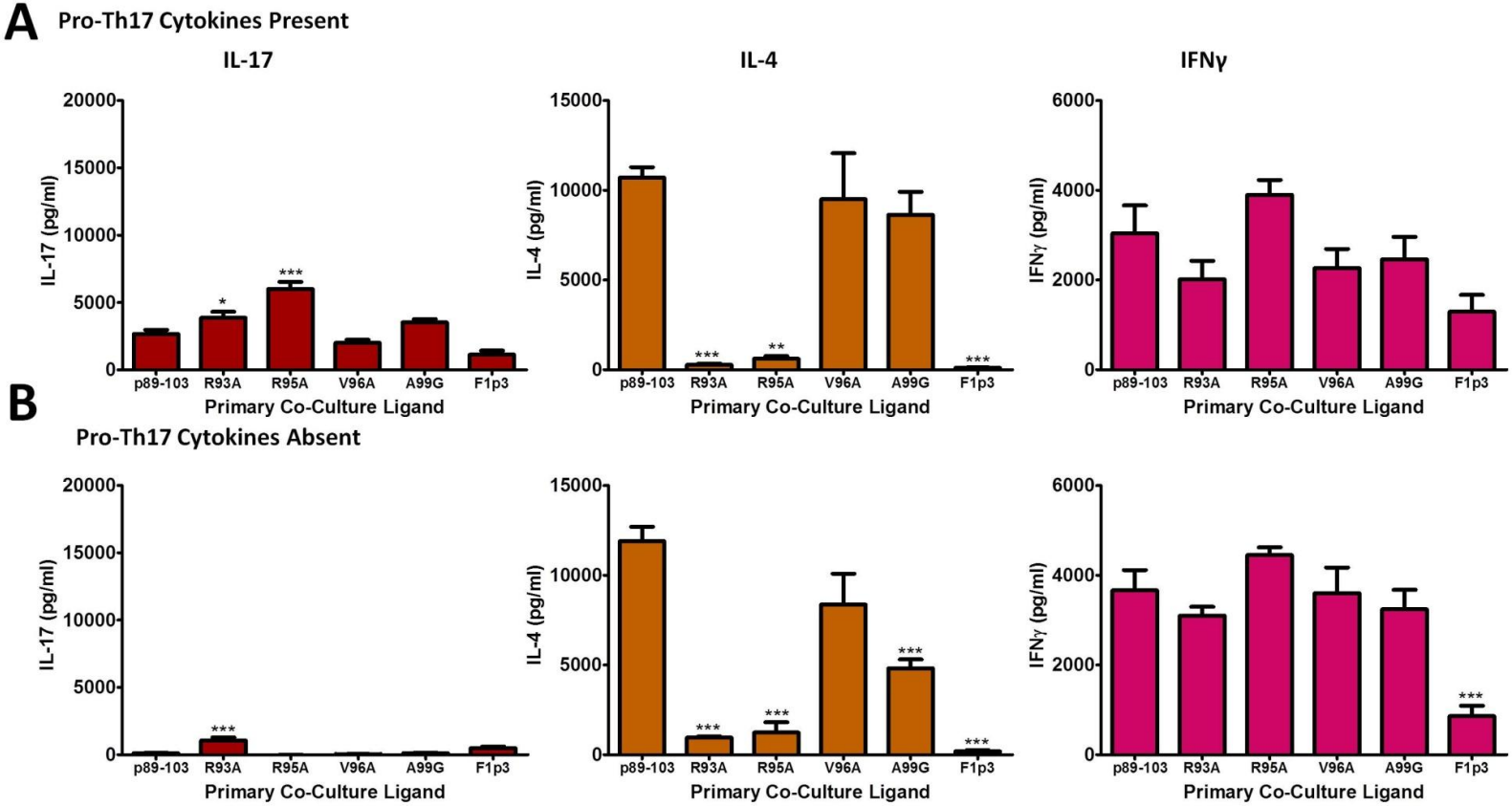

Figure 35 - Effect of Alanine APLs on cytokine response. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established across with either p89-103 or various Alanine APLs at 2µM. After 5 days cells were harvested, washed and re-plated with $4x10^4$T cells, $2x10^3$ 'fresh' mDC and 2µM p89-103. Assays were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. After a further 48h supernatants were harvested and stored at -20°C prior to analysis. ELISAs for IL-17, IL-4 and IFNγ were performed according to the manufacturer's instructions Data represents the mean of three independent biological experiements (three technical samples per biological repeat) ± SEM. Significance relative to p89-103 was determined by one-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001).

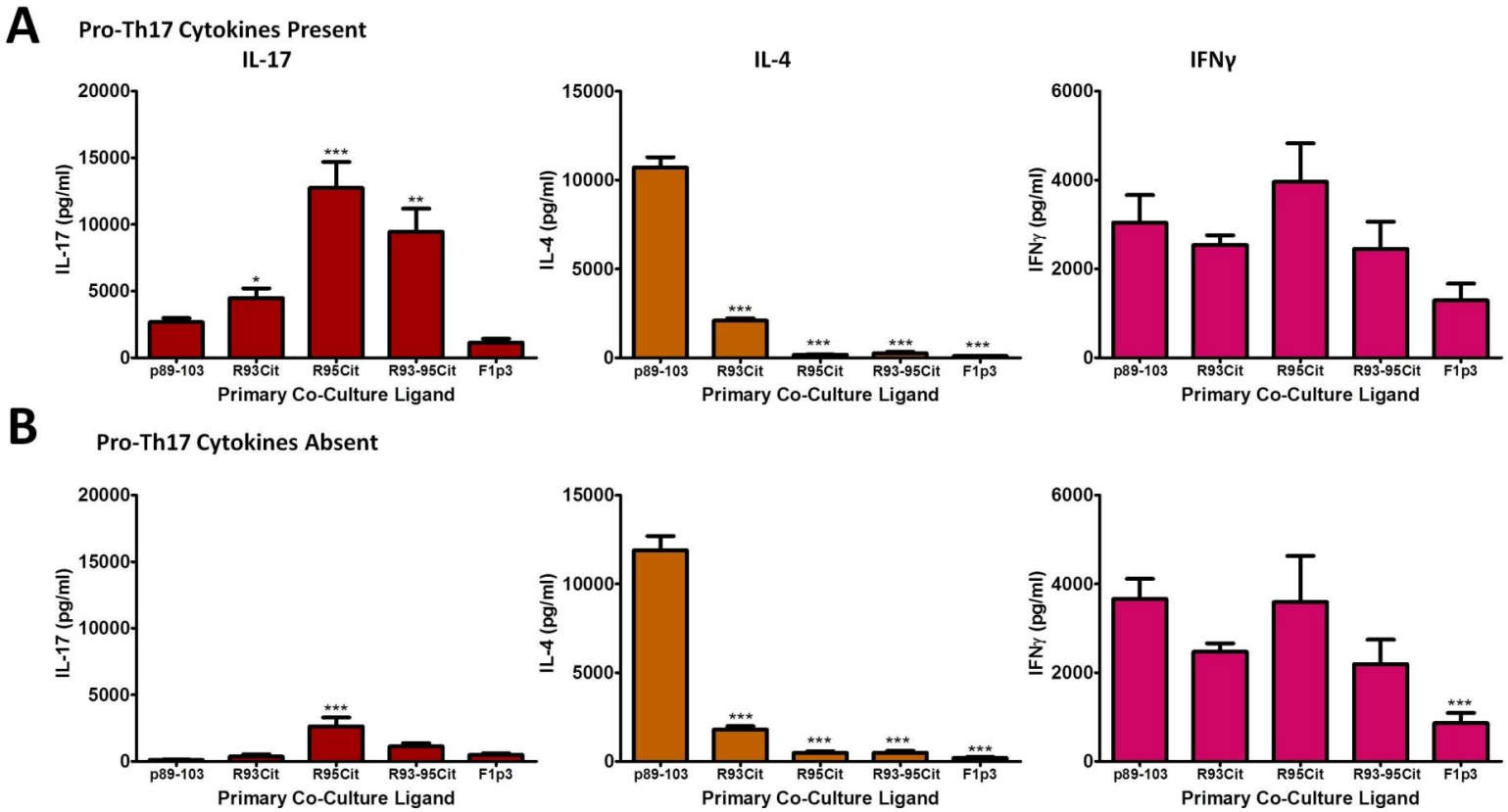

Figure 36 - Citrullinated APLs enhance IL-17 release at the expense of IL-4. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established across with either p89-103 or various citrullinated APLs at 2µM. After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2µM p89-103. Assays were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. After a further 48h supernatants were harvested and stored at -20°C prior to analysis. ELISAs for IL-17, IL-4 and IFNγ were performed according to the manufacturer's instructions. Data represents the mean of three independent biological experiements (three technical samples per biological repeat) ± SEM. Significance relative to p89-103 was determined by one-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001).

4.4 Proliferative response to citrullinated APLs

The association between citrullination and inflammatory arthritis is relatively well-characterised (393). Given the profound shift in the T cell response induced by the citrullinated forms of p89-103, I sought to examine the T cell response in more detail. Firstly, it was important to ascertain the proliferation profile of 5/4E8 TCR Tg T cells over a range of doses and time points. Therefore it was important to assess proliferation over an extended time course. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were activated with three forms of the citrullinated peptide and pulsed with 3H-Thymidine at either day 3, 5 or 7. **Figure 37A** shows that throughout the time course assessed any modification that included the second Arginine (R95) failed to induce proliferation any greater than the negative control. In contrast, modification to the TCR contact, R93Cit, resulted in a significantly greater T cell expansion than other citrullinated APLs tested, with a peak response at day 5. If compared to the cognate peptide, as shown in the previous chapter, although significantly reduced at day 3, a similar peak response was reached. Thus, the data suggest that the R93Cit peptide was a partial agonist for the 5/4E8 TCR.

With the lack of T cell proliferation in response to the R95cit peptide across the time course and likely alteration to the ability of this peptide to bind to MHC cII, the focus of the rest of this chapter will be on the impact of the partial agonist R93Cit. Moreover, by citrullinating a putative TCR contact point, it is more likely that any changes are due to alteration in affinity for TCR. To further understand the response to R93Cit, T cell proliferation was determined over a range of peptide concentrations (see **Figure 37B**). In line with the reduced affinity of the R93Cit peptide, the dose response showed a shift in the curve towards the right if compared to the cognate peptide over the same concentration range (R93Cit EC50 - 0.3244µM compared to p89-103 EC50 - 0.005µM). However, at cell counts at day 5 indicate similar number of cells in the primary cultures stimulated with p89-103 or R93Cit (see **Figure 38**). Similarly, viability was significantly greater than F1p3 co-cultures for both p89-103 and all forms of citrullianted peptides.

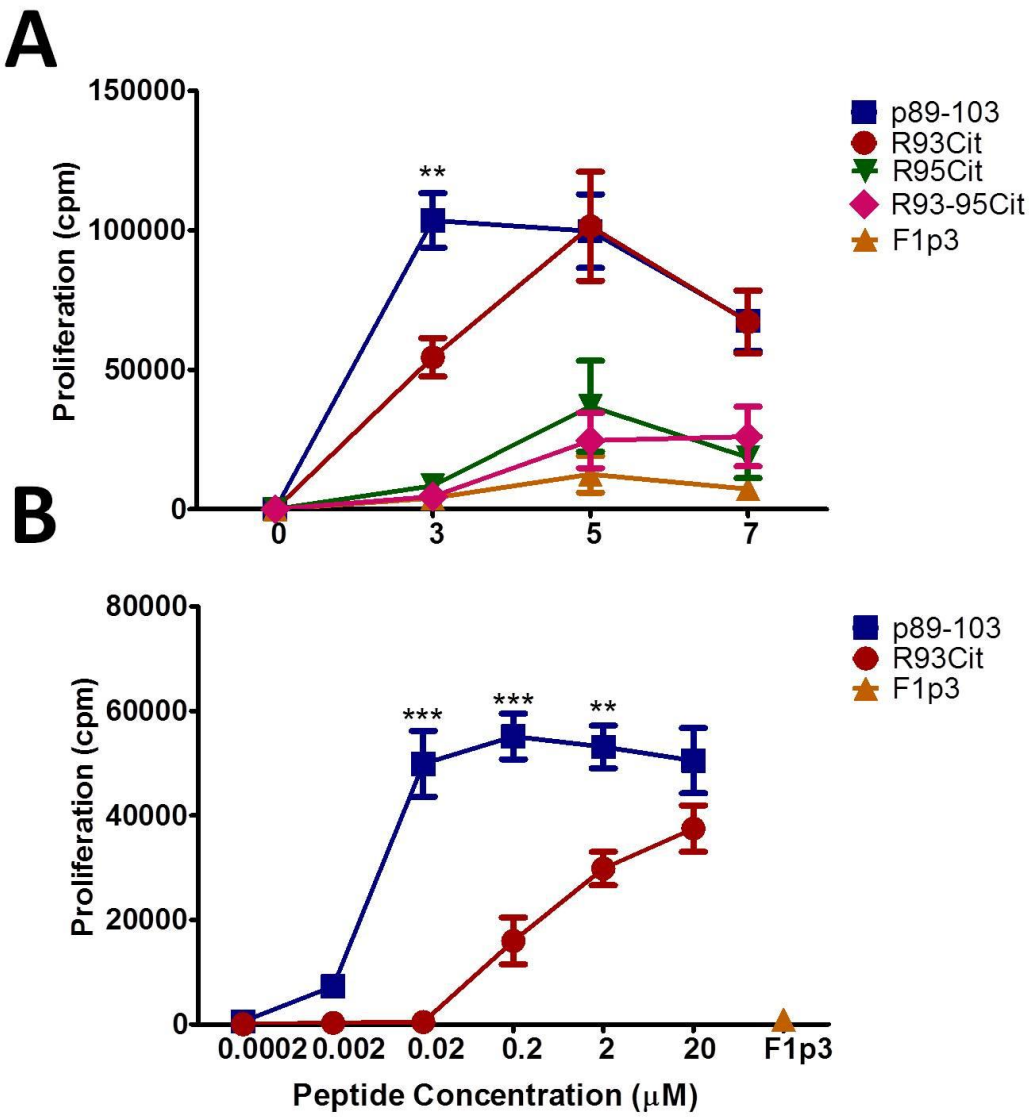

Figure 37 - Kinetics of the proliferative response to citrullinated forms of p89-103. 4x10^4 naïve 5/4E8 TCR Tg T cells and 2x10^3 mDCs were co-cultured with 2μM of p89-103 or citrullinated APLs as indicated or F1p3 (control) in flat bottom 96-well plates for either 3, 5 or 7 days (**A**). A range of R93Cit and p89-103 peptide concentrations were also established (0.0002-20μM) with results analysed after 72h (**B**). Co-cultures were pulsed with radioactive thymidine (3H) for the last 12h of co-culture. At the respective timepoint DNA was harvested onto glass fibre filters prior to counting with a scintillation counter and counts per minute (cpm) determined. Data represents the mean of three independent biological experiements (two technical samples per biological repeat) ± SEM. Significance was determined by two- way ANOVA - Bonferroni post t-test (*p= <0.05, ** p=<0.01 *** p=<0.001).

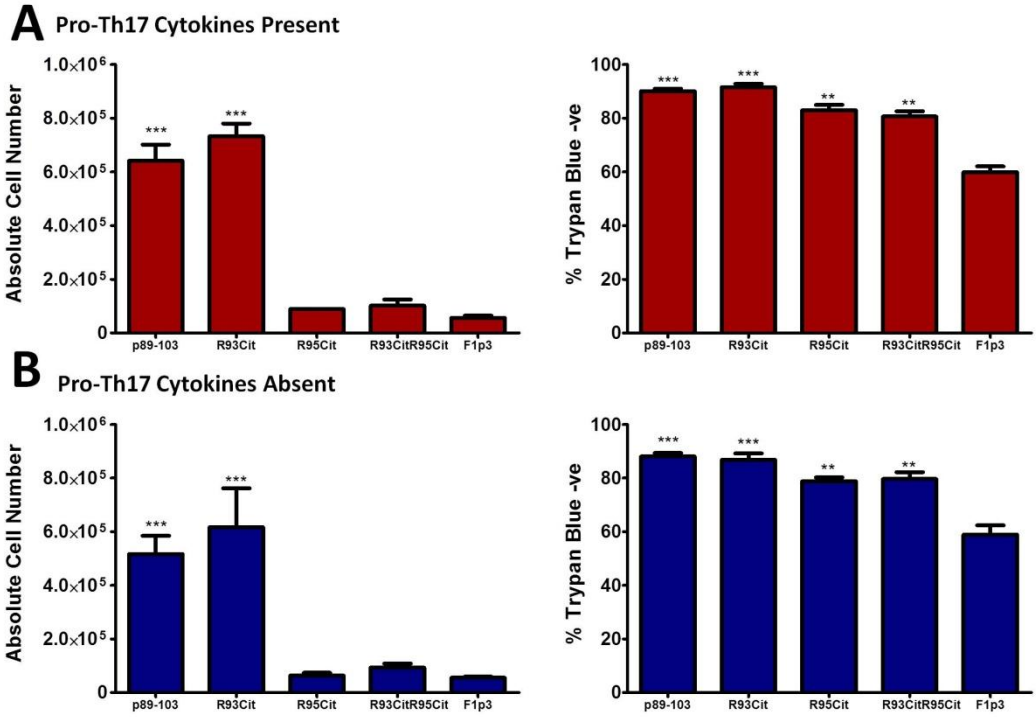

Figure 38 - Absolute cell numbers and viability of 5/4E8 TCR Tg T cells activated with citrullinated peptides . Co-cultures of $1.25x10^5$ 5/E4 TCR Tg T cells and $6.25x10^3$ mDCs were established with either p89-103, citrullinated APLs or F1p3 (all 2µM). Assays were established in the presence (**A**) and the absence (**B**) of pro-Th17 cytokines. After 5 days cells were harvested, washed and cell numbers determined using a neubauer haemocytometer. Trypan blue was used to assess cell viability with trypan positive cells being determined as dead. Data shows mean of three independent biological experiments (four technical counts per biological repeat) ± SEM. Significance relative to F1p3 was determined by one-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001)

4.5 Citrullination enhances IL-17 release

Building on the proliferation data above it was important to understand how the cytokine response also changed over an extensive titration of a lower affinity peptide such as R93Cit. To assess the impact of citrullination of a TCR contact point, the two-step co-culture system was established as previously described with a range of R93Cit concentrations (0.002-20µM). After the initial 5 days of co-culture, cell numbers were normalised and secondary cultures re-stimulated with 2µM of the cognate peptide, p89-103. 48h later supernatants were collected and levels of IL-17, IL-4 and IFNγ assessed by ELISA.

Unlike the response detected with the higher affinity p89-103 as seen in the previous chapter, the response of IL-17 across the dose ranged was reversed with a positive increase with concentration (see **Figure 39**). This is reflected in the positive slope value as determined by linear regression. As seen beforehand pro-Th17 cytokines were required to detect high levels of IL-17 in the secondary cultures. In contrast, Th cells activated with R93Cit fail to produce large quantities of IL-4 in the presence of pro-Th17 cytokines. With the removal of exogenous cytokines, the IL-4 response was increased showing a positive correlation with increasing peptide concentrations.

4.6 Frequency of Th subsets is also altered by citrullination

Whilst the cytokine production from secondary cultures provides an understanding of the type of immune response, further assays were required to quantify the frequency of cytokine expressing cells. To gain further insight into the type of response induced by the R93Cit peptide, ICS was carried out over the same dose titration. Following 5 days co-culture with mDCs and various doses of R93Cit, 5/4E8 TCR Tg T cells were re-stimulated with PMA/I for 5h with BFA added for the last 4h. These parallel assays were carried out both in the presence and absence of pro-Th17 cytokines.

The results as shown in **Figure 40** demonstrate a very similar outcome to that obtained by ELISA. With regards to IL-17, expression correlated with increasing concentrations of the citrullinated form of the peptide. This is also reflected by linear regression showing positive slopes values. However, without the pro-Th17 cytokines the development of Th17 cells barely rises above that of the negative control population. With regards to IL-4 expression, the frequency of IL-4+ cells did not increase compared to the negative control at any of the R93Cit concentrations tested. In the absence of pro-Th17 cytokines, the frequency of IL-4- producers did increase with increasing R93Cit concentrations. Interestingly, the frequency of IFNγ-producers, although relatively unresponsive to changes in dose with pro-Th17 cytokines, did rise without these cytokines. Thus, these data demonstrate how

changes in affinity can alter the effector response of naive Th cells in terms of their cytokine production profile.

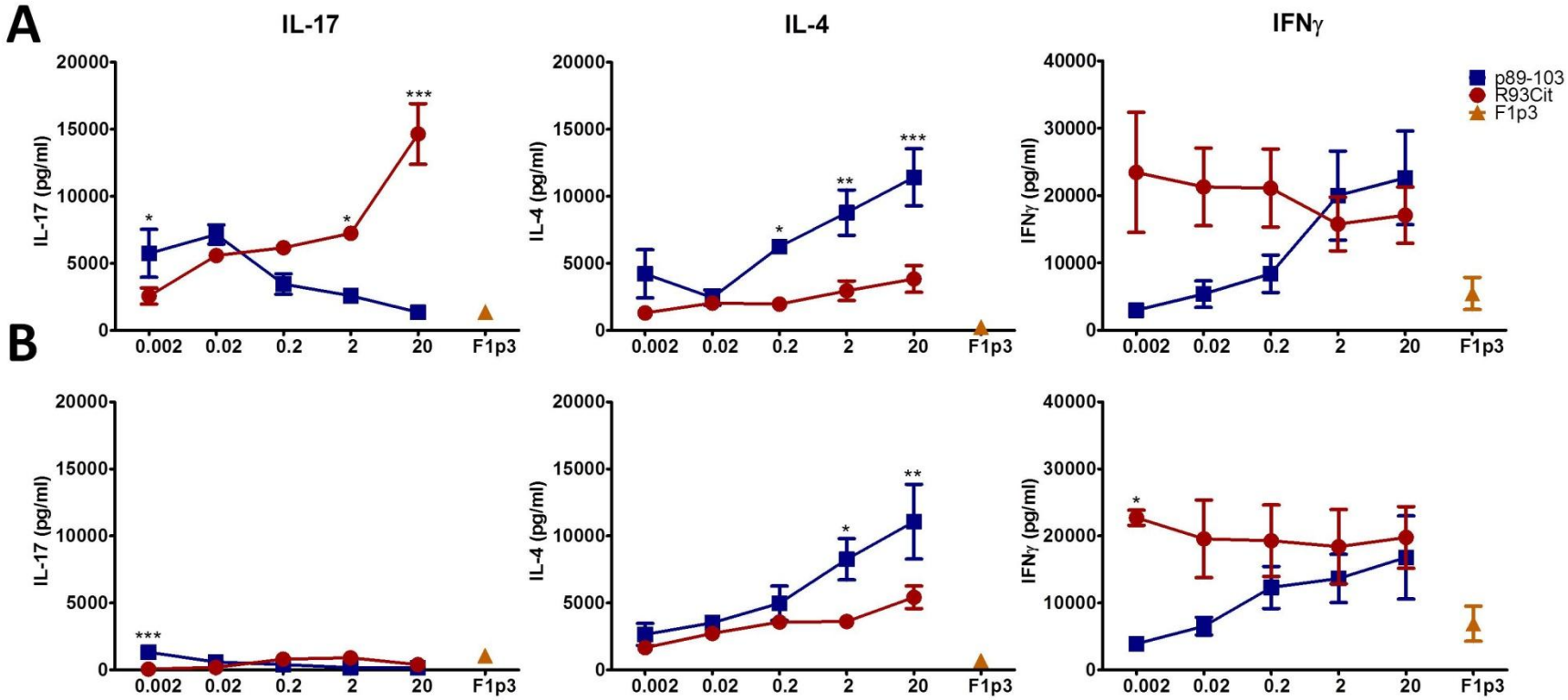

Figure 39 - Cytokine production by R93Cit activated 5/4E8 TCR Tg T cells. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established across a range of p89-103 or R93Cit concentrations (0.002-20μM) or F1p3 (2μM). ELISAs were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2μM of p89-103. After a further 48h supernatants were harvested and stored at -20°C prior to analysis. ELISAs for IL-17, IL-4 and IFNγ were preformed according to the manufacturer's instructions. Data shows mean of three independent biological experiments (three technical replicates per biological repeat) ± SEM. Significance was determined by two- way ANOVA - Bonferroni post t-test (*p= <0.05, ** p=<0.01 *** p=<0.001).

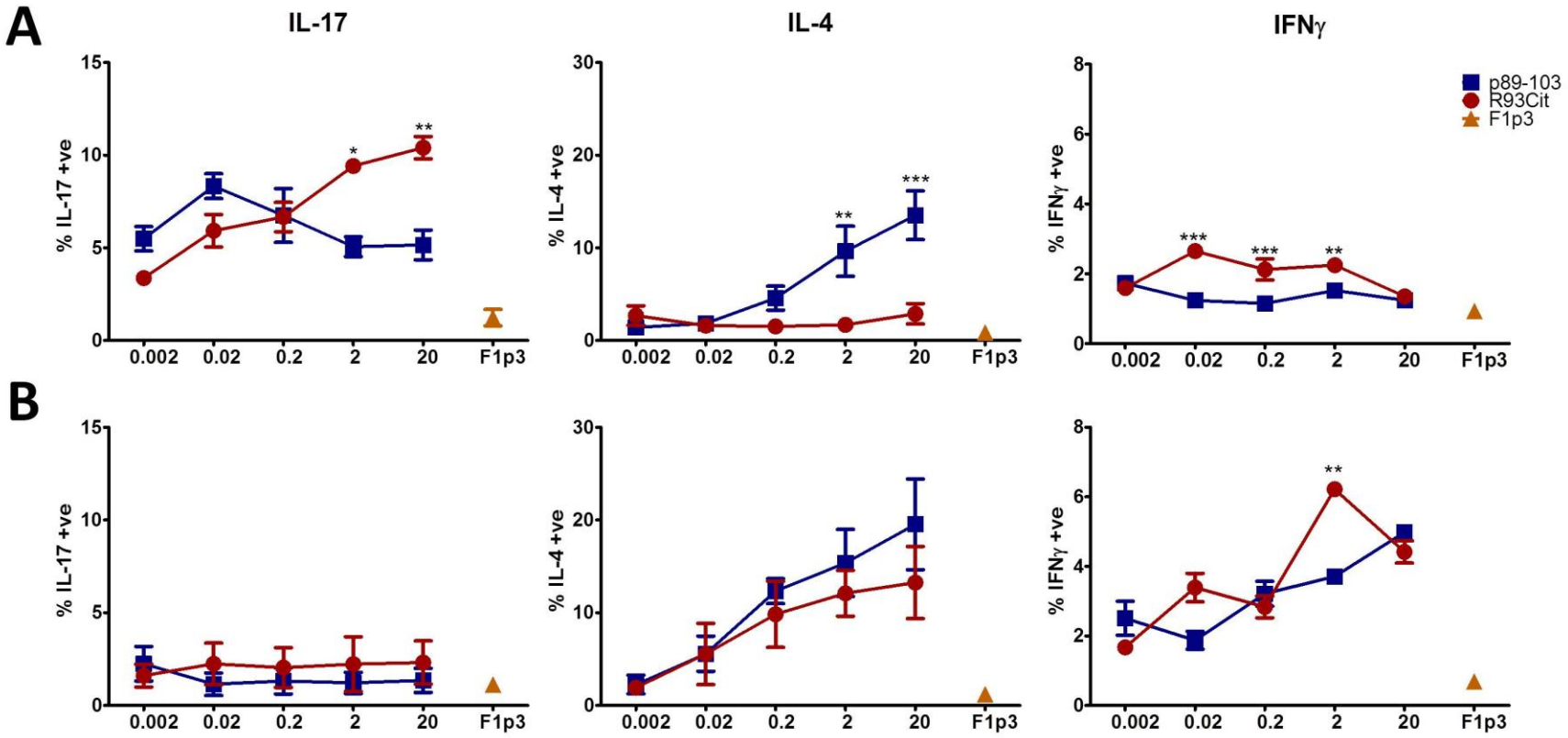

Figure 40 - R93Cit alters the frequency of Th cells expressing effector cytokines. Co-cultures of 5/4E8 TCR Tg T cells and mDCs were established across a range of p89-103 or R93Cit concentrations (0.002-20μM) or F1p3 (2μM). Assays were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of IL-17, IL-4 and IFNγ was determined following gating on the CD4. Data shows mean of three independent biological experiments (single technical replicate per biological repeat) ± SEM. Significance was determined by two- way ANOVA - Bonferroni post t-test (*p= <0.05, ** p=<0.01 *** p=<0.001).

4.7 Transcription factor expression induced by R93Cit

Given the distinct cytokine profile and proliferative response induced by the R93Cit peptide it was important to determine if the levels of ROR-γT, GATA-3, T-bet and FOXP3 would be also be altered. Therefore, cultures were activated with either p89-103 or R93Cit at the same concentration for 5 days. PMA/I was added for the remaining 5h of culture and BFA for the last 4h. Expression of the relevant transcription factor was determined by flow cytometry. These as with prior experiments were carried out in the presence and absence or Pro-Th17 cytokines.

The results demonstrate that transcription factor expression was less sensitive to changes in peptide affinity than seen with cytokine data (see **Figure 41**). GATA-3 was relatively widely expressed if either p89-103 or R93Cit was used to activate naive 5/4E8 TCR Tg T cells. This was despite the significant decline in IL-4 release found when cultures were stimulated with R93Cit compared to cognate peptide. With regards to ROR-γT, the levels of this transcription factor were found to be significantly enhanced by the weaker affinity ligand when compared to the same concentration of p89-103. However, if pro-Th17 cytokines were removed, levels of ROR-γT were reduced drastically regardless of which peptide was used during co-culture. Moreover, there was no significant difference between naive 5/4E8 TCR Tg T cells activated with either peptide. Consistent with previous data, co-expression of IL-17 and ROR-γT was detected (see **Figure 42**). The majority IL-17+ cells stained positive for ROR-γT as expected. However, not all ROR-γT+ cells stained for IL-17. A similar relationship was found to occur between IL-4 and GATA-3 (see **Figure 43**).

In terms of T-bet and FOXP3 the frequency of expression remained relatively low regardless of which peptide was used to activated 5/4E8 TCR Tg T cells (see **Figure 44**). There was no significant difference that could be detected following activation with either p89-103 or R93Cit. Thus, taken together, these data indicate that unlike the cytokine expression and production, changes in affinity affect only the expression of ROR-γT in the presence of pro-Th17 cytokines.

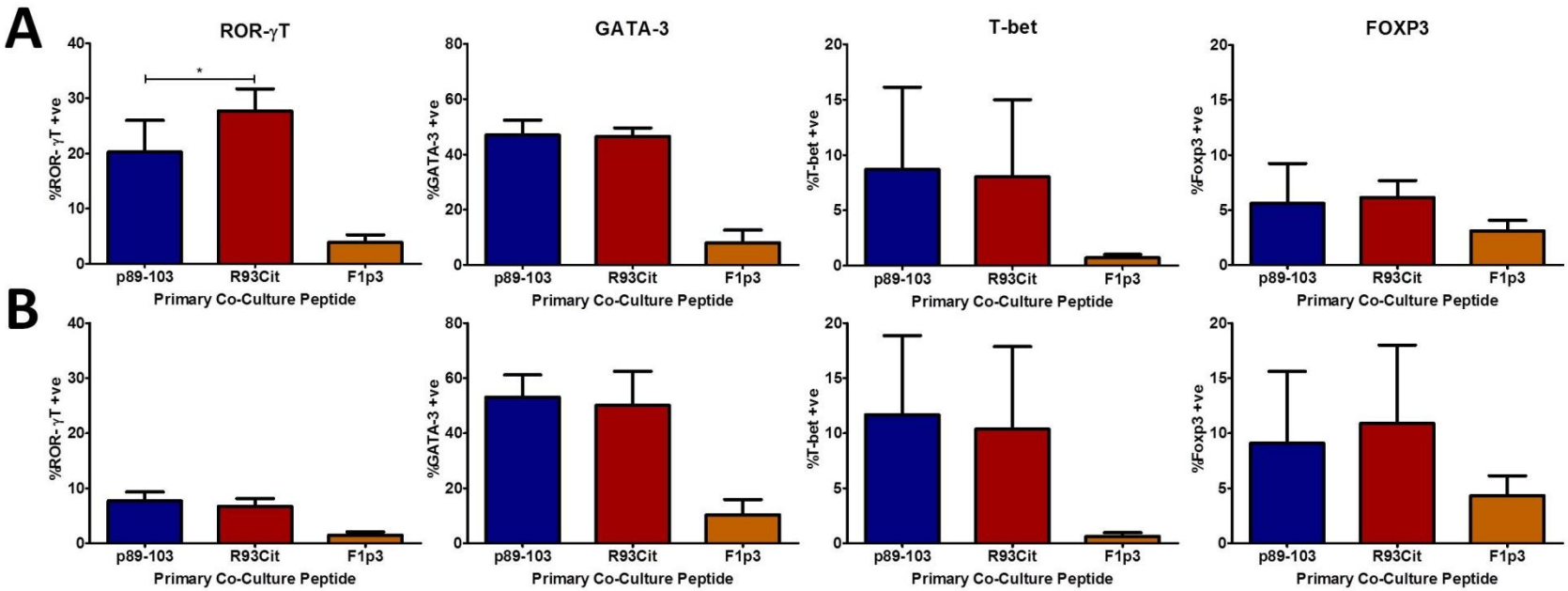

Figure 41 - R93Cit-induced transcription factor expression. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of R93Cit or F1p3 (2µM). Assays were carried out in both the presence (**A**) and absence (**B**) of Pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of ROR-γT, GATA-3, T-bet and Foxp3 positive cells following gating on the CD4. Data shows mean of three independent biological experiments (single technical replicate per biological repeat) ± SEM. Significance was determined by *t*-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001)

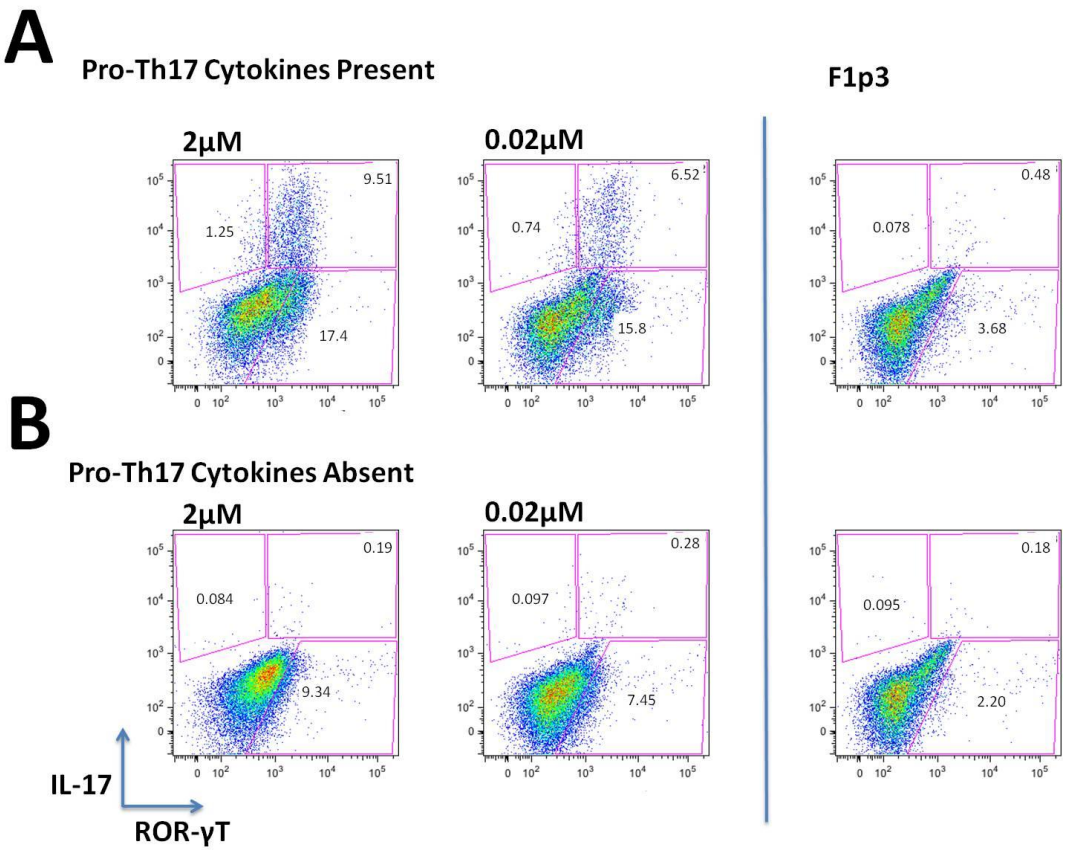

Figure 42 - Co-expression of IL-17 and ROR-γT in R93Cit-activated T cells. Example of R93Cit activated cells stained for CD4, IL-17 and ROR-γT. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of R93Cit or F1p3 (2µM). Cells were stimulated in either the presence (**A**) or absence (**B**) of Pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Data are a representative example of three independent experiments.

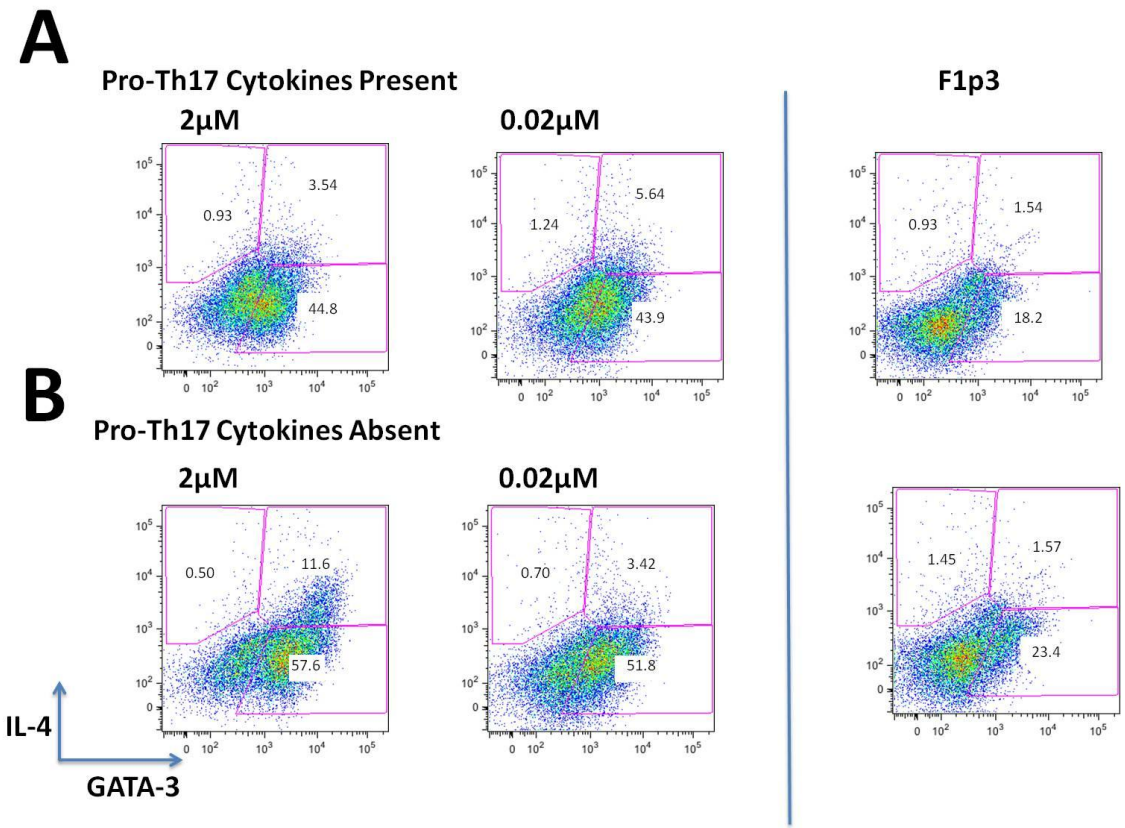

Figure 43 - Co-expression of IL-4 and GATA-3 in R93Cit activated populations. Example of R93Cit activated cells stained for CD4, IL-4 and GATA-3. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of R93Cit or F1p3 (2µM). Cells were stimulated in either the presence (**A**) or absence (**B**) of Pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Data are a representative example of three independent experiments.

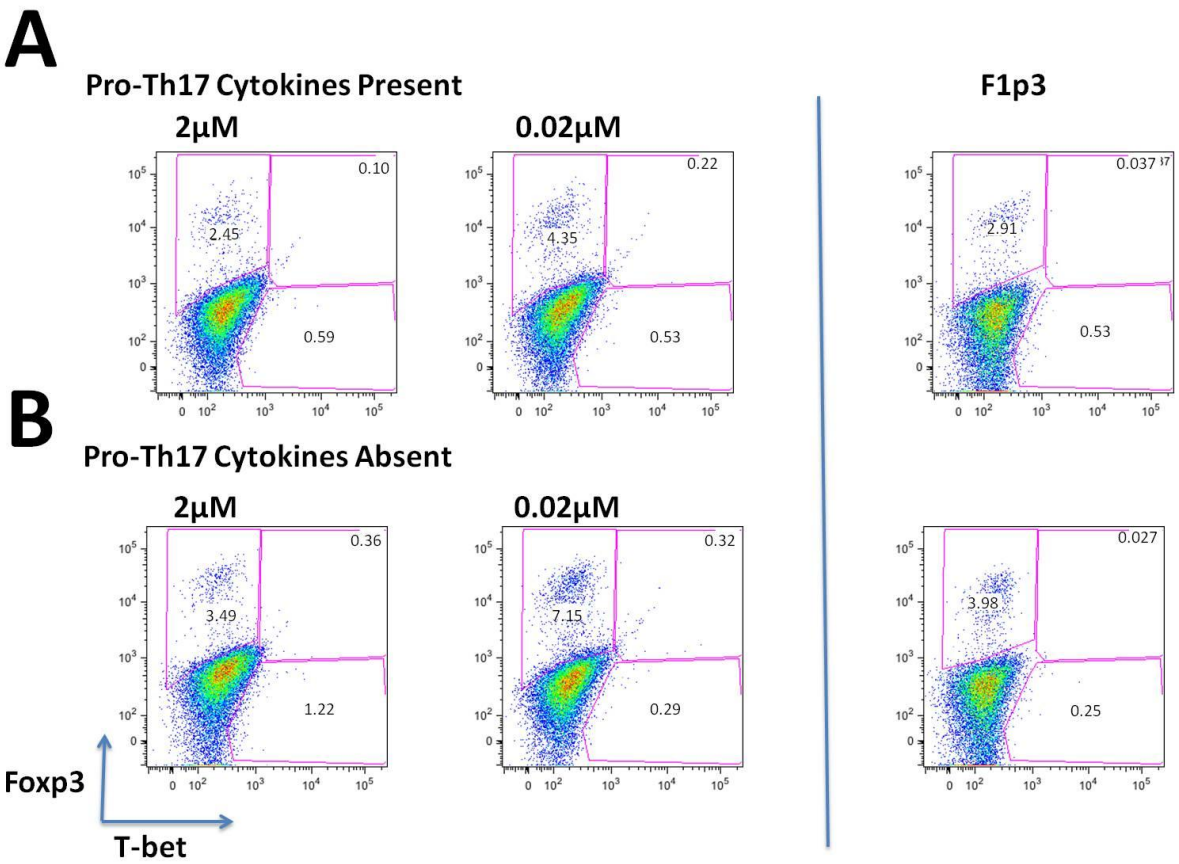

Figure 44 - Foxp3 and T-bet expression in R93Cit-activated T cells. Example of R93Cit activated cells stained for CD4, FOXP3 and T-bet. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established at either a high (2µM) or low (0.02µM) concentration of R93Cit or F1p3 (2µM). Cells were stimulated in either the presence (**A**) or absence (**B**) of Pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Data are a representative example of three independent experiments.

4.8 Pattern of surface marker expression is altered by citrullination

With the reduced proliferation and distinct cytokine profile it was important to determine if a weaker affinity peptide would alter the expression of a range of surface markers. If citrullination was reducing the potency of the interactions with 5/4E8 TCR it was expected that the down regulation in TCR and CD4 would also be reduced as compared to the cognate peptide. In contrast, I hypothesised that upregulation of CD69, CD25 and CD40L would be reduced when compared to p89-103.

Figure 45 indicates that, broadly, the hypothesised response was true. Focusing on the expression of TCRβ although a slight reduction occurred at day 3, the levels of expression are almost identical when p89-103 activated 5/4E8 TCR Tg T cells were compared to R93Cit at the same peptide concentration. A very similar response occurs with regards to the expression CD4 with no significant variation between any of the groups evaluated. However, expression of Vβ4, used by the 5/4E8 TCR, was significantly reduced after 3 days of co-culture only if by activation with the higher affinity cognate peptide. Thus, these data fit with the concept that the R93Cit is a weaker affinity peptide that induces less of a downregulation in 5/4E8 TCR over the time course tested.

The proportion of cells expressing activation markers, CD69, CD25 and CD40L, was also determined for over the same time course as shown in **Figure 46**. With regards to CD69 and CD25 both show a similar pattern of expression over time. As expected, in those cultures activated with p89-103 there was significantly greater induction of both of these markers at early timepoints. In the case of CD25, this difference between p89-103 and R93Cit activated 5/4E8 TCR Tg T cells was evident at every timepoint tested. CD40L was induced at frequencies far less than either CD69 or CD25. Indeed, the only significant difference in CD40L between co-cultures activated with either cognate or citrullinated forms occurred in the absence of pro-Th17 cytokines.

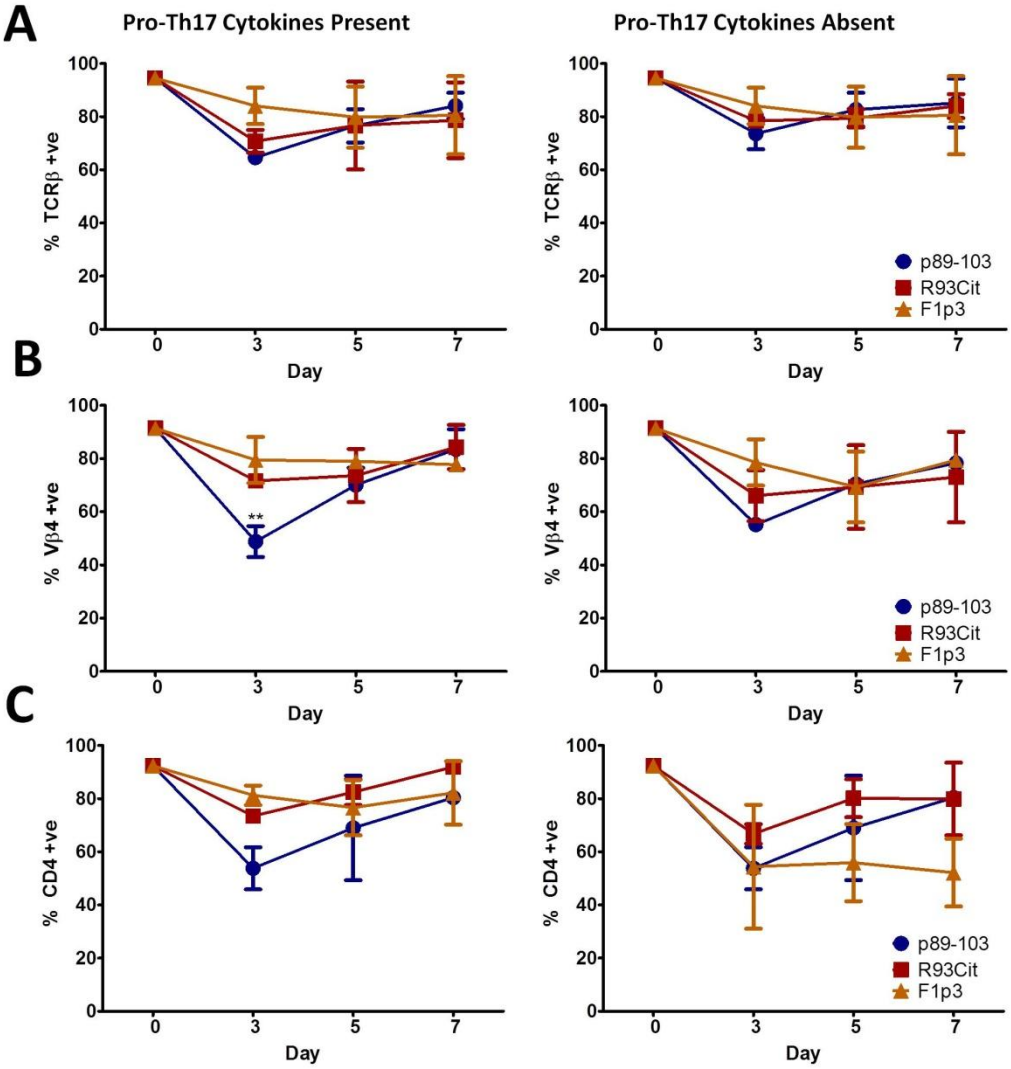

Figure 45 - The effect of citrullination on TCR and CD4 expression. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3. All peptides were present at the same concentration (2μM). At day 0, 3, 5, and 7 cells flow cytometry was used to determine the proportion of TCRβ (**A**), Vβ4 (**B**) and CD4 (**C**) positive cells. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM. Significance was determined by two- way ANOVA - Bonferroni post t-test (*p= <0.05, ** p=<0.01 *** p=<0.001).

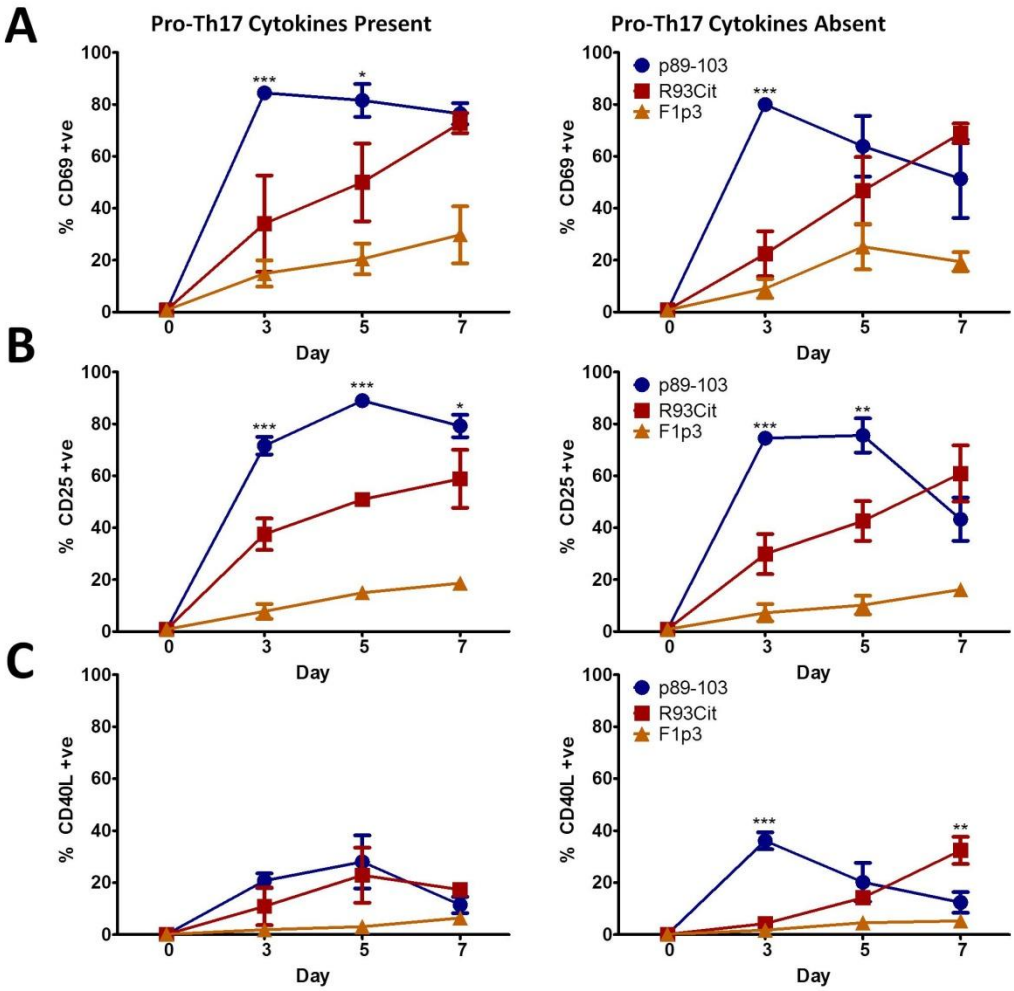

Figure 46 - Citrullination induces lower expression of activation markers relative to cognate peptide. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3. All peptides were present at the same concentration (2µM). At day 0, 3, 5, and 7 cells flow cytometry was used to determine the proportion of CD69 (**A**), CD25 (**B**) and CD40L (**C**) positive cells following gating on the CD4. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM. Significance was determined by two- way ANOVA - Bonferroni post t-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001).

4.9 Conversion of naive cells unaffected by citrullination

With the apparent differences in the T cell responses induced by the lower affinity citrullinated peptide R93Cit, it was important to determine if it was capable of inducing the conversion of naive ($CD4^{+}CD62^{hi}CD44^{lo}$) to memory cells ($CD4^{+}CD62^{lo}CD44^{hi}$). As is apparent by the data presented in **Figure 47** only at the higher concentration does R93Cit stimulate the loss of the naive phenotype with majority of cells up regulating CD44 while losing expression of CD62L. However, when compared to the cognate peptide at the equivalent concentration, no significant difference in the induction of memory cells could be found (see **Figure 47C** and **D**). Thus, while peptide affinity can alter the timing of expression of activation markers, the conversion of naive 5/4E8 TCR Tg T cells was not significantly changed by the weaker affinity R93Cit peptide.

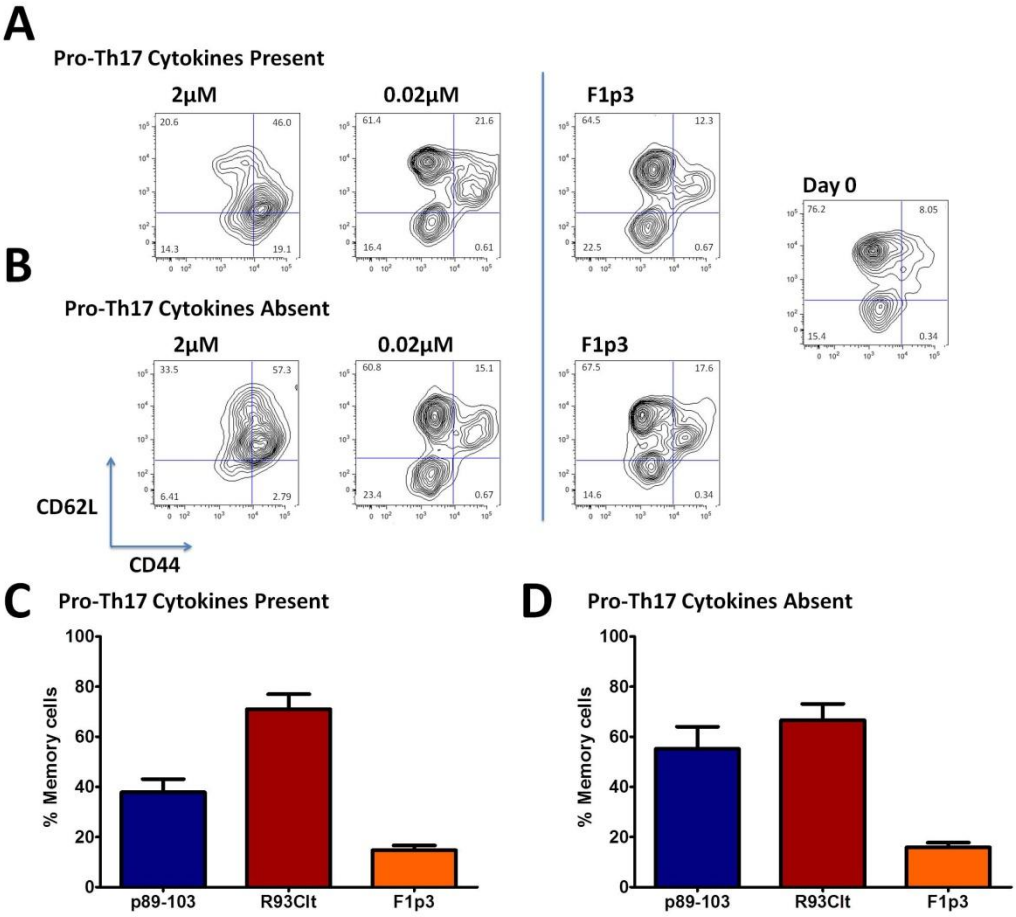

Figure 47 - Citrullination induces memory response at similar levels to cognate peptide. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3 (all 2µM). After 5 days cells flow cytometry was used to assess the expression of CD44 and CD62L following gating on CD4. An example of the gating is shown in both the presence (**A**) and the absence (**B**) of pro-Th17 cytokines. The frequency of memory cells from three independent experiments is summarised in presence (**C**) and absence (**D**) of pro-Th17 cytokines. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM. Significance was determined by *t-test* (*p= <0.05, ** p=<0.01 *** p=<0.001).

4.10 Pathogenic-associated cytokines are also regulated by citrullination

With R93Cit inducing an increase in Th17 development it was important to assess if the production of GM-CSF and IL-22 was also regulated by peptide affinity. Primary cultures were established as previously described with either cognate or citrullinated peptide at the equivalent concentration. After 5 days these cells were harvested and re-plated at a fixed cell density. These secondary cultures were incubated for a further 48h with 2μM of p89-103 before levels of GM-CSF and IL-22 were determined by sandwich ELISA. **Figure 48** indicates that the citrullination has a positive effect on the levels of both IL-22 and GM-CSF produced in secondary cultures. Indeed, R93Cit stimulated cultures released significantly increased levels of both cytokines in both the presence and absence of pro-Th17 cytokines.

When assessing the frequency of GM-CSF+ T cells R93Cit relfected the cytokine concentrations assessed in the supernatant of secondary cultures (see **Figure 49**). Again, those induced by R93Cit express GM-CSF at a significantly greater frequency than found in cultures stimulated with the higher affinity p89-103. However, in the absence of pro-Th17 cytokines difference in the levels of GM-CSF+ T cells was found not to be significant. Thus, taken together these data indicate that as well as supporting greater Th17 induction, R93Cit significantly enhances release of those cytokines associated with the pathogenic subset of the Th17 phenotype.

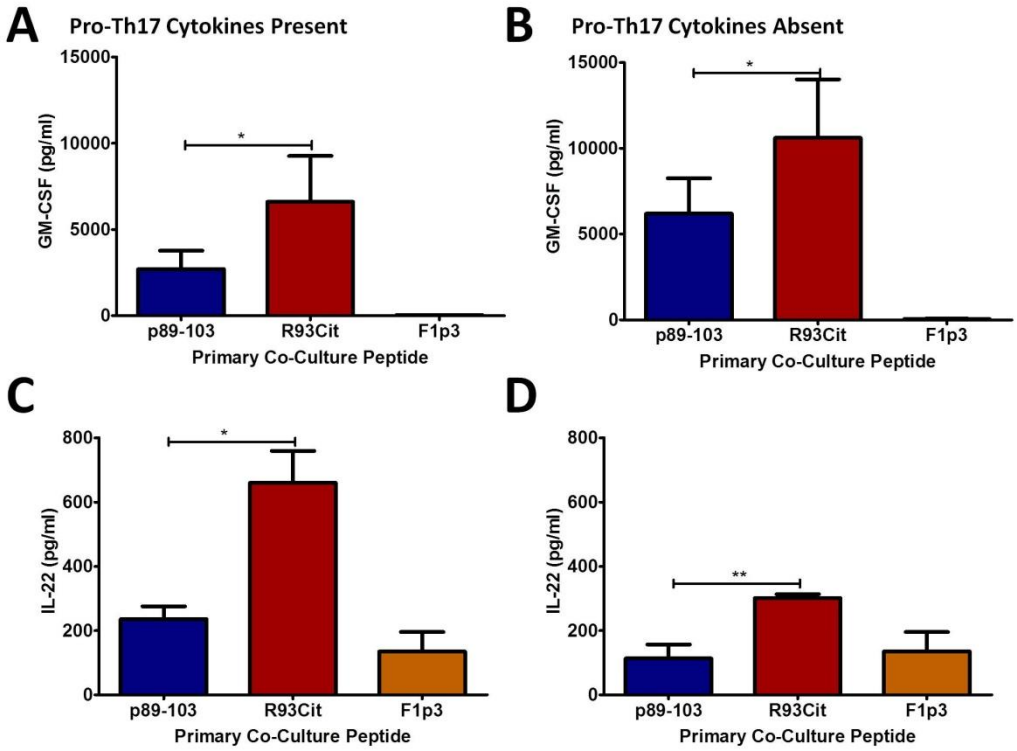

Figure 48 - Citrullination enhances the release of Th17 associated cytokines. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at the same concentration of p89-103, R93Cit or F1p3 (2µM). After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2µM of p89-103. After a further 48h supernatants were harvested and stored at -20^{o}C prior to analysis. ELISAs for GM-CSF and IL-22 were preformed according to the manufacturer's instructions. These assays were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. Data represents the mean of three independent biological experiements (three technical sample per biological repeat) ± SEM. Significance was determined by *t*-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001)

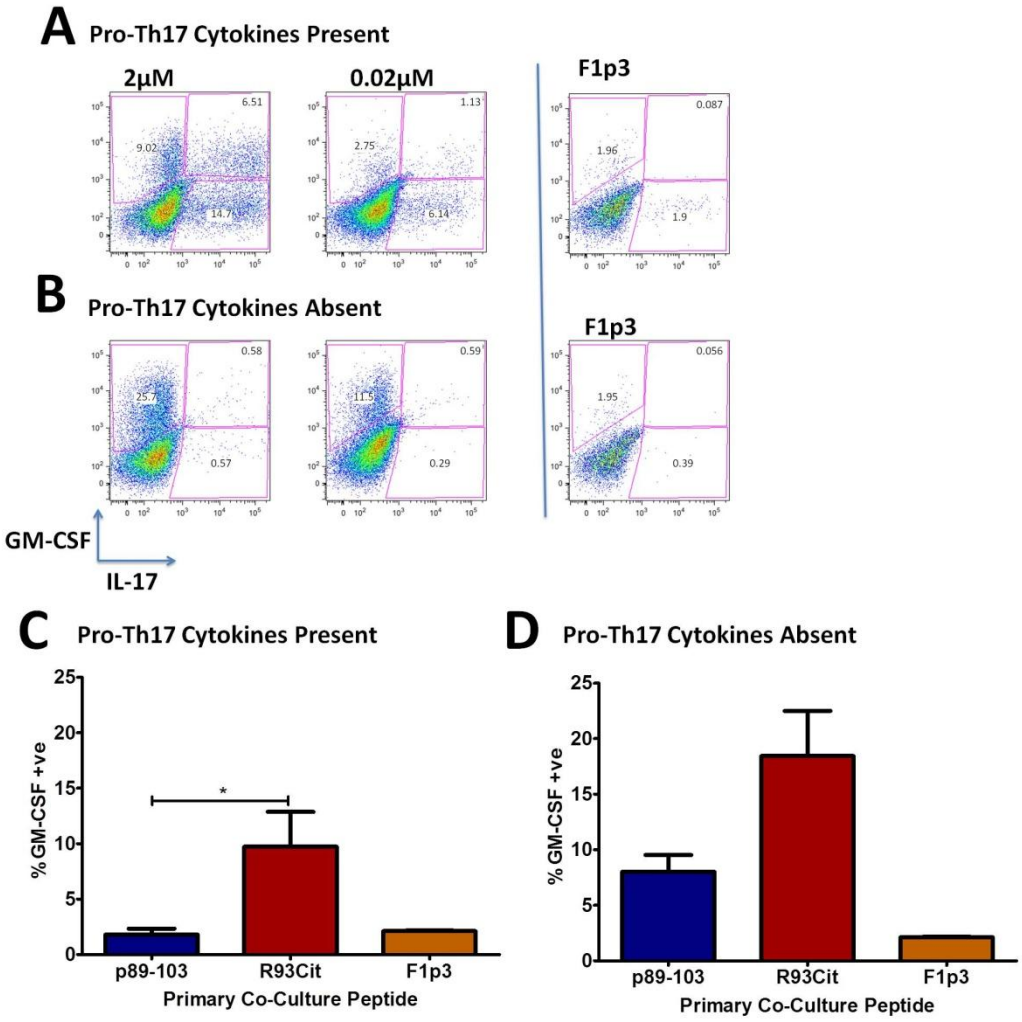

Figure 49 - The frequency of GM-CSF+ cells is enhanced by citrullination. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established at the same concentration of p89-103, R93Cit or F1p3 (2µM). After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Cells were initially gated in CD4 before the frequency of IL-17+ and GM-CSF+ cells was determined. An example of the gating is shown in both the presence (**A**) and the absence (**B**) of Pro-Th17 cytokines. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat) ± SEM is summarised in presence and absence of these exogenous cytokines (**C** and **D**) respectively. Significance was determined by *t*-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001)

4.11 Re-stimulation of secondary cultures with R93Cit enhances IL-17 release

Given the enhanced IL-17 response in those cultures primed with R93Cit, it was of interest to determine if re-stimulation with the weaker affinity peptide could also impact the cytokine response detected in secondary cultures. Therefore, primary

cultures of naïve 5/4E8 TCR Tg T cells and mDCs with either cognate or citrullinated peptide were established at a high (2µM) or low concentration (0.02µM). Unlike previous assays, the secondary cultures were re-stimulated with 2µM of either cognate peptide or the citrullinated form R93Cit. Following 48h of secondary co-cultures supernatants were collected and the levels of IL-17, IL-4 and IFNγ analysed by ELISA.

Figure 50 indicates that following primary cultures stimulated with cognate peptide, a weaker affinity peptide can still influence cytokine responses detected in secondary cultures. The use of a weaker R93Cit in secondary cultures caused a significant increase in IL-17 release. In contrast, re-stimulation with the citrullinated peptide failed to induce IL-4 even from those cells exposed to a high concentration of cognate peptide in primary cultures. There was also a decline in IFNγ at the lower peptide dose if R93Cit was used in secondary cultures.

In those populations stimulated in primary co-cultures with R93Cit the shifts in cytokine production were similar to that seen if p89-103 was used to activate secondary cultures (see **Figure 51**). In the case of IL-17, if secondary cultures are stimulated with the citrullinated peptide there is a significant rise in production. As expected the IL-4 levels were not enhanced by re-stimulation with R93Cit. There was no significant alteration in IFNγ whether cognate or citrullinated peptide was used in the secondary culture. Taken together these data confirm the positive influence of R93Cit on the production of IL-17 from 5/4E8 TCR Tg T cells.

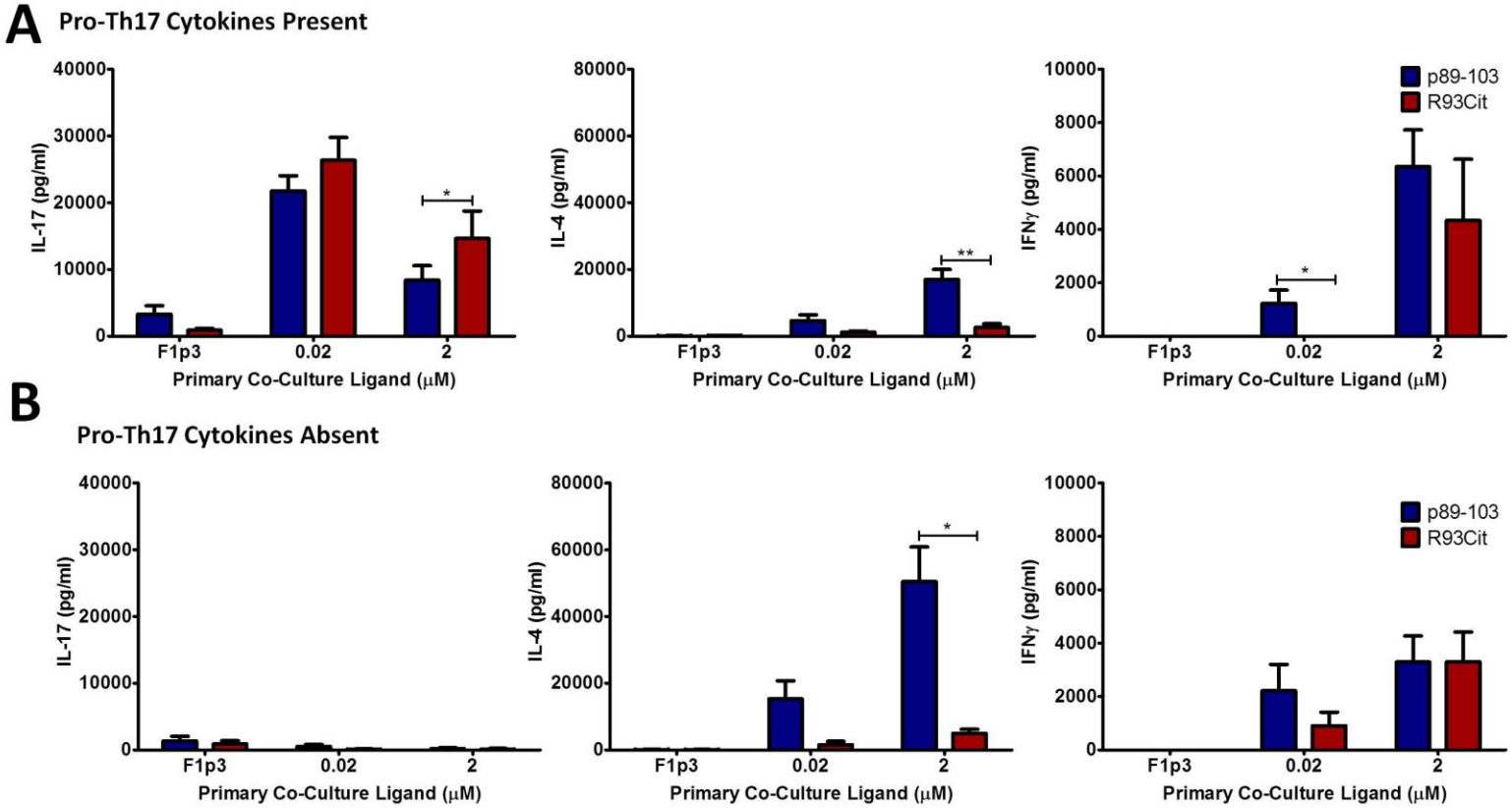

Figure 50 - IL-4 production can be reduced in p89-103 activated primary co-cultures re-stimulated with R93Cit. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established at either a high (2μM) or low (0.02μM) concentration of p89-103 or F1p3 (2μM). Primary co-cultures were established out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2μM p89-103 or R93Cit. After a further 48h supernatants were harvested and stored at -20°C prior to analysis. ELISAs for IL-17, IL-4 and IFNγ were performed according to the manufacturer's instructions. Data represents the mean of three independent biological experiements (three technical sample per biological repeat) ± SEM. Significance was determined by *t*-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001).

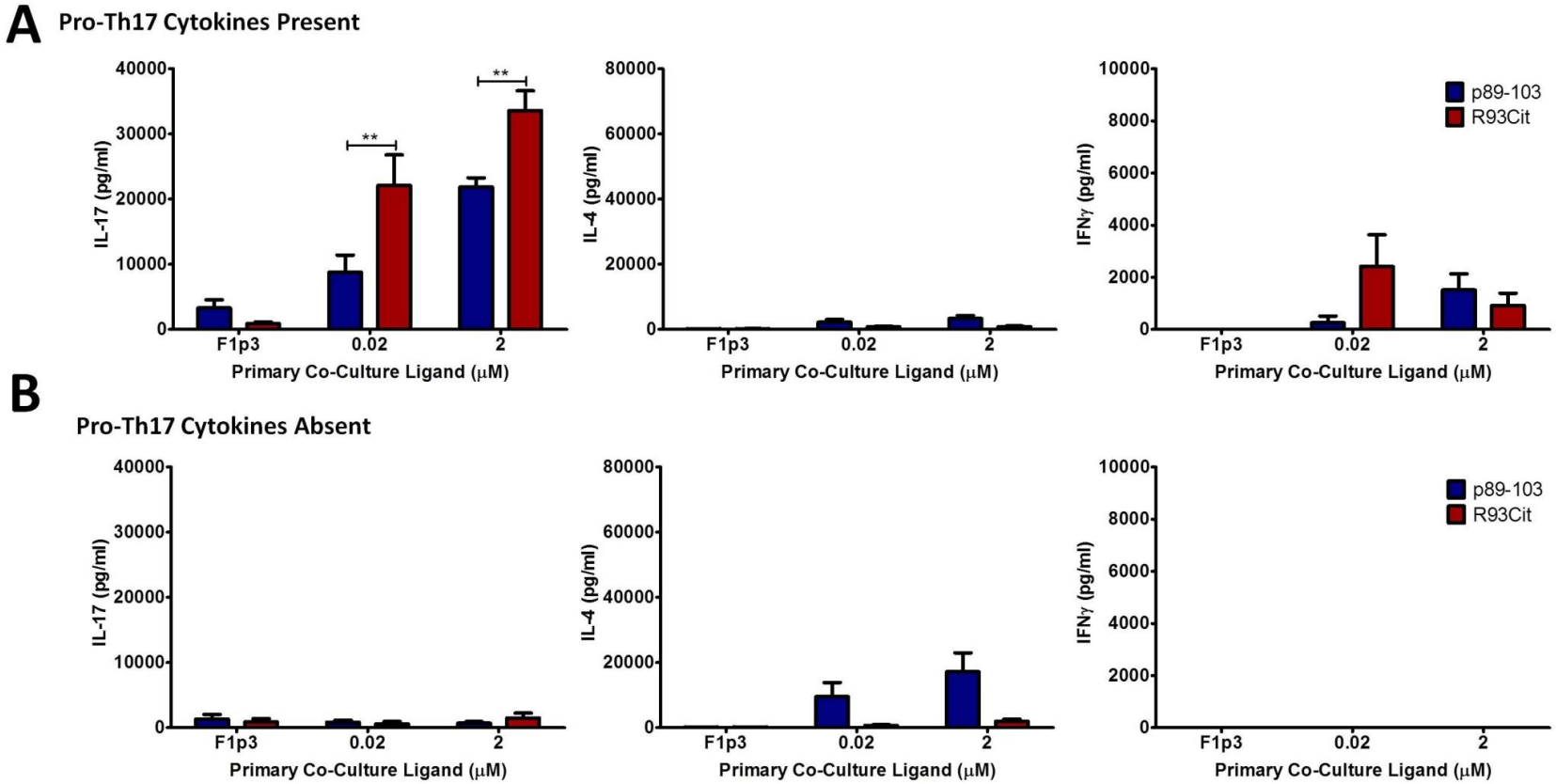

Figure 51 - IL-17 production can increased in R93Cit activated primary co-cultures re-stimulated with R93Cit. Co-cultures of naïve 5/4E8 TCR Tg T cells and mDCs were established at either a high (2μM) or low (0.02μM) concentration of R93Cit or F1p3 (2μM). Primary co-cultures were established out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2μM p89-103 or R93Cit. After a further 48h supernatants were harvested and stored at -20°C prior to analysis. ELISAs for IL-17, IL-4 and IFNγ were performed according to the manufacturer's instructions. Data represents the mean of three independent biological experiements (three technical sample per biological repeat) ± SEM. Significance was determined by *t*-test (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001)

4.12 Discussion

TCR recognition of pMHC is critical to the generation of adaptive immune responses to both foreign and self antigens. Although the binding of TCR to pMHC is relatively weak when compared to antibody:antigen interactions, the affinity of this interaction has been shown to influence the type of T cell response (273). Numerous studies have begun to characterise how subtle changes in the sequence of antigenic peptide can influence the affinity of this interaction (159). Thus, it was of interest to determine how single amino acid substitutions to the core 9-mer of aggrecan p89-103 could influence the response of naive 5/4E8 TCR Tg cells both in terms of the magnitude and type of response. The data presented in this chapter shows how some alterations, particularly those involving either Arginine-93 or 95, can significantly shift the response of naive 5/4E8 TCR Tg T cells both in terms of proliferation and effector cytokine production. Despite the concentration of peptide being equivalent during the primary culture, the production of IL-17 and IL-4 were significantly altered. Citrullination also led to an increase in IL-17 with R95Cit peptide inducing by far the greatest level in secondary cultures. Of the APLs screened, none were found to significantly inhibit IL-17 release relative to the cognate peptide. Studies by Iwanami *et al* have shown that APLs to GPI-6 325-339 can reduced IL-17 production from antigen specific Th17 cells (394). Indeed, co-immunisation of antagonistic APLs can prevent *in vivo* disease onset (395). It would be interesting if a larger panel of APLs were screened if any of these would significantly impair IL-17 production relative to cognate peptide. Thus, an APL that antagonised IL-17 might prove to be useful in ameliorating joint damage *in vivo*.

Despite the increased release of IL-17 in secondary co-cultures, further examination of the T cell response to R95Cit failed to induce any significant degree of expansion over an extended time course. Indeed, the proliferative response fits that of a null peptide with the response not significantly greater than the negative control. Given its likely status as an MHC cII contact point, the changes in Th response could result in altered peptide binding to the MHC molecule which in turn would affect the peptide concentration presented to T cells. Other studies have also examined the impact of alterations at MHC contact points. Modification to the Haemoglobin

peptide (Hb (p64-76)) at the P6 MHC anchor lead to a thousand fold reduction in proliferation of Hb specific 3.L2 TCR Tg cells (396). Similarly, APLs altering MHC contacts in the collagen type II epitope, 261-273, were found to completely abrogate IL-2 release of four antigen specific hybridomas when APL was presented by HLA-DRB1*0401 (397). *In vivo* modifications at MHC sites can attenuate contact between the T cell and the respective APC (273). In the context of EAE, Ford *et al* have shown that an APL involving the switching of the a MHC contact at P6 position could induce IFNγ and autoantibody production (398). However, these levels were insufficient to induce any clinical signs of EAE or optic neuritis. Thus, the lack of proliferation with R95Cit is broadly in line with previous examples were a MHC contact is substituted.

It was important to further evaluate the Th response to R93Cit given this modification affected a putative TCR contact. Moreover, citrullination is a process common to many forms of inflammatory arthritis, therefore greater understanding of how such modifications can shape the type of Th response may offer a valuable insight into disease aetiology. Confirmation of the change to TCR affinity would require future studies using fluorescently-labelled tetramers for both the cognate and citrullinated forms of aggrecan peptide, thus allowing for calculation of on/off rates as well as K_d values.

The data presented in this chapter are in agreement with the hypothesis that a reduced TCR signalling intensity favoured induction of the Th17 phenotype. When compared to the equivalent dose of cognate peptide, the lower affinity R93Cit peptide significantly enhanced both the frequency of IL-17+ cells and production of IL-17 in secondary cultures. Interestingly, when R93Cit was titrated over an extended dose range, the pattern was reversed when compared to that induced by the cognate peptide with positive increase in IL-17 with higher concentrations of R93Cit. Furthermore, there was a complete abrogation of IL-4 production in cultures stimulated with the citrullinated form. This contrasts with observations by Tao *et al* using the APLs derived from the MCC 81-104 (399). The K99R APL (significantly less potent than the cognate peptide at inducing proliferation of antigen specific T cells) was capable of inducing large quantities of IL-4 in secondary

cultures (159). In contrast, the response to cognate peptide was dominated by IFNγ with minimal levels of IL-4 detected. Thus, with regards to the influence TCR signal intensity, this conflicts with the data presented in this chapter. However, differences in TCR affinity, genetic background of the mice, APC type and ratio could all influence the type of Th2 response observed meaning direct comparisons between TCR Tg models is challenging.

A pertinent question arises as to whether the qualitative or quantitative changes in TCR signal strength are interchangeable or mutually exclusive in terms of their effects on the type of Th responses. Studies by Gottschalk et al have shown that for induction of the Treg phenotype a low density of a high affinity peptide was optimal (271). Further, work by the Allison lab has indicated that some features of TCR signalling can be compensated by increasing the concentration of a lower affinity ligand (400). Other pathways remain independent i.e changes in concentration cannot overcome the reduced affinity of a particular peptide. Interestingly, the Th17 response induced by the higher dose of R93Cit was very similar to the lower dose of p89-103 in terms of IL-17 production, the frequency of IL-17+ and ROR-γT+ 5/4E8 TCR Tg T cells. This suggests that with regards to the Th17 phenotype that concentration and peptide affinity can compensate for one another. Further analysis by Gottschalk *et al* demonstrated a signature of genes that could be regulated by either avidity of affinity while another set were exclusively influenced by peptide affinity (400). It would be of interest in future studies to undertake the same genetic analysis to determine if the same 'gene signature' could be observed in 5/4E8 TCR Tg T cells following stimulation with either cognate or citrullinated peptides. Genes such as *Il7r, Klf2* and a number of G protein receptors were found to be dependent on affinity of a TCR ligand (400). Increasing concentrations of weaker affinity peptides could not compensate for the reduced affinity for a particular TCR. Thus, it would be expected that even at a high dose of R93Cit expression of those genes (e.g. *Il7r* or *Klf2*) would not be similar to those T cells activated with the p89-103.

Other studies have assessed the impact of TCR affinity *in vivo* using APLs. Using TCR Tg T cells that recognised the MCC peptide 81-103, APLs of varying affinities were

found to influence the interaction of naive TCR Tg T cells with APCs within lymph nodes (274). With high affinity pMHC complexes, rapid engagement and arrest of T cells for several hours with the partner APC occurred. In contrast, a lower affinity APL failed to alter T cell motility within the lymph node with less time spent interacting with APCs. Interestingly, CD69 expression was not affected by changes in peptide affinity with APLs of varying affinities inducing a similar level (274). This contrasts with the data presented in this project in which cognate and R93Cit induced significantly differing levels of CD69 expression *in vitro.*

Gottschalk *et al* also used two-photon imaging to examine the differences between peptide density and affinity (400). These data indicated a distinct pattern of interaction between the T cells and DC *in vivo*; both in terms of the duration of T cell-DC contacts and the stability of these interactions that was primarily dependent on the affinity of the TCR-pMHC binding rather than the peptide concentration used during immunisation. While T cell:DC interactions were not addressed in this project, it would be of interest in future to understand if 5/4E8 TCR Tg T cells interacted with cognate or citrullinated pulsed DCs in a similar manner. It would be expected that DCs presenting the higher affinity cognate peptide would form more stable contacts with 5/4E8 Th cells than those presenting R93Cit.

As well enhancing the development of the Th17 cells, the citrullinated peptide induces significantly greater production of Th17 associated cytokines IL-22 and GM-CSF. Further examination showed the frequency of GM-CSF+ cells at a high concentration of R93Cit. There was also an increase in the frequency of GM-CSF+ cells that co-express IL-17. Taken together the data shows that not only does citrullination expand Th17 development especially when compared to equivalent dose of cognate peptide, but the population that develops also has the potential to become more pathogenic *in vivo*. It would be of interest in future studies to confirm that citrullination enhanced Th17 pathlogy *in vivo.* Others have shown that administration of citrullinated peptides *in vivo* can exacerbate ongoing arthritis following intraarticular immunisation with citrullinated peptides (401). Hill *et al* have shown that citrullination of aggrecan p280-292 increased, by a hundred fold, its affinity for HLA DRB1 (402). Furthermore, citrullination enhances the binding of

vimentin to HLA-DR , with only the citrullinated analogue capable of inducing a T cell response. The data presented in this chapter show that citrullination of an immunogenic candidate autoantigen can impact on the type of T cell response. It would be interesting to evaluate if citrullinated APLs, such as R93Cit, could induce or exacerbate inflammation in the context of an *in vivo* disease model such as PGIA.

The source of citrullinated antigen *in vivo* would also be an area of interest. Expression of PAD2 and -4 can be found in patients and closely correlates with joint inflammation (403). In both acute and chronic animal models of inflammatory arthrtis, PAD expression can also be detected. Interestingly, in streptococcal cell wall (SCW) induced arthritis and CIA PAD2 is expressed but not transcribed while PAD4 protein can be found only within inflamed joints, primarily expressed by neutrophils infiltrating the synovium (404). Given that the PGIA can be induced in 5/4E8 TCR Tg mice, it would be of interest to assess intraarticular PAD expression and if a T cell response occurs to citrullinated epitopes during disease induction.

Citrullination can also alter the response of T cells in other autoimmune diseases such as MS. MBP can be citrullinated at 19 distinct site throughout the 18.5kDa protein (312). Studies using adoptive transfer have shown that citrullination widens the pool of T cells capable of reacting. Indeed, PAD expression and citrullination correlate with disease progression in a transgenic spontaneous demyelination disease model. In Marburg's syndrome, a form of MS, in which 18 of the 19 sites are modified in patients. The citrullinated form of MBP can be used to reactivate EAE pathology in rats immunised with unmodified MBP. It would be of interest to understand if the modified form of MBP could also induce changes in the effector type of Th response.

In summary, the data presented in this chapter demonstrated that modification of the first TCR contact in aggrecan p89-103 has profound effects on both the magnitude and type of T cell response. The citrullinated form of this peptide behaves as a partial agonist for 5/4E8 TCR Tg T cells as defined by proliferation. Comparison over an extended titration shows a loss of the Th2 phenotype while enhancing Th17 cell development. Indeed, re-stimulation of secondary co-cultures

with the R93Cit peptide enhanced IL-17 release still further. R93Cit-stimulated cells fail to up regulate a range of activation markers including CD69 and CD25 to the with the same rapidity as those stimulated with cognate peptide. The weaker affinity of the R93Cit also induces a potentially more pathogenic population of T cells with an enhanced release of both IL-22 and GM-CSF. Thus, the data agree with the hypothesis that a reduced TCR signal enhances the Th17 development in cooperation with an appropriate cytokine environment. The focus on the next chapter will be the understanding of the potential mechanism that underpins the relationship between TCR signal intensity and the Th17 response.

5 IL-2 - the link between TCR signal intensity and Th responses?

5.1 Introduction

One of the major consequences of TCR signalling is the engagement of the IL-2 system resulting in both IL-2 cytokine production and expression of the high affinity α chain (CD25) of the IL-2 receptor (405). Traditionally defined as a key mitogen for naive T cells, IL-2 has been routinely added to *in vitro* T cell assays and is an important reagent in establishing T cell clones (406). The three IL-2R subunits can form two classes of receptor- either a dimer of IL-2Rβ-γ_c or a trimer consisting of IL-2Rα-β-γ_c (407). Alone, each receptor component has a relatively weak affinity for IL-2 (IL-2Rα - K_d 10^{-8}M, IL-2Rβ - K_d 10^{-6}M in combination with γc K_d - 10^{-9}M) yet the trimeric form has much greater affinity (K_d 10^{-11}M) with rapid on/off rates (408). After engagement of the IL-2R, activation of multiple signalling pathways occurs via the associated JAK1 and JAK3, which activate STAT5 that subsequently traffics to the nucleus (see **Figure 52**) (407). IL-2 transcription is primarily regulated by NFAT proteins which cooperate with AP-1 to bind the IL-2 promoter following TCR signalling (409) (410). Loss of NFAT prevents IL-2 induction following TCR signalling (117). Indeed, this pathway is a major target of immunosuppressive drugs such as cyclosporine A and FK-506, used to prevent rejection of transplanted organs (411). In an intrinsic form of negative feedback, IL-2 induces BLIMP-1 protein expression that in turn serves to bind to regions in the IL-2 and c-FOS promoters preventing further transcription (412). FOXP3 can also serve to negatively regulate IL-2 production while still inducing the expression of CD25 and CTLA-4 in Tregs (409).

It has become increasingly apparent that IL-2 can also modulate Th cell effector functions (413-415). With regards to Th2 cells the STAT5 signal stimulated by IL-2 is needed to modify the chromatin landscape at the *Il4* locus in conjunction with c-maf (416, 417). IL-4Rα expression is also promoted by IL-2 therefore enhancing the sensitivity of the cell to further IL-4 stimulation (418). The mechanism proposed to inhibit Th2 development after a strong TCR signal is dependent on sustained ERK activation preventing efficient IL-2 signal transduction during the early stages of Th2

differentiation by prevention of STAT5 phosphorylation (419). Similarly, other STAT5 inducing cytokines also promote IL-4 and IL-4Rα expression such as IL-7 and IL-15 (420).

The impact of IL-2 on the Th17 phenotype has been shown by the O'Shea group to be profoundly negative (421). Indeed, adoptive transfer of T cells derived from *Il2*$^{-/-}$ mice results in enhanced Th17 induction when compared to WT T cells. The explanation for the precise mechanism differs between groups although these are by no means mutually exclusive. The hypothesis advanced by the O'Shea group is that the presence of a strong IL-2 signal leads to competition between STAT3 and STAT5 at the *il17a* locus as shown by CHiP data (422). Thus, these cells, despite expressing ROR-γT, fail to produce significant levels of IL-17. However, others have shown that IL-2 inhibits the expression of IL-6Rα and gp130 necessary for STAT3 signalling within Th17 cells (309). Retroviral transduction of gp130 was capable of partially restoring Th17 development within the model. Moreover, other evidence shows that Th17 cells express negative regulators of IL-2 release including transcriptional repressors such as Aiolos and IL-4 induced gene-1 (IL4G1) that bind the *Il2* promoter (423). Aiolos is found to be induced by STAT3 and AHR signalling and is found to be expressed exclusively in Th17 cells. In humans IL4G1 limits IL-2 release and is found to be expressed at greater levels in the Th17 subset (424). Moreover, an anti-proliferative protein, Tob1, is found to limit the expansion of human Th17 cells following TCR stimulation (425). The expression levels are far greater than those found in Th1 cells both in resting cells and following TCR activation. Furthermore, it has been proposed that Th17 cell survival may in fact be enhanced by Tregs due to their high expression of CD25 (426). Thus, Tregs deplete the local microenvironment of IL-2 and help to sustain Th17 cells. However, Fuijmara *et al* have postulated that IL-2 had no negative effects on Th17 compared to Th1 cells when comparing absolute cell numbers both *in vitro* and *in vivo* (427). Thus, the role of IL-2 with regards to Th17 cells remains an area needing further study.

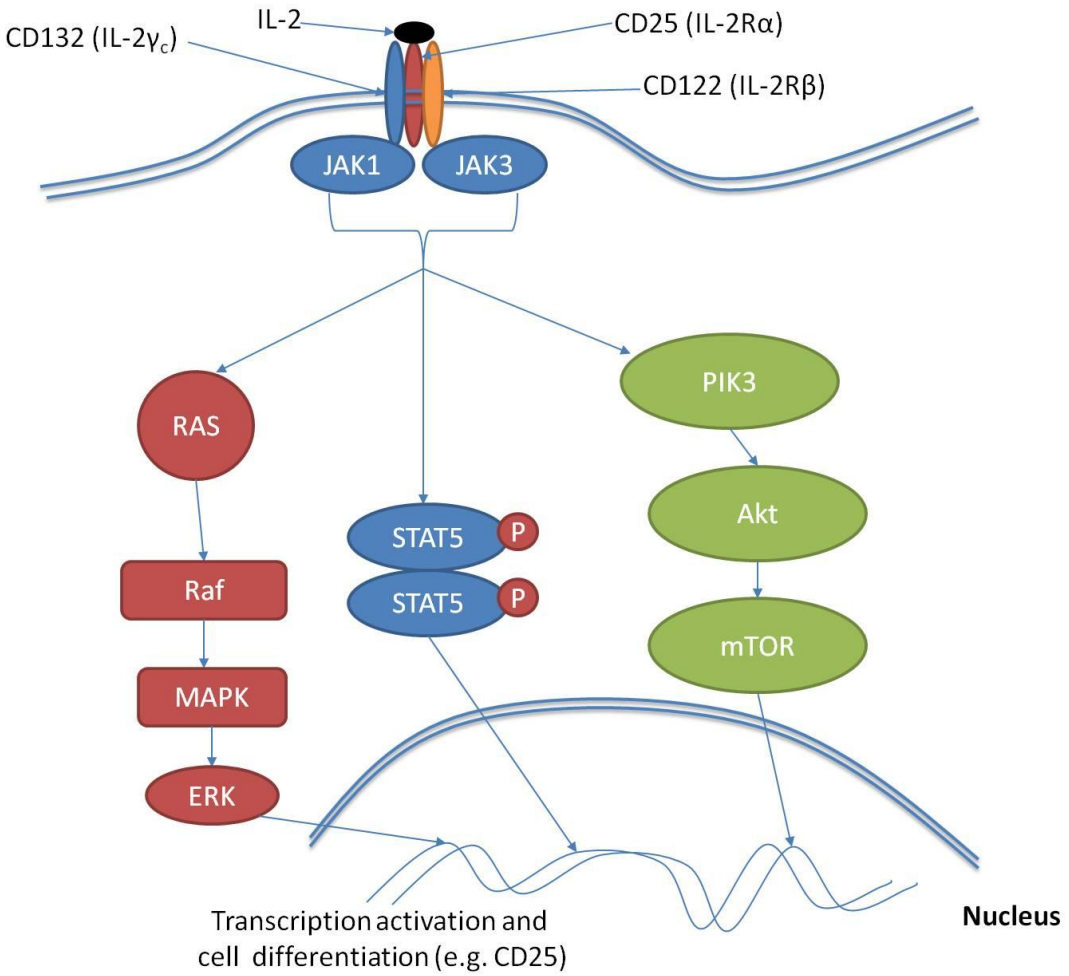

Figure 52 - Overview of IL-2 signalling. The trimeric form of the IL-2 receptor has the greatest affinity for IL-2 (K_d 10^{-11}M). Although not shown the pathway can be triggered by less efficient combinations of IL-2R chains (409). Once bound, IL-2 induces binding and activation of JAK1/3 kinases that serve as the key adaptors for the three main downstream pathways. Together these pathways promote cell proliferation, survival and further expression of genes involved in IL-2 signalling such as CD25.

5.2 Chapter aims

Given that the IL-2 pathway is one of the major targets engaged by TCR signalling and the growing data implicating IL-2 in the regulation of Th17 cell development it was important to explore this area further. The use of naïve 5/4E8 TCR Tg T cells as our starting population allowed modulation of signal strength in a more precise manner using a disease-relevant antigen. Thus, it was possible to determine the contribution that changes to TCR signalling had upon both IL-2 production and IL-2 receptor expression. Utilising these tools the aims of this chapter were to:

- Determine if IL-2 production and signalling were altered by changes to TCR signal strength
- Assess the impact of changes to activity of the IL-2 pathway on the T cell response to a high or low affinity TCR ligand
- Determine the relationship between the STAT3 and STAT5 phosphorylation levels and the intensity of TCR signalling

5.3 Citrullination of aggrecan peptide leads to reduced IL-2 release

Initial experiments sought to define if the production of IL-2 varied between cultures stimulated with either cognate or R93Cit peptides. Parallel cultures of naive 5/4E8 TCR Tg T cells were established with mDCs and the same concentration of either the cognate p89-103 or the R93Cit peptide. At 24h intervals supernatants were collected and IL-2 levels assessed by sandwich ELISA. **Figure 53** shows the responses from three replicate experiments carried out in both the presence and absence of pro-Th17 cytokines.

Although the exact kinetics and level of IL-2 release varied between each replicate experiment, the analysis indicated that cognate peptide induced consistently more IL-2 than those cultures stimulated with R93Cit. Assessment using two-way ANOVA showed significant differences especially at earlier time points. Moreover, the peak response of T cells stimulated with R93Cit was also a lower value than that induced by the cognate peptide. When comparing naive 5/4E8 TCR Tg T cells activated in the presence of pro-Th17 cytokines or without, the same pattern occurred with greater IL-2 release in T cells activated by the cognate peptide. However, levels of IL-2 were consistently higher in the absence of exogenous cytokines. Taken together, these data demonstrate that changes to TCR signalling can alter the production of IL-2 within these cultures.

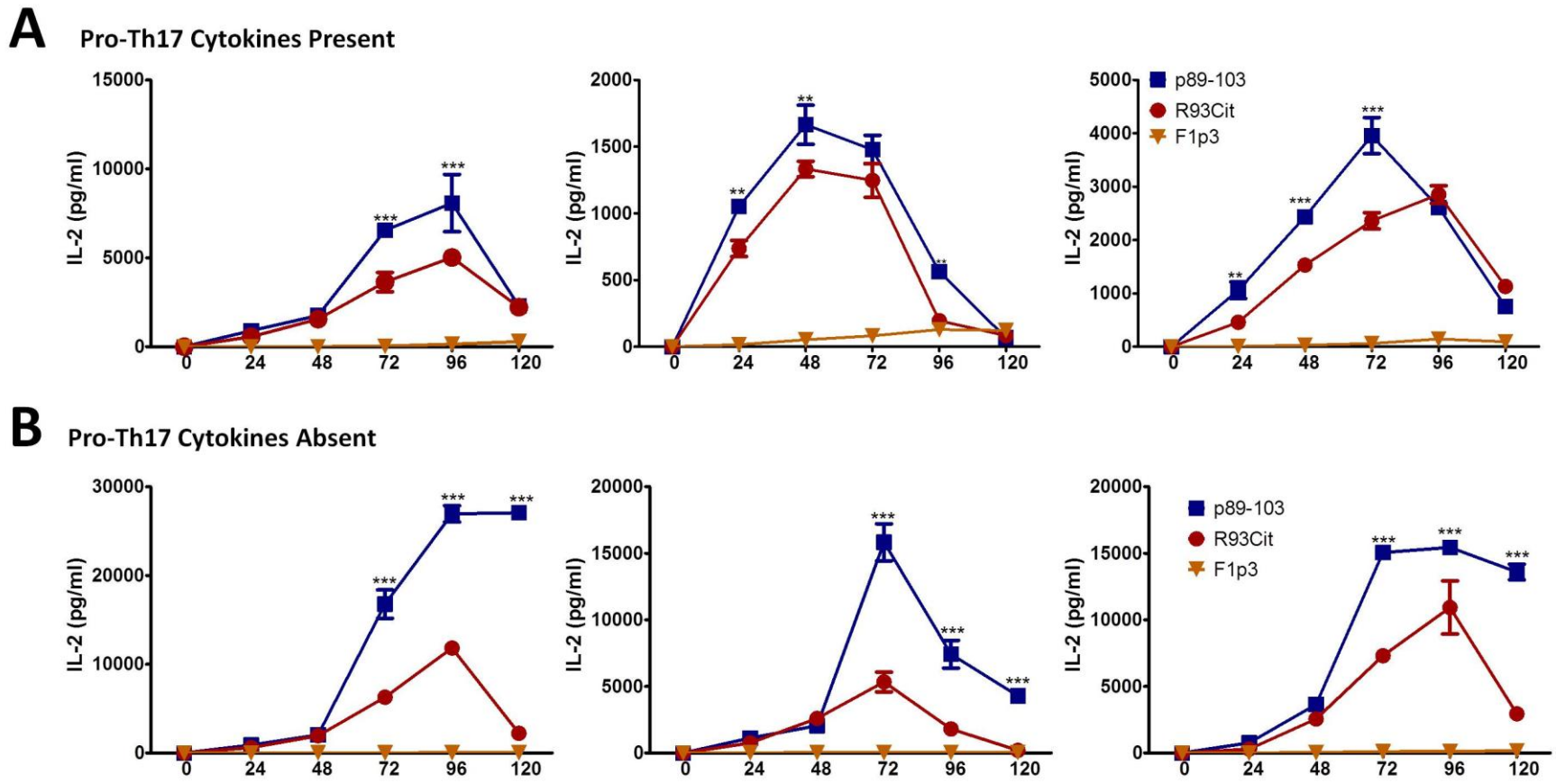

Figure 53 - IL-2 release during primary co-cultures. Parallel co-cultures of $1.25x10^5$ naive 5/4E8 TCR Tg T cells and $6.25x10^3$ LPS mDC were established with 2µM of either p89-103, R93Cit or F1p3. Assays were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. At each respective time point supernatants were harvested and stored at -20°C prior to analysis. The level of IL-2 in each sample was determined by sandwich ELISA according to the manufacturer's instructions. All three biological replicates (two technical repeats per biological sample) are shown with error bars indicating ± SEM. Significance was determined by two-way ANVOA test (*p= <0.05, ** p=<0.01 *** p=<0.001)

5.4 IL-2Rα expression is affected by citrullination of aggrecan peptide

Given the apparent differences in IL-2 production, it was also important to determine if these populations could respond to the IL-2 present in the local microenvironment. As such the expression of the high affinity component of the IL-2R complex, CD25, was determined using flow cytometry. **Figure 54** shows the summary of both the percentage CD25 positive cells and the degree of CD25 expression as determined by median fluorescence intensity (MFI) for T cells activated with either cognate or R93Cit peptides after 5 days of co-culture with mDCs. Unsurprisingly, CD25 expression both at the population and single cell level was altered by TCR signal strength as demonstrated by the significant differences between 5/4E8 TCR Tg T cells activated with cognate and R93Cit. Both peptides induced CD25 at levels far greater than that of the control population exposed to the irrelevant F1p3 peptide. However, the difference in MFI values indicates that stimulation with cognate peptide induced significantly greater levels of CD25. Given that CD25 is regulated by TCR signalling it was expected that those stimulated with the weaker affinity R93Cit would express far less CD25. This finding has implications for both the Th17 and Th2 response given that IL-2, a potent driver of STAT5, is an important brake on IL-17 release in the former while being a critical factor in IL-4 release. The distinct CD25 levels coupled with the differential IL-2 release compounds the differences in T cell phenotypes that develop within each population.

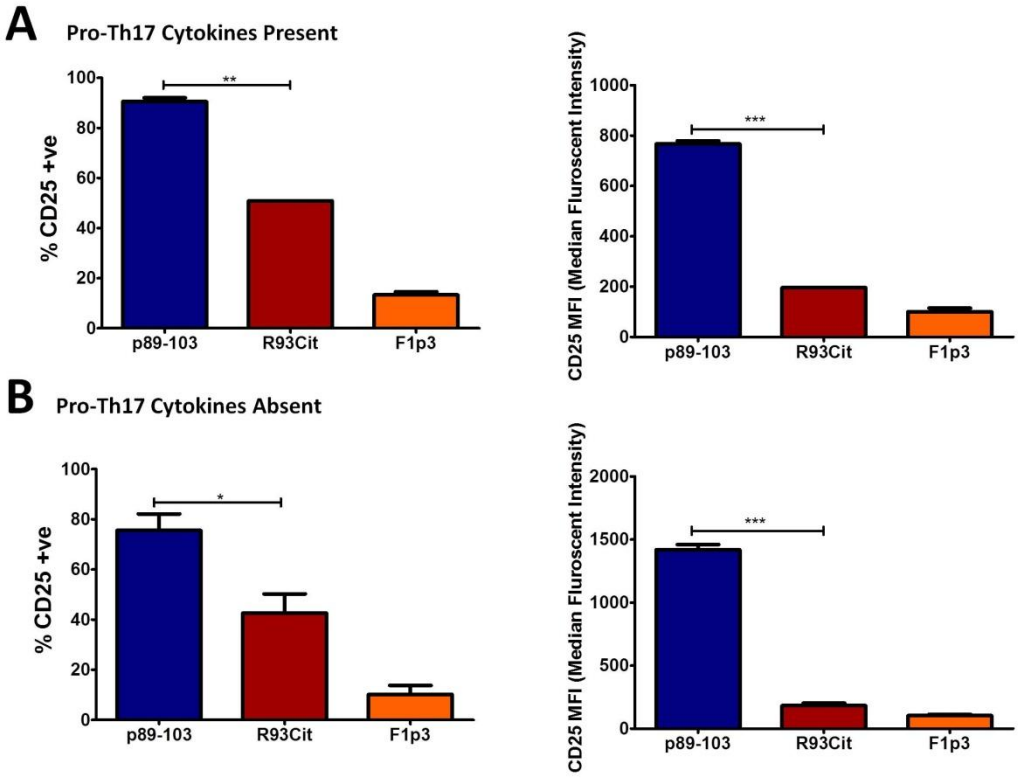

Figure 54 - Citrullination diminishes the expression of CD25. Co-cultures of $1.25x10^5$ naive 5/4E8 TCR Tg T cells T cells and $6.25x10^3$ LPS mDC were established with 2µM of either p89-103, R93Cit or F1p3. Assays were carried out in both the presence (**A**) and absence (**B**) of pro-Th17 cytokines. After 5 days of co-culture cells were harvested and stained for CD4 and CD25. Following gating on CD4, both the percentage positive and MFI were determined for each population. Data represents the mean of three independent biological experiements (a single technical sample per biological repeat). Error bars show ± SEM with significance assessed by *t-test* (*p= <0.05, ** p=<0.01 *** p=<0.001).

5.5 Addition of IL-2 can reduce IL-17 release

With the significant differences in both the production of IL-2 and the expression of the high affinity receptor component CD25 it was important to confirm the influence of this pathway through experimental manipulation. The initial focus was on how the addition of IL-2 would affect those stimulated with the citrullinated peptide (R93Cit). Given IL-2 is a potent T cell mitogen I hypothesised that proliferation would increase with increasing levels of rhIL-2 (420). Firstly, the degree of proliferation was assessed following the addition of rhIL-2 over a range of concentrations. Therefore, co-cultures of naive 5/4E8 TCR Tg T cells were activated with a range of exogenous rhIL-2 for 5 days before pulsing with tritiated Thymidine for the remaining 12h of co-culture (see **Figure 55**). Interestingly, the addition of rhIL-2 had no effect on proliferation with no change occurring at any concentration of rhIL-2 added with either peptide.

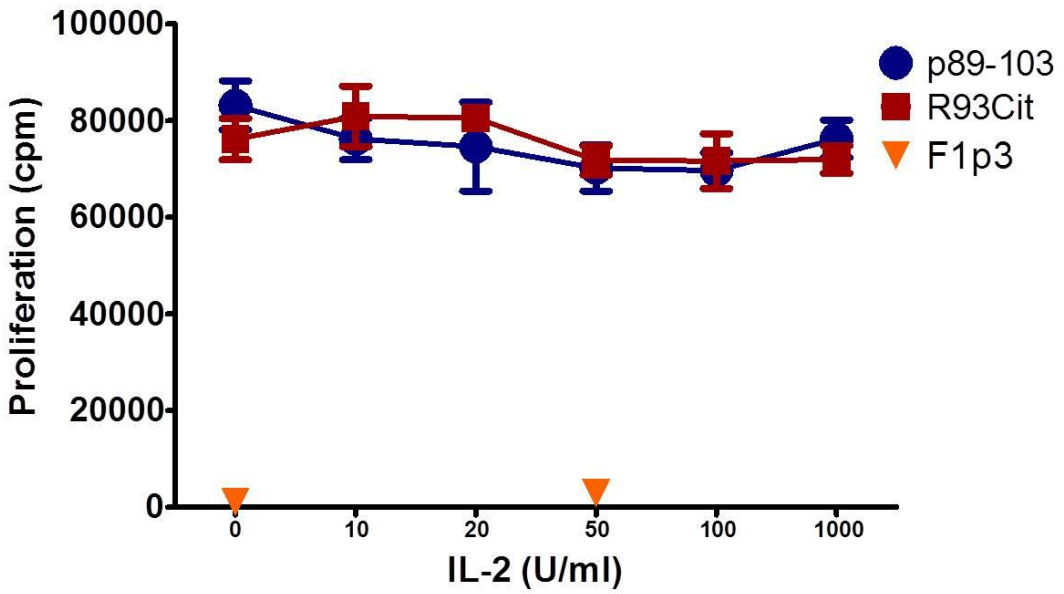

Figure 55 - The effect of rhIL-2 on T cell proliferation. $4x10^4$ naive 5/4E8 TCR Tg T cells were co-cultured with $2x10^3$ LPS-mDCs and 2µM of either p89-103, R93Cit or F1p3. Parallel co-cultures were established with a range of rhIL-2 (0-1000IU/ml). In the case of F1p3 only 50IU/ml or no rhIL-2 was evaluated. After 5 days 120µl of supernatant was removed and cells pulsed with 3H Thymidine for a further 12h prior to DNA harvesting. 3H Thymidine incorporation was measured using a scintillation counter and the cpm determined for each well. Data represents the mean of three independent biological experiements (three technical repeats per biological sample) ±SEM.

Despite the lack of change in terms of proliferation, it was important to determine if the addition of further rhIL-2 could change cytokine release following stimulation with the weaker affinity citrullinated peptide R93Cit. After primary co-cultures in the presence or absence of rhIL-2 (50IU/ml) cells were washed and secondary cultures were established for a further 48h with cognate peptide before supernatants collected. The production of IL-17, IL-4 and IFNγ were assessed by ELISA (see **Figure 56**).

In the presence of pro-Th17 cytokines, rhIL-2 had the expected effect of suppressing the levels of IL-17 released. However, the addition of rhIL-2 had no significant effect on the levels of either IL-4 or IFNγ. Flow cytometric analysis was also performed following re-stimulation with PMA/I after 5 days of primary culture (see **Figure 57**). In contrast to the ELISA data, the addition of rhIL-2 to these cultures failed to suppress the frequency of IL-17-producers. The expression of the Th17 specific transcription factor ROR-γT was also unaltered by exposure to rhIL-2.

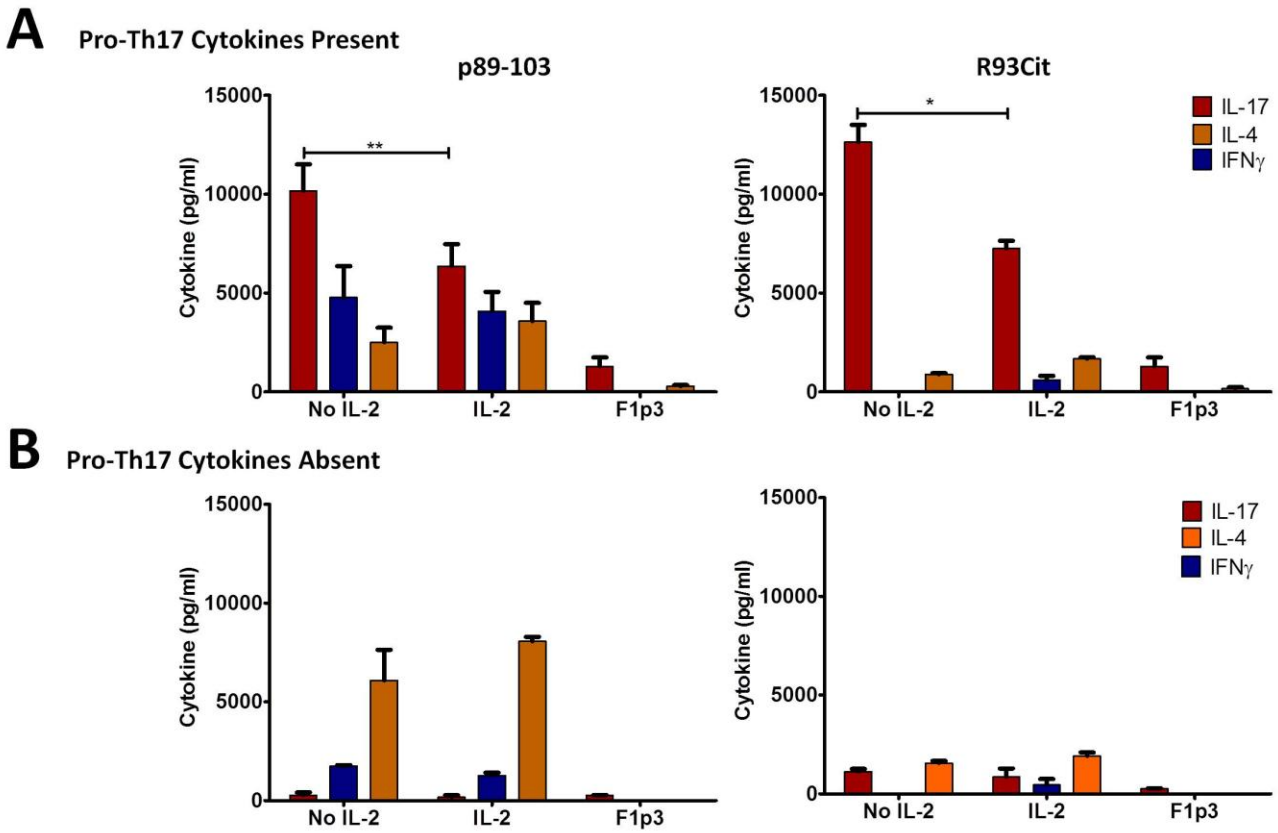

Figure 56 - rhIL-2 suppresses the release of IL-17. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or the F1p3 control peptide. rhIL-2 (50 IU/ml) was added to some cultures from the onset. Assays were carried out in both the presence (**A**) and absence (**B**) of Pro-Th17 cytokines. After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2µM of p89-103. After a further 48h supernatants were harvested and stored at -20°C prior to analysis. ELISAs for IL-17, IL-4 and IFNγ were performed according to the manufacturer's instructions. Data represents the mean of three independent biological experiements (three technical repeat per biological sample) ±SEM. Significance was determined by *t*-test (*p= <0.05, ** p=<0.01 *** p=<0.001)

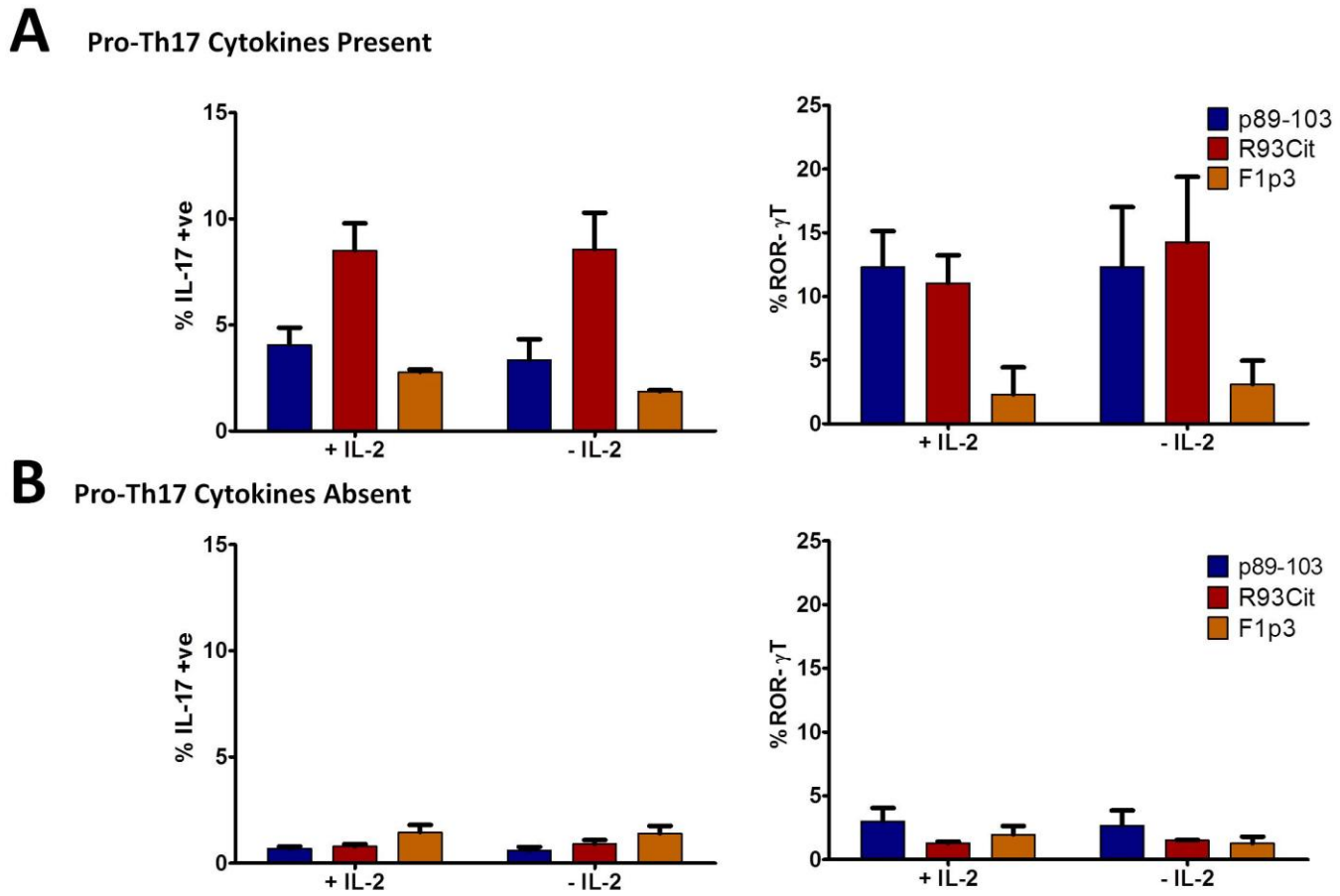

Figure 57 - rhIL-2 has no effect of the frequency of Th17 cells. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3. rhIL-2 (50 IU/ml) was added to some cultures from the onset. Assays were carried out in both the presence (**A**) and absence (**B**) of Pro-Th17 cytokines. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of IL-17 and ROR-γT expression CD4+ T cells. Data represents the mean of two independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by *t*-test (*p= <0.05, ** p=<0.01 *** p=<0.001)

5.6 Anti IL-2 enhances Th17 development after stimulation with high affinity peptide

Whilst the addition of rhIL-2 had an influence on the amount of IL-17 released, this approach is complicated by the endogenous production of IL-2 and the ability of cells to respond to the addition of IL-2. Another approach was to examine the role of IL-2 by using anti-IL-2 IgG2a monoclonal antibody S4B6. This is derived from the semi-purified supernatant from the rat hybridoma S4B6 -1 and is specific to mouse IL-2. This clone has been used in previous studies, including Laurence *et al*, to successfully block IL-2 in murine T cell studies (421).

Initial studies focused on the influence of increasing dilutions of the anti-IL-2 supernatant on T cell proliferation within cultures activated with 2µM of cognate peptide. After 5 days of primary co-culture, cells were pulsed with 3H-Thymidine for the remaining 12h of culture. As expected, given the importance of IL-2 to T cell expansion, higher concentrations of S4B6 significantly impaired the rate of 3H-Thymidine incorporation relative to an isotype-matched control supernatant (see **Figure 58**). However, it was not completely blocked, remaining greater than that of T cells exposed to the negative control peptide F1P3. The isotype control showed similar response to those without S4B6 indicating the response was mostly due to targeting of IL-2 and not due to other components found in the semi-purified medium from the hybridoma.

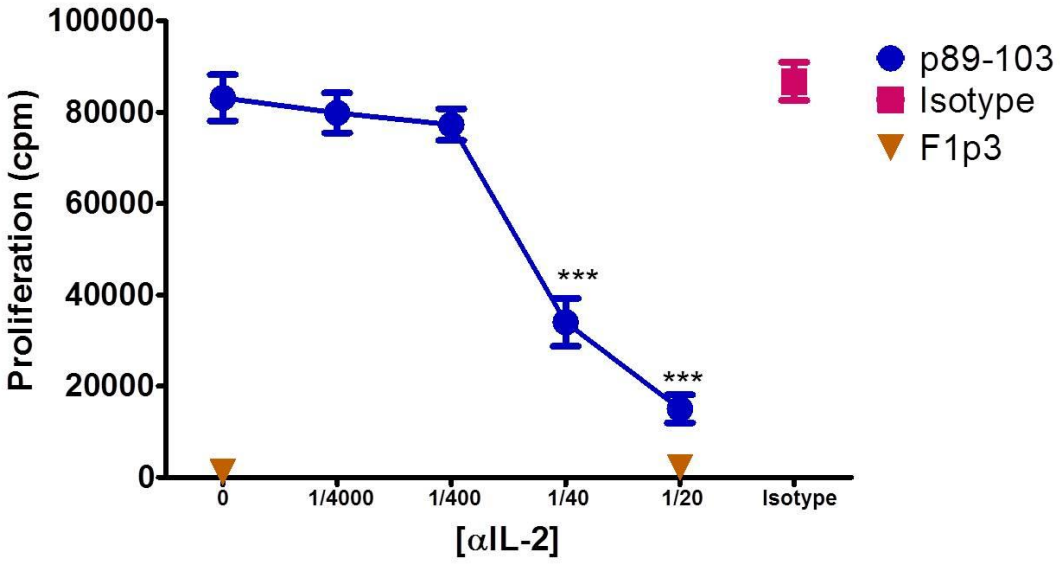

Figure 58 – Anti-IL-2 inhibits T5/4E8 TCR Tg T cell proliferation in a dose-dependent manner. $4x10^4$ naive 5/4E8 TCR Tg T cells were co-cultured with $2x10^3$ LPS mDCs and 2μM of p89-103 or F1p3. Parallel co-cultures were established with a range of anti IL-2 antibody, S4B6, dilutions (1/4000 - 1/20). An isotype control was also included at a 1/20 dilution. After 5 days 120μl of supernatant was removed and cells pulsed with 3H Thymidine for a further 12h prior to DNA harvesting. 3H Thymidine incorporation was measured using a scintillation counter and the counts per minute (cpm) determined for each well. Data represents the mean of three independent biological experiements (three technical repeats per biological sample) ±SEM. Significance was determined by one-way ANOVA relative to Isotype (*p= <0.05, ** p=<0.01 *** p=<0.001).

The next stage was to determine if targeting of the IL-2 pathway could alter the type of cytokine response detected. It was hypothesised that blockade of IL-2 during T cell activation would lead to increased IL-17 release. In line with other studies it was expected that despite the changes in IL-17 expression, levels of ROR-γT would remain unchanged by such treatment. As shown in **Figure 59** co-cultures were established with varying dilutions of the anti IL-2 antibody S4B6. After 5 days, secondary cultures were re-stimulated with cognate peptide and supernatants collected after a further 48h. Analysis of the ELISA data shows a clear change in cytokine profile in the presence of anti-IL-2 with an increase in IL-17 and a loss of IL-4. In contrast, IFNγ release was generally unaltered by the introduction of anti-IL-2 with no significant shift when compared to those activated in the presence of the isotype control.

The increase in IL-17 can also be detected in those T cell cultures activated without any pro-Th17 cytokines. Even at the lowest dilution of anti-IL-2 tested, IL-17 release

was greater than in cultures in which IL-2 was not blocked. IL-4 release was also reduced by the introduction of anti-IL-2. Unexpectedly, at the lower anti-IL-2 concentrations IL-4 was significantly enhanced relative to the isotype control. Again there was no significant alteration in IFNγ release as compared to the isotype.

As well as determining the amount of cytokine released, the frequency of IL-17- and ROR-γT-expressing T cells was also determined by flow cytometry. The introduction of anti-IL-2 also led to a significant increase in the frequency of IL-17 and ROR-γT positive cells (see **Figure 60** and **Figure 61**). When the same assay was carried out without pro-Th17 cytokines the only significant rise in IL-17 and ROR-γT frequencies occurred in those cultures stimulated with the R93Cit peptide.

5.7 The effect of IL-2 on GM-CSF expression

Given that IL-2 was capable of negatively regulating Th17 generation it was of interest to determine if IL-2 affected GM-CSF production from these cells as well. Primary co-cultures were established with the same concentration of either cognate or citrullinated peptide in either the presence or absence of the anti-IL-2 antibody S4B6. After 5 days of co-culture these were assessed by flow cytometry and the frequency of IL-17 and GM-CSF expressing cells determined. As shown earlier the levels of GM-CSF were again higher in those cultures stimulated with the citrullinated peptide (see **Figure 62** and **Figure 63**). In the absence of pro-Th17 cytokines, the frequency of GM-CSF cells was also higher possibly due to the absence of TGFβ. In contrast to IL-17, the addition of anti-IL-2 had no significant effect on the frequency of GM-CSF. Thus, regulation of GM-CSF may be independent of IL-17.

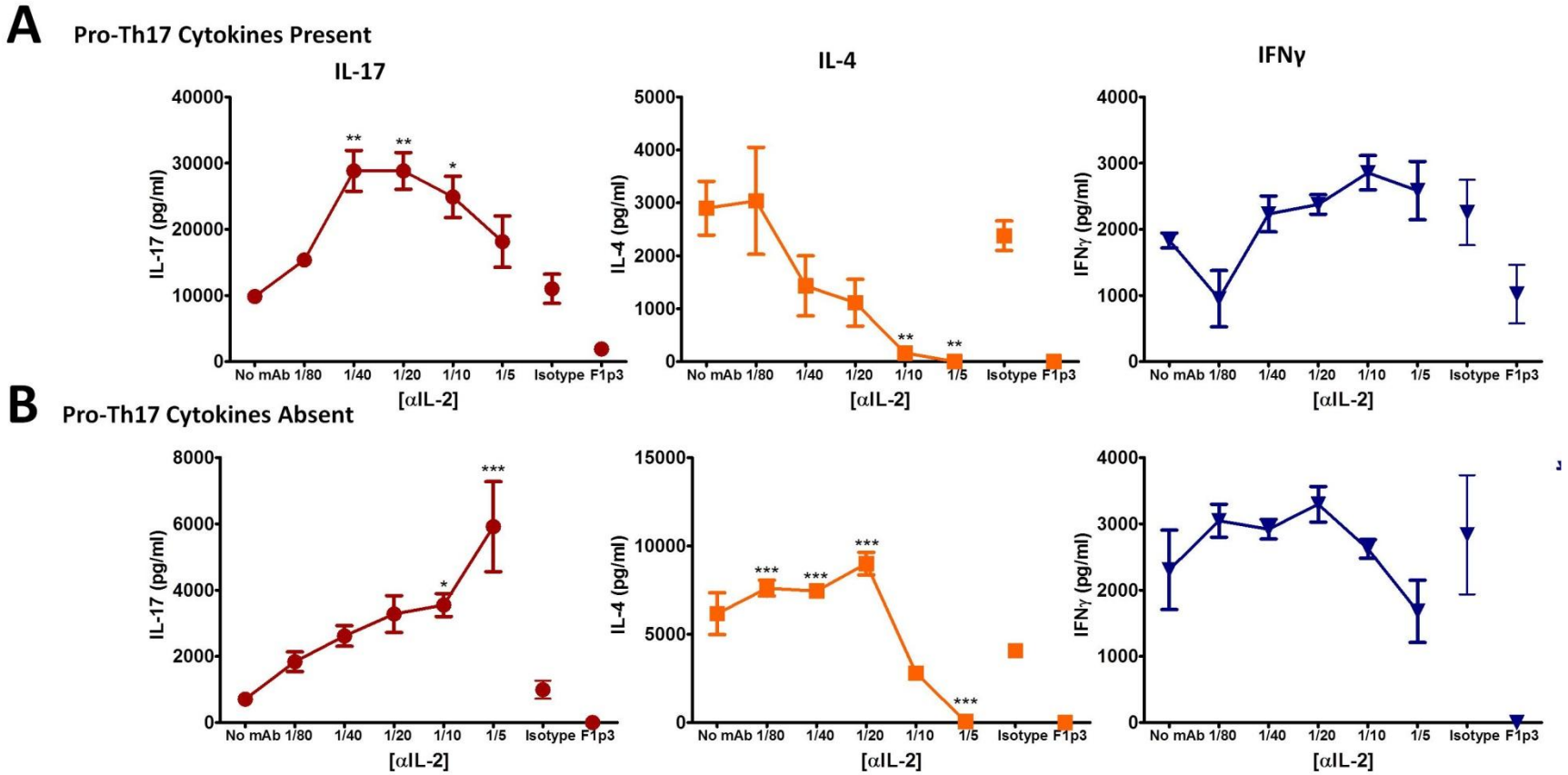

Figure 59 – Anti-IL-2 has differential affects on cytokine production. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103 or F1p3. A range of S4B6 dilutions (1/5 -1/80) added to some cultures from the onset. An isotype control was also established. Assays were carried out in both the presence (**A**) and absence (**B**) of Pro-Th17 cytokines. After 5 days cells were harvested, washed and re-plated with $4x10^4$ T cells, $2x10^3$ 'fresh' mDC and 2µM of p89-103. After a further 48h supernatants were harvested and stored at -20°C prior to analysis. ELISAs for IL-17, IL-4 and IFNγ were preformed according to the manufacturer's instructions. Data represents the mean of three independent biological experiements (three technical repeats per biological sample) ±SEM. Significance relative to Isotype was determined by one-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001).

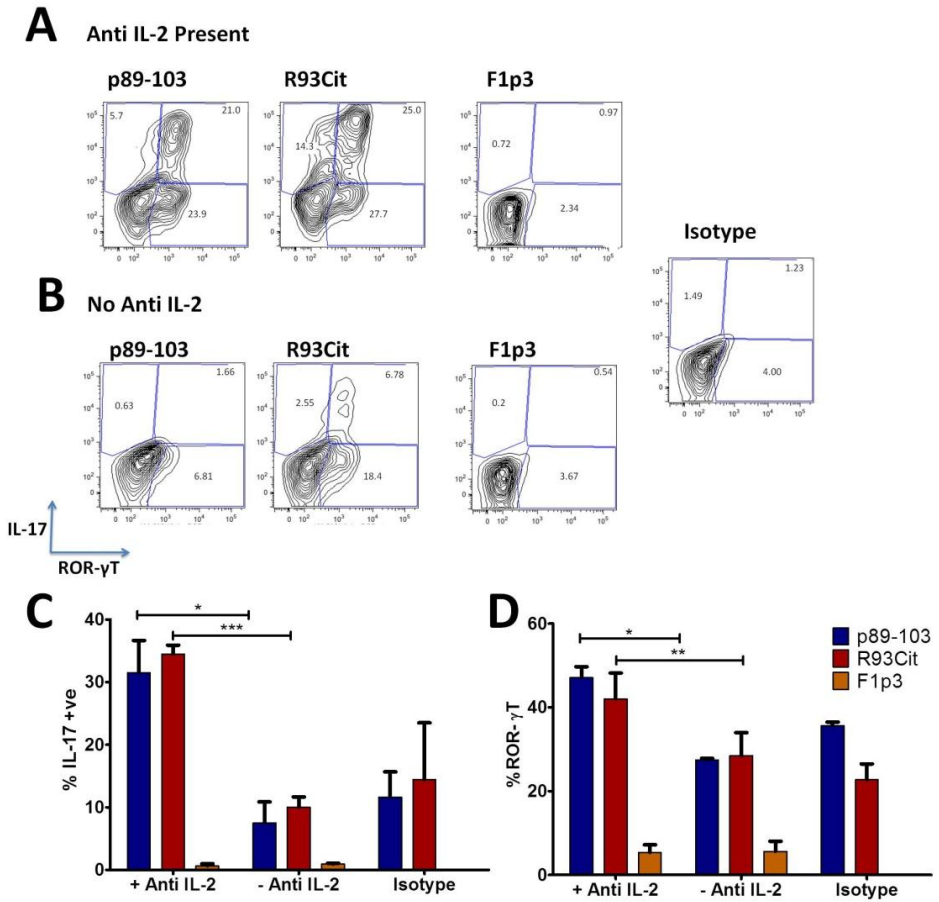

Figure 60 – Anti-IL-2 significantly enhances Th17 generation in the presence of pro-Th17 cytokines. Co-cultures naive 5/4E8 TCR Tg T cells and mDCs were established with 2µM of either p89-103, R93Cit and F1p3 in the presence of Pro-Th17 cytokines. S4B6 (1/40) was added to some cultures from the onset. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of IL-17 and ROR-γT postive cells determined following gating on the CD4. Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by *t*-test (*p= <0.05, ** p=<0.01 *** p=<0.001)

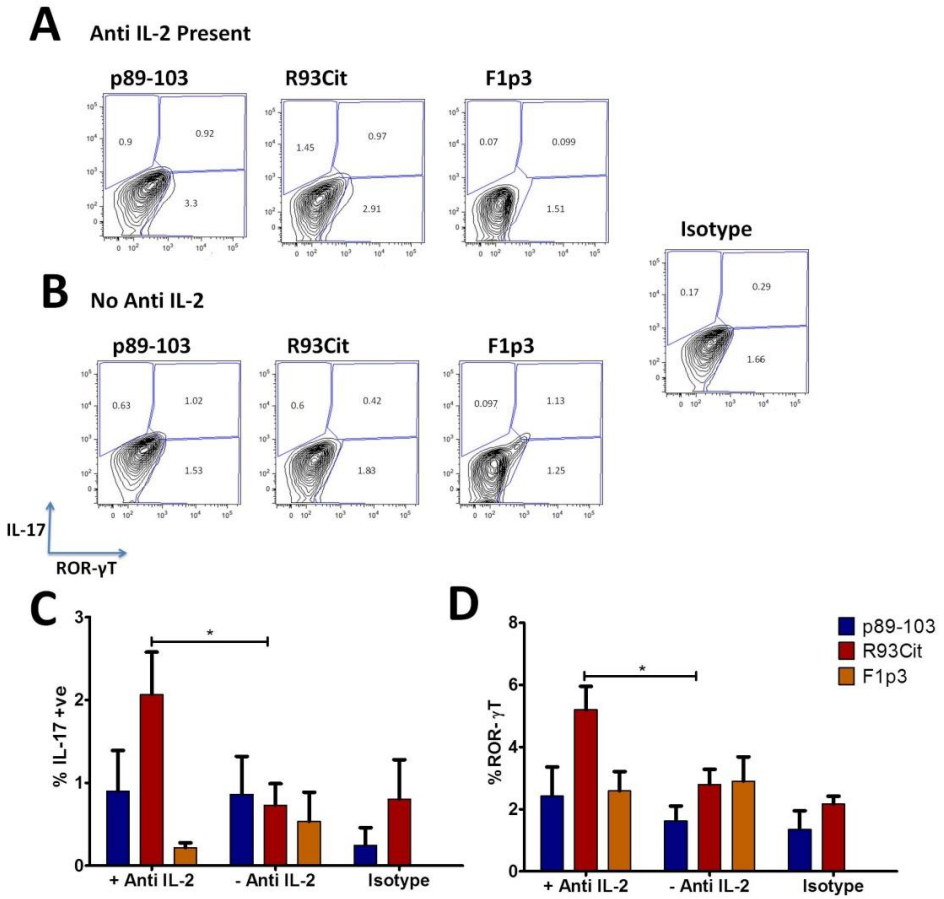

Figure 61 - Anti IL-2 supports Th17 generation in the absence of pro-Th17 cytokines. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with 2µM of either p89-103, R93Cit and F1p3 in the absence of Pro-Th17 cytokines. S4B6 (1/40) was added to some cultures from the onset. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of IL-17 and ROR-γT positive cells determined following gating on the CD4. Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by *t*-test (*p= <0.05, ** p=<0.01 *** p=<0.001)

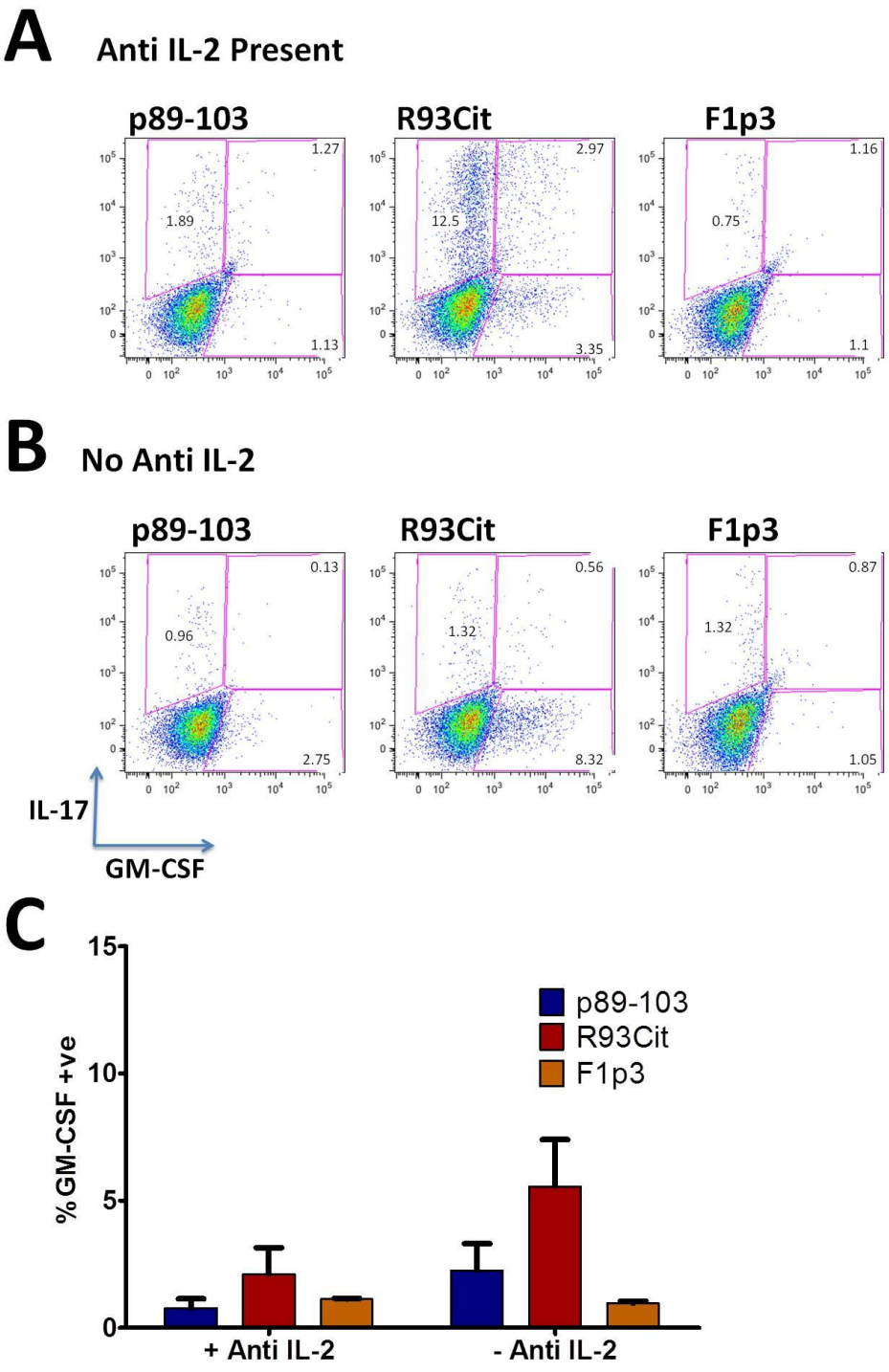

Figure 62 – IL-2 has no significant affect on GM-CSF expression in the presence of pro-Th17 cytokines. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with 2µM of either p89-103, R93Cit or F1p3 in the presence of Pro-Th17 cytokines. S4B6 (1/40) was added to some cultures from the onset. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of IL-17 and ROR-γ determined following gating on the CD4. Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by *t*-test (*p= <0.05, ** p=<0.01 *** p=<0.001)

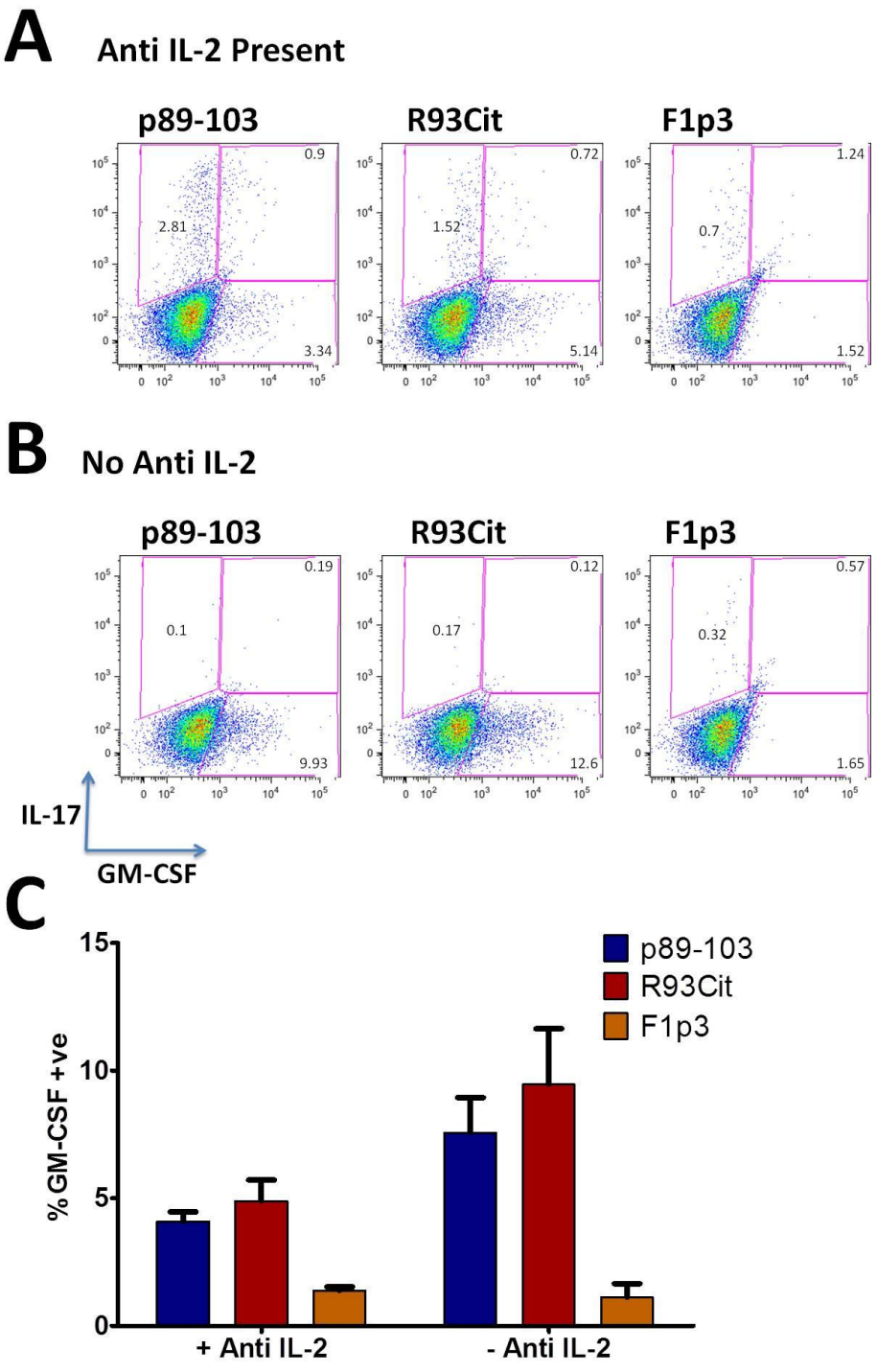

Figure 63 - IL-2 has no significant affect on GM-CSF expression in the absence of Pro-Th17 cytokines. Co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with 2µM of either p89-103, R93Cit or F1p3 in the absence of Pro-Th17 cytokines. S4B6 (1/40) was added to some cultures from the onset. After 5 days cells were re-stimulated with PMA/I for a further 5h with BFA present for the final 4h. Flow cytometry was used to determine the proportion of IL-17 and ROR-γ determined following gating on the CD4. Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by *t*-test (*p= <0.05, ** p=<0.01 *** p=<0.001)

5.8 Citrullination results in changes to the pSTAT3(Y705)/ pSTAT5(Y694) balance

With the profound effect that IL-2 had on the development of Th17 cells, it was important to further understand how these changed the balance between STAT3 and STAT5. STAT3 is critical for the expression of IL-17 and ROR-γT that are essential to the definition of Th17 cells. Phosphorylation at tyrosine-705 has been shown to be critical for dimerisation, nuclear translocation and DNA binding of STAT3 (428, 429). In contrast STAT5 is the primary transducer of IL-2 signalling. Y694 is an obligate phosphorylation site for STAT5 activation with loss preventing translocation and DNA binding (430, 431). Moreover, the relative balance between STAT5 and STAT3, has been postulated to control the development of Th17 cells by IL-2 (248). Thus, it was important in the context of this project to confirm the role of IL-2 by analysing the pSTAT3 and pSTAT5 levels in each population.

Other studies in our group have demonstrated that the degree of STAT3 phosphorylation was important to Th17 development especially at early stages of activation (432). Therefore parallel DC:T cell co-cultures were established with the higher affinity cognate peptide p89-103 or the lower affinity citrullinated peptide R93Cit. Every 24h T cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) assessed by phosflow (see **Figure 64** and **66**). The data shown in **Figure 65** and **Figure 67** indicate that with regards to STAT3 phosphorylation, there was no difference both in terms of the percentage positive cells and the MFI values between either p89-103 or R93Cit stimulated T cell populations. Indeed, the percentage of pSTAT3 positive cells was similar even in those cells exposed to the negative control F1p3. In contrast to the near identical pSTAT3 levels, there were significant differences in the pSTAT5 signals between p89-103- and R93Cit peptide-stimulated T cells. When both frequency of pSTAT5+ cells and the MFI values are compared it is apparent that p89-103 induces a significantly greater signal. This distinction was especially apparent at early time points and only reached parity after 96h. Similarly to the IL-2 ELISA data, the peak pSTAT5 response was lower in

terms of MFI values and occurred at a later time point in those T cells stimulated with the weaker affinity peptide, R93Cit.

It was important to clarify the role of IL-2 in generating the different pSTAT5 signals. Thus, further assays aimed to determine if blockade of IL-2 in populations activated with p89-103 caused a similar shift in the pSTAT3/pSTAT5 balance as occurs when using citrullinated peptide. While the lower IL-2 release was one mechanism that could be responsible, it was possible that these cells could fail to respond to IL-2 due to the significantly reduced expression of CD25. Therefore, it was important to determine if the addition of exogenous IL-2 could be transduced by STAT5 in these cells. Therefore, cultures were activated with or without exogenous rhIL-2 for 24h due to the importance of early STAT signalling. The levels of pSTAT3 and pSTAT5 were determined as previously described. In **Figure 68** and **Figure 69** it was apparent that the addition of rhIL-2 had no effect on the pSTAT5 signal in naive 5/4E8 T cell populations activated with either the cognate or R93Cit peptides. These data could mean that R93Cit-activated T cells failed to respond due to a lack of CD25. However, STAT5 phosphorylation was expected to rise in those T cells activated with p89-103. It may be that endogenous IL-2 is saturating in this population thus mitigating the effect of exogenous IL-2.

A similar time course was established as above with naïve 5/4E8 TCR Tg T cells activated with cognate peptide in the presence or absence of the anti-IL-2 mAb S4B6 (see **Figure 70** and **71**). Rather unexpectedly, when comparing those T cells co-cultured in the presence of S4B6 to the isotype control there was a slight drop in pSTAT3 signal in the presence of anti-IL-2. This was most pronounced at the level of MFI were the pSTAT3 was almost as low as those stimulated with the negative control F1p3. As expected, the pSTAT5 signal was suppressed to a far greater extent both in terms of the frequency of pSTAT5+ cells and the MFI values within the T cell population exposed to the anti-IL-2 antibody. Thus it was apparent that the use of S4B6 targeting of IL-2 could lead to a reversal in the pSTAT3/pSTAT5 balance in those T cells activated with the higher affinity cognate peptide.

The shift in pSTATs becomes clearer when the ratio of pSTAT3 relative to pSTAT5 was determined as shown in **Figure 72**. In those T cells activated with the R93Cit peptide this ratio was shifted towards greater pSTAT3 levels as compared to cognate peptide-stimulated T cells, especially at the 24h time point. Moreover, when IL-2 was blocked using S4B6, this ratio increased, despite the relative decline in pSTAT3.

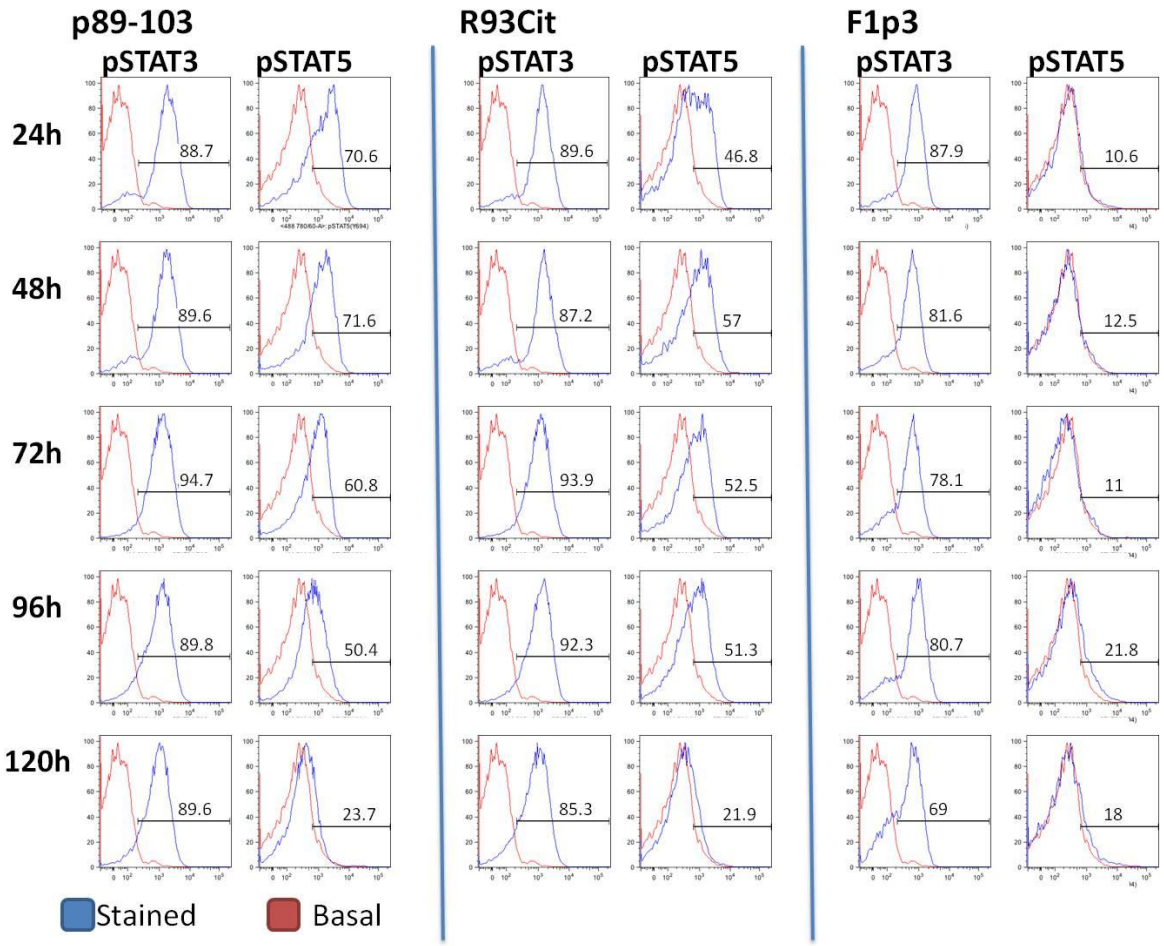

Figure 64 - Citrullination shifts the pSTAT3/pSTAT5 balance. Parallel co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3 in the presence of Pro-Th17 cytokines. At each time point cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) determined by phosflow. Levels of pSTAT3/5 were determined using FMO controls following gating on CD4. Data is representative of three independent biological replicates.

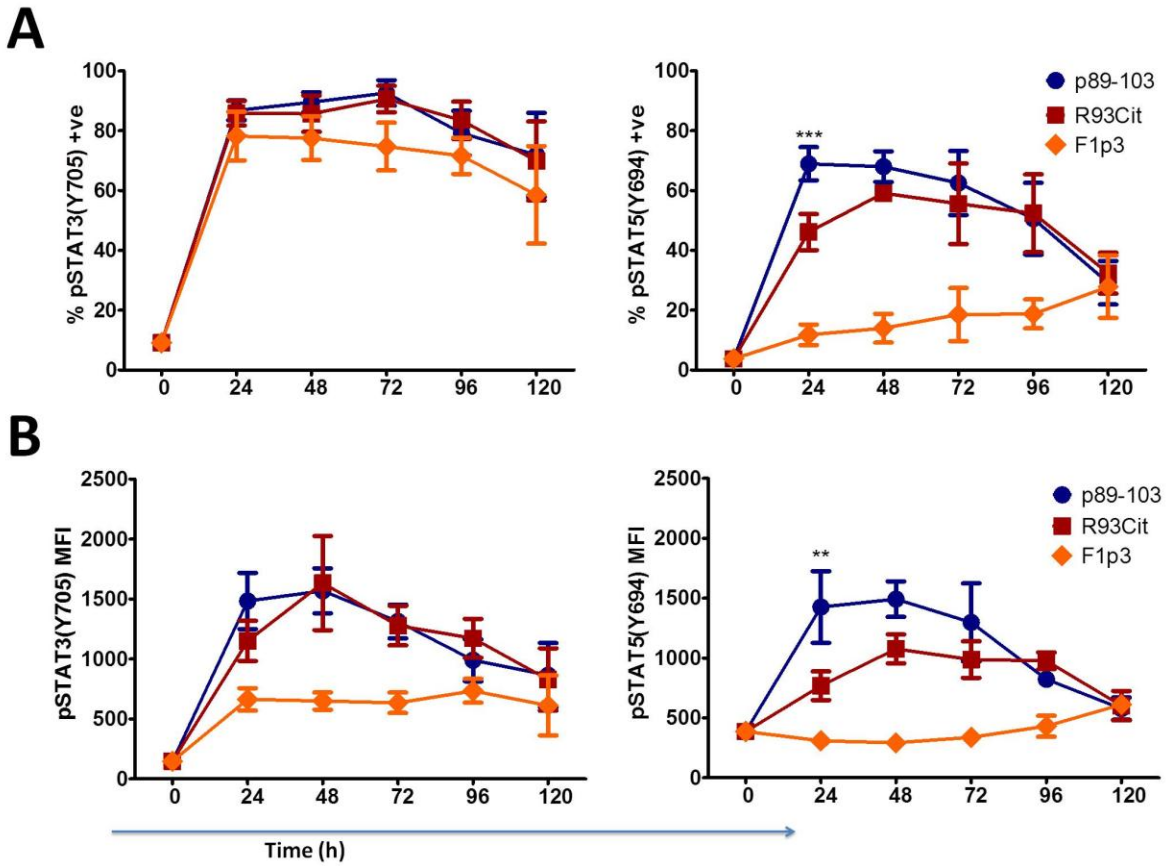

Figure 65 - Frequency and MFI of pSTAT3/pSTAT5 altered by citrullination. Parallel co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3 in the presence of Pro-Th17 cytokines. At each time point cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) determined by phosflow. The percentage positive (**A**) and MFI (**B**) are shown for both pSTAT3(Y705) and pSTAT5(Y694). Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by two-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001).

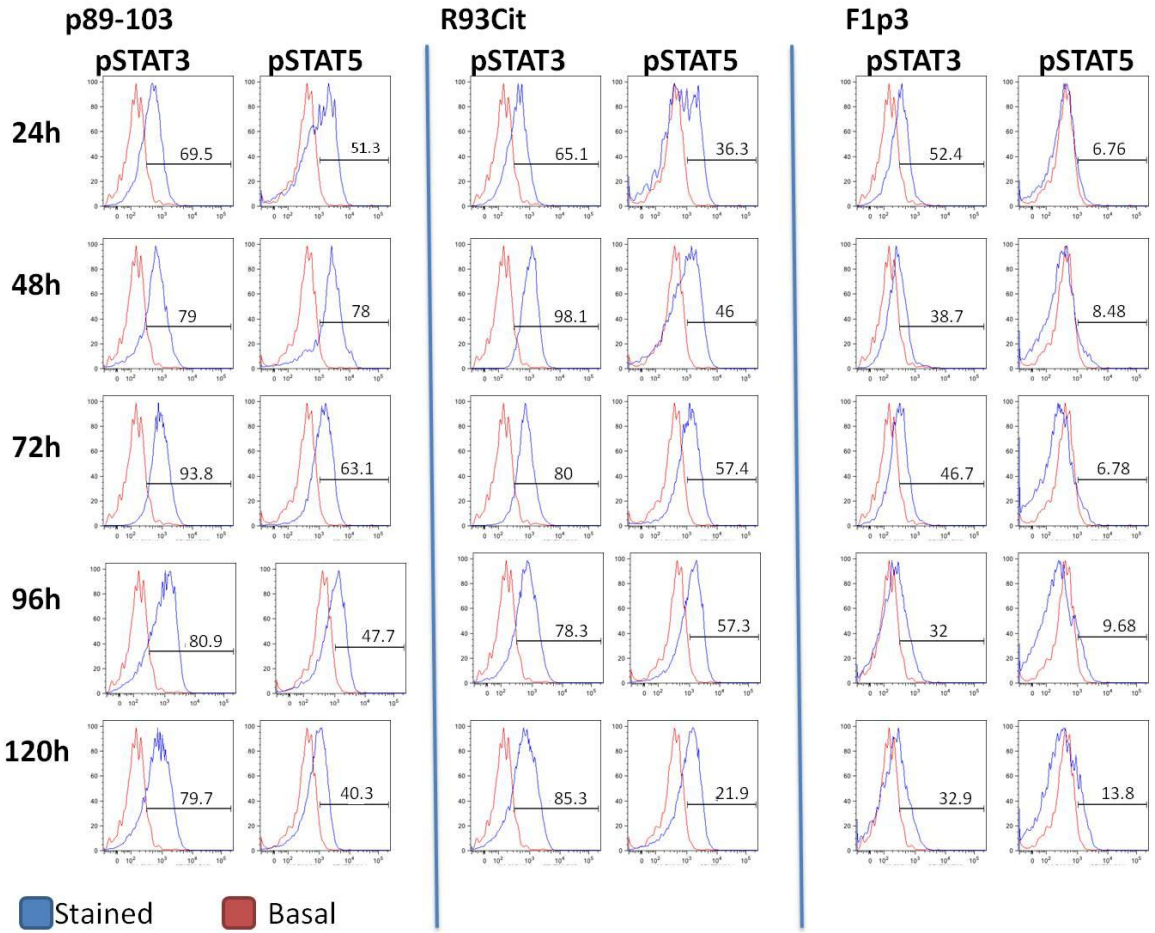

Figure 66 - Citrullination reduced pSTAT5 levels in the absence of pro-Th17 cytokines. Parallel co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit and F1p3 in the absence of Pro-Th17 cytokines. At each time point cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) determined by phosflow. Levels of pSTAT3/5 were determined using FMO controls following gating on CD4. Data is representative of three independent biological replicates.

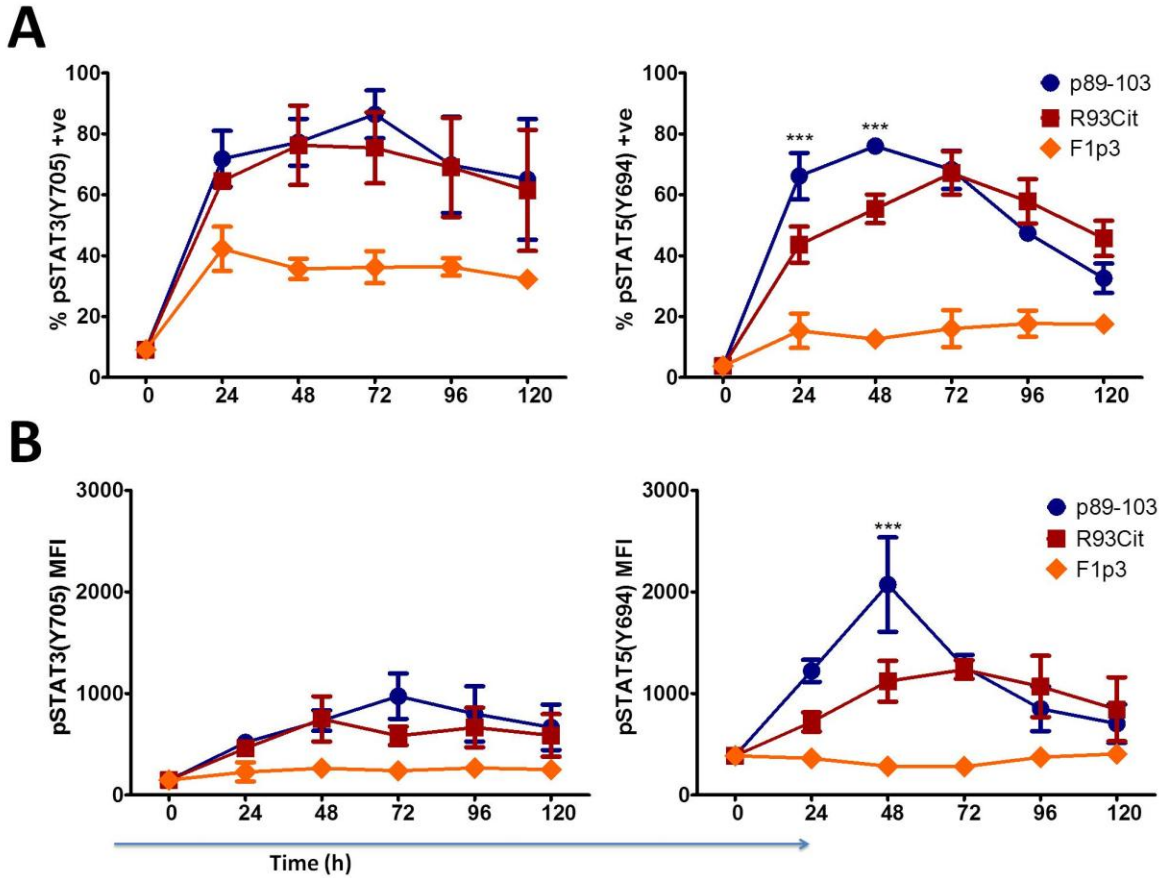

Figure 67 - Citrullination reduced pSTAT5 levels in the absence of pro-Th17 cytokines. Parallel co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3 in the absence of Pro-Th17 cytokines. At each time point cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) determined by phosflow. The percentage positive (**A**) and MFI (**B**) are shown for both pSTAT3(Y705) and pSTAT5(Y694). Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by two- way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001)

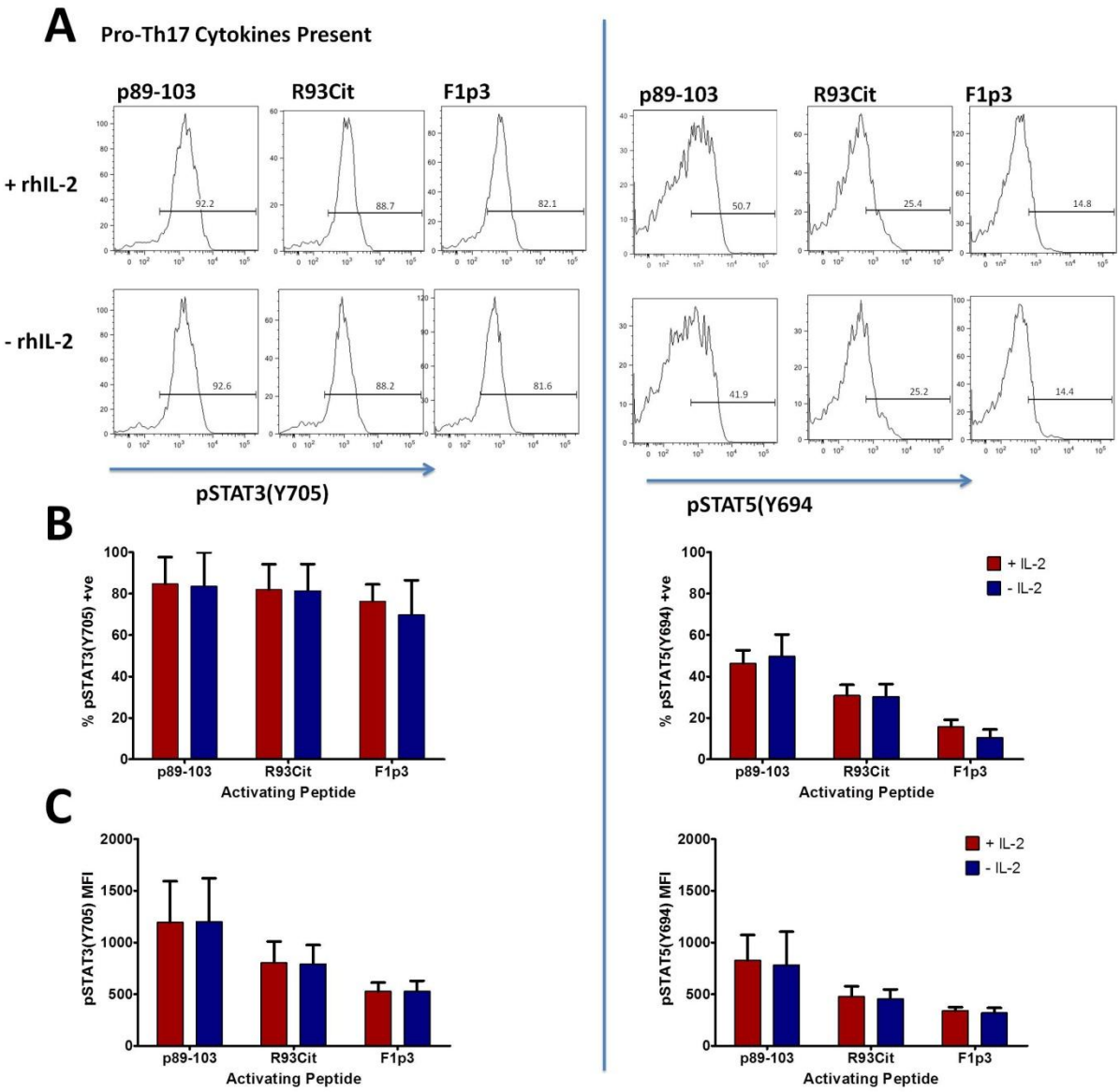

Figure 68 - The addition of rhIL-2 has no effect on the early pSTAT3/5 balance. Parallel co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3 in the presence of Pro-Th17 cytokines. After 24h cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) determined by phosflow after gating on CD4. (**A**) shows a representative data set gating based on the FMO controls. The frequency of pSTAT3/5 (**B**) and MFI values (**C**). Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM.

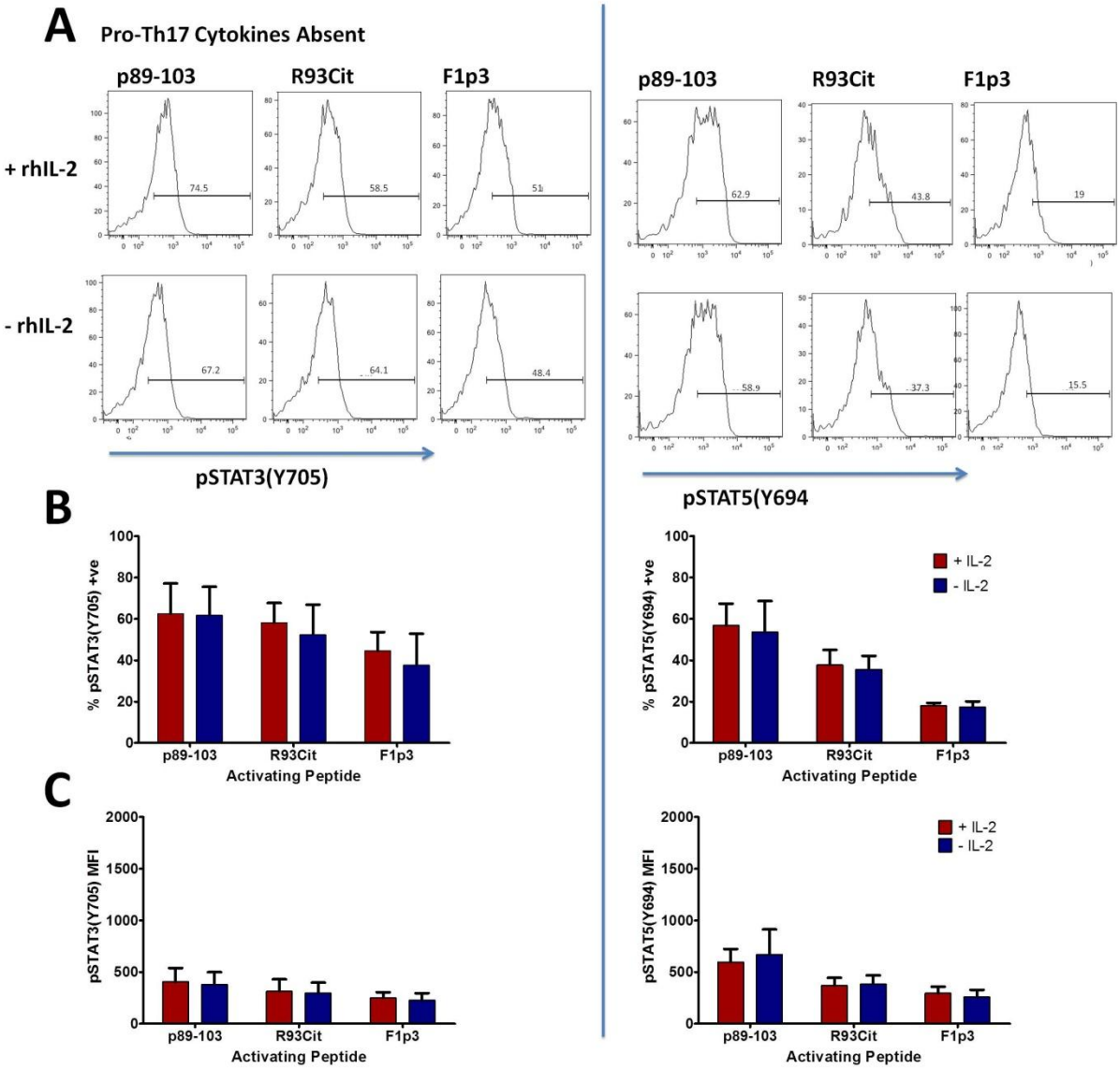

Figure 69 - The addition of rhIL-2 has no effect on the early pSTAT3/5 balance in the absence of pro-Th17 cytokines. Parallel co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3 in the absence of Pro-Th17 cytokines. After 24h cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) determined by phosflow after gating on CD4. (**A**) shows a representative data set gating based on the FMO controls. The frequency of pSTAT3/5 (**B**) and MFI values (**C**). Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM.

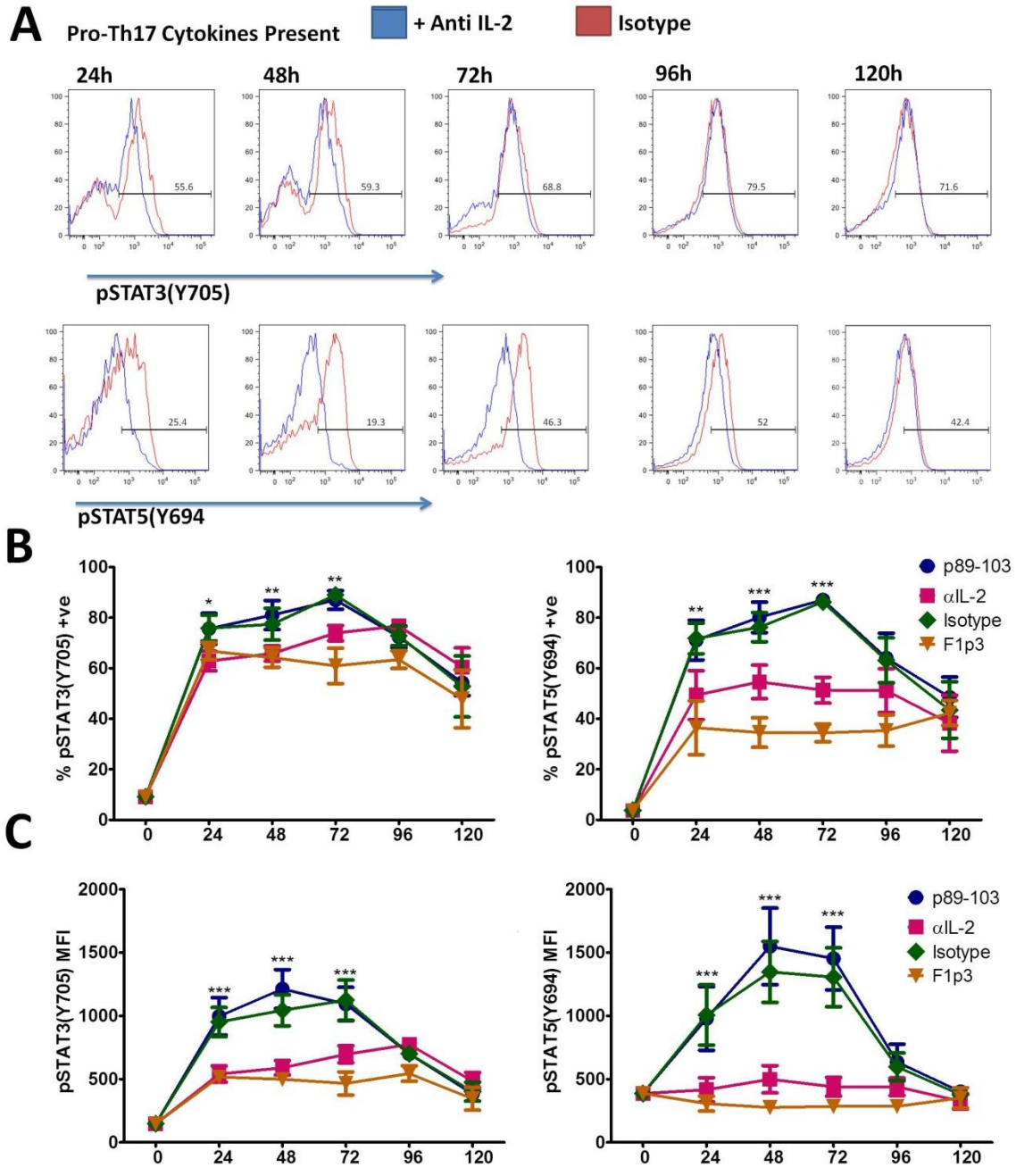

Figure 70 - Anti IL-2 attenuates the intensity of the pSTAT5 signal. Parallel co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit or F1p3 in the presence of Pro-Th17 cytokines. Some cultures were established with the addition of S4B6 (1/40) or isotype. At each timepoint cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) determined by phosflow. A representative example is shown in (**A**). The percentage positive (**B**) and MFI (**C**) are shown for both pSTAT3(Y705) and pSTAT5(Y694). Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by two-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001).

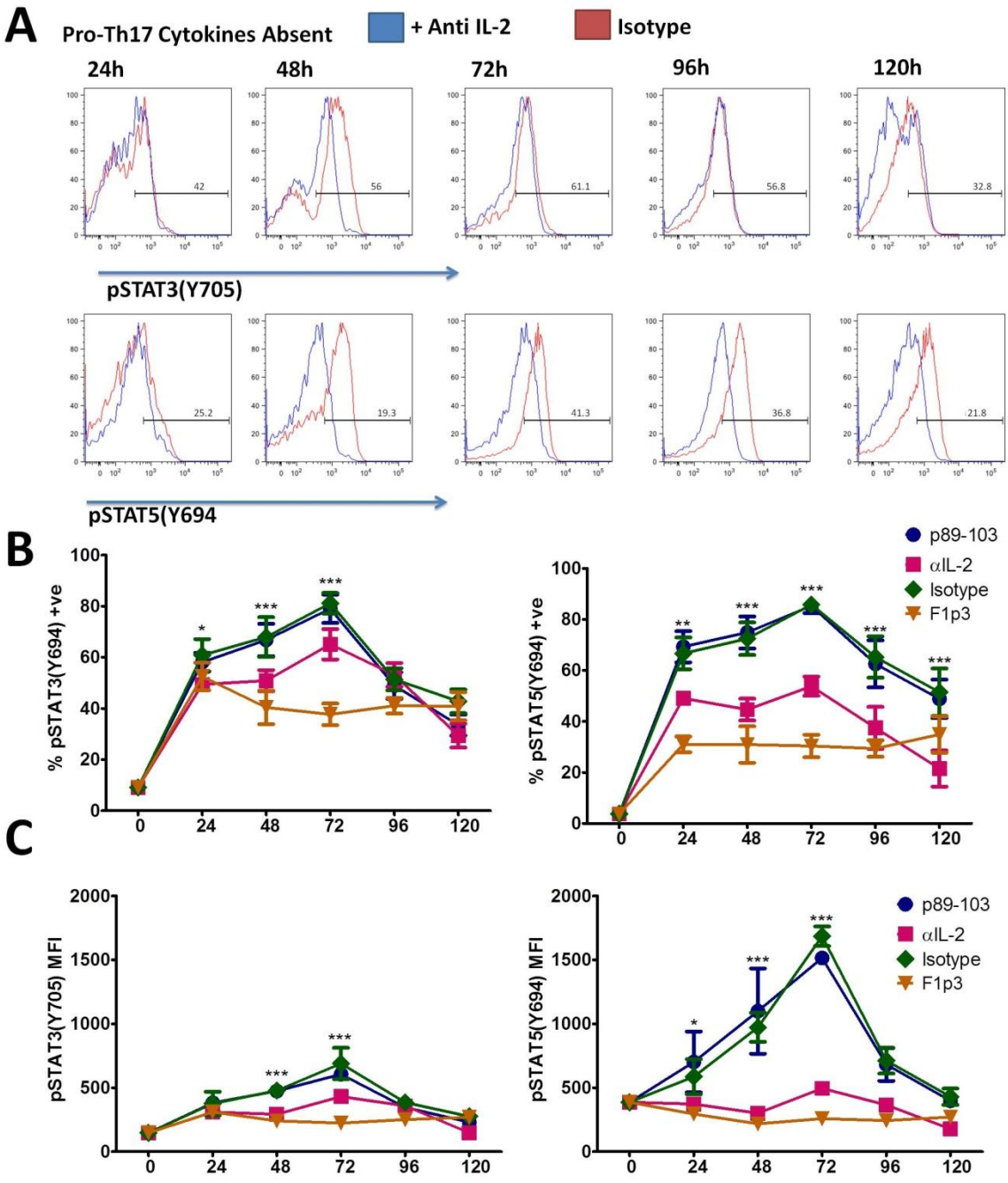

Figure 71 - Anti IL-2 attenuates the intensity of the pSTAT5 signal in the absence of pro-Th17 cytokines. Parallel co-cultures of naive 5/4E8 TCR Tg T cells and mDCs were established with either p89-103, R93Cit and F1p3 in the absence of Pro-Th17 cytokines. Some cultures were established with the addition of S4B6 (1/40) or isotype. At each timepoint cells were harvested and the levels of pSTAT3(Y705) and pSTAT5(Y694) determined by phosflow. A representative example is shown in (**A**). The percentage positive (**B**) and MFI (**C**) are shown for both pSTAT3(Y705) and pSTAT5(Y694). Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by two-way ANOVA (**p*= <0.05, ** *p*=<0.01 *** *p*=<0.001).

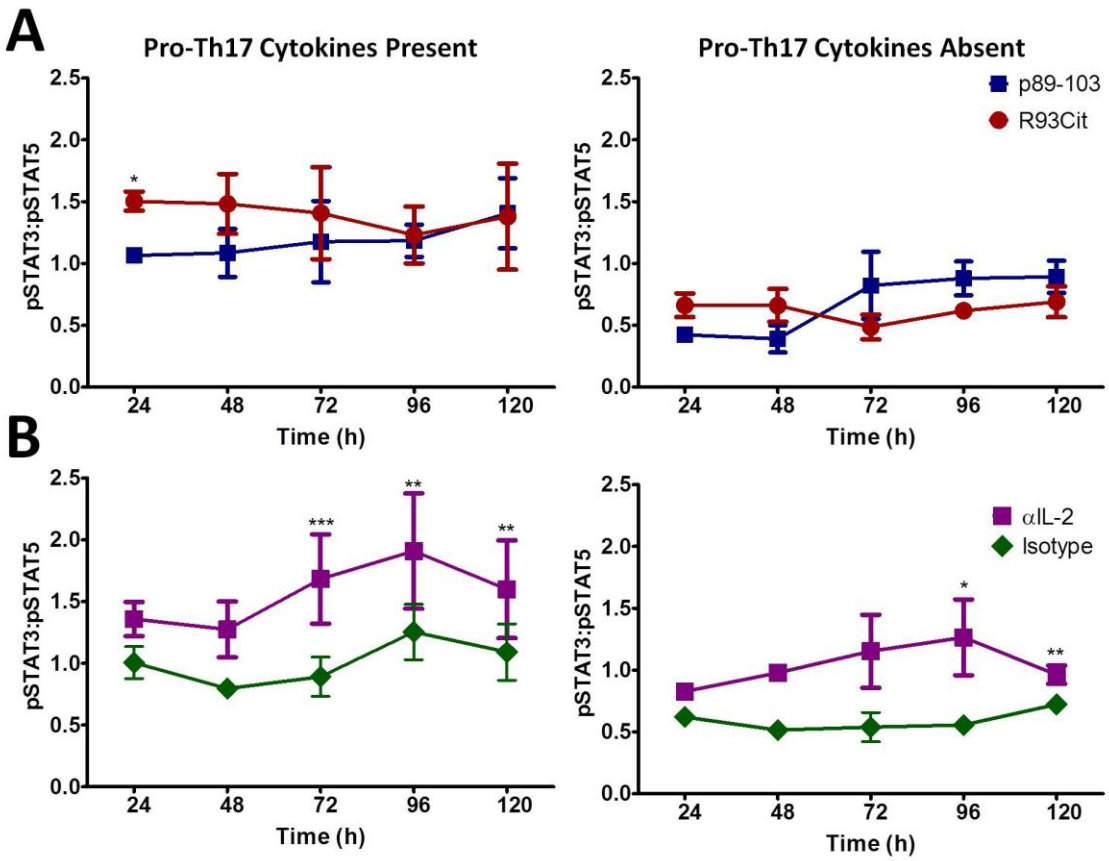

Figure 72 - Citrullination enhances the pSTAT3:pSTAT5 ratio. Using the MFI values as presented the ratio of pSTAT3 relative to pSTAT5 can be determined for each time point. (**A**) indicates pSTAT3:pSTAT5 ratio of 5/4E8 TCR Tg T cells activated with 2µM of p89-103 or R93Cit. (**B**) shows the pSTAT3:pSTAT5 ratio of 5/4E8 TCR Tg T cells activated with 2µM of p89-103 with either S4B6 or isotype control. Data represents the mean of three independent biological experiements (single technical repeat per biological sample) ±SEM. Significance was determined by two-way ANOVA (*p= <0.05, ** p=<0.01 *** p=<0.001).

5.9 Discussion

The data present in this chapter investigated a possible mechanism by which the a change in TCR signal intensity can promote the development of Th17 cells as observed in Chapters 3 and 4. As IL-2 is one the major genes induced by TCR signalling, it was important to determine if differential IL-2 signalling could be responsible for the different levels of Th17 responses induced by cognate and citrullinated forms of p89-103. These studies utilised the *in vitro* model developed in the previous chapter in which the lower affinity R93Cit peptide induced significantly enhanced Th17 generation when compared to the equivalent concentration of cognate peptide. Based on the previous data from studies by Laurence *et al*, I hypothesised that IL-2 could be responsible for the distinct Th responses observed in response to high versus low affinity peptides (421, 433). The reduced IL-2 release coupled with a reduced expression of CD25 indicated a less active IL-2 signalling induced by R93Cit stimulation. The negative influence of IL-2 was confirmed by, firstly, the addition of exogenous IL-2 that had a significant effect in reducing IL-17 production. Secondly, through the addition of the anti-IL-2 mAb S4B6; blocking of IL-2 in those T cells stimulated with the strong IL-2 inducer, p89-103, lead to a significant increase in Th17 development within these cultures. The pSTAT3 signal was near identical in both p89-103 and R93Cit stimulated cultures. As expected if IL-2 was the key influence, the pSTAT5 signal, especially at early time points, was significantly greater in naive 5/4E8 TCR Tg T cells activated with p89-103 than R93Cit stimulated cultures. Taken together these data highlight the influence of IL-2 as a negative influence on Th17 cell development that can be influenced by the degree of TCR signalling.

It has been established that IL-2 is one of the major products of TCR stimulation. Indeed, both NFκB and NFATC1 are potent initiators of IL-2 transcription and both are primary downstream effectors of TCR signals (434). Since the studies of Laurence *et al,* it has been suggested that IL-2 serves as a negative regulator of Th17 cell function (421). However, the exact mechanism remains disputed with other studies suggesting alterative mechanisms by which IL-2 can influence the Th17 phenotype. Others have suggested that IL-2 exerts a negative influence through impaired IL-6R expression (309). Whilst in this chapter the level of either IL-6Rα or gp130 was not directly assessed, the strong pSTAT3 signal implies that IL-6 signalling was competent. It has

been suggested by Fujimara *et al* that IL-2 does not have a direct effect on Th17 cells suggesting rather the Th17 phenotype was not dependent on its presence compared to Th1 cells (427). However, in this project, cell numbers were normalised after primary culture. Indeed, the secondary cultures are re-stimulated with the same stimulation (2µM of p89-103) meaning the only difference was the modification to IL-2 signal within the primary culture.

Broadly the data presented agree with the mechanism proposed by the O'Shea group in which the level of pSTAT3 relative to pSTAT5 influences the generation of Th17 cells (435). However, in this project, the targeting of IL-2 by S4B6 leads, not only, to an increase in IL-17 release and IL-17+ cell frequency but also in terms of cells expressing ROR-γT (433). Whether the differences are due to genetic background of the mice, the concentration of S4B6 used (due to the nature of purification it is difficult to directly compare) or different kinetics remains unclear. Moreover, the studies by the O'Shea group did not vary the strength of the initial TCR signal. My study is the first to link differential TCR affinities to IL-2 signalling that in turn influences Th17 generation.

While the addition of exogenous rhIL-2 did inhibit IL-17 production from naive 5/4E8 TCR Tg T cells in this project, it had no effect on the frequency of Th17 cells assessed by ICS as found by Laurence *et al* (421). Again this may be attributed to the different strains of mice or the use of anti CD3/CD28 instead of pMHC by the O'Shea group. It could also be a result of the complications of using human IL-2. In terms of sequence homology human and murine IL-2 share 65% of the 133aa that constitute the mature protein (436). Despite the differences at the level of protein sequence, studies have shown a similar efficacy in the ability of human IL-2 to induce proliferation of murine T cells (437). It may also have been that endogenous IL-2 was saturating in the model presented in this chapter negating the addition of further IL-2. Clarification would require the genetic ablation of IL-2 in 5/4E8 TCR Tg T cells to eliminate the influence of IL-2.

The data presented also highlight the cooperation between the cytokine milieu and the TCR signalling intensity and its effects on Th17 differentiation. It was likely that the addition of IL-6 and IL-23 accounts for the strong STAT3 signal. In contrast, the STAT5 signal was dependent on the degree of IL-2 signalling that was distinct between those

T cells activated with p89-103 and the R93Cit peptides. Blockade of IL-2 lead to a significant rise in both IL-17 and ROR-γT expression even in the absence of exogenously supplied pro-Th17 cytokines. Interestingly, this observation only occurred in those stimulated with the weaker citrullinated peptide. This may be due to a weaker TCR-induced IL-2 signal. Whilst this study was able to interfere with IL-2 signalling both by the addition of exogenous IL-2 and by blocking with S4B6 it would be interesting to confirm these responses in mice lacking IL-2, CD25 and also STAT5 to confirm the observation of this study. It would also be of merit in future studies to utilise the power of semi quantitative imaging techniques, such as ImageStream, to determine the localisation of STAT3/STAT5 within each individual cell as this would add further evidence as to the relative balance between these STATs. Absolute confirmation would require CHIP analysis of pSTAT3/5 binding to the *il17a* locus.

Recent reports by van Panhuys *et al* emphasised the influence of TCR signals on subsequent cytokine receptor expression (284). TCR signals were found to operate upstream of the receptor expression and so altering the ability of cells to respond to their local cytokine environment. However, these studies focussed primarily on the Th1/ Th2 paradigm. The data presented in this chapter can be interpreted in a similar manner in which the reduced TCR signals leads to a failure to upregulate the high affinity component of the IL-2R in those T cells stimulated with R93Cit. The van Panhuys study also highlighted the influence of co-stimulatory molecules through various adjuvants. Whilst out with the parameters of this study, it would be of interest to determine if modifications to the co-stimulatory pathway could have a similar outcome as CD28 is also known to be capable of influencing NFκB and NFAT activity at the *il2* locus (438). Thus, it is plausible that different adjuvants could also have pro-Th17 actions via a similar mechanism. This may have especial relevance *in vivo* with possible implication for vaccine designs targeting Th17 immunity.

It was not only the production of IL-17 that was affected but IL-4 levels were also sensitive to changes in IL-2 signalling. Although the addition of IL-2 lead to a non-significant effect on IL-4 release, the blockade of IL-2 led to a dose dependent inhibition of IL-4. This was in stark contrast to IFNγ levels, which were not significantly altered by either treatment. In the context of other studies, the data presented fit with those that emphasise a role for IL-2 during the initiation of Th2 responses (416). While

not the primary focus of this study, the ability of cells to produce IL-4 in secondary cultures was affected by the presence of anti-IL-2 antibody. The lack of IL-4 response to the addition of IL-2, may perhaps indicate an existing abundance of endogenous IL-2 within these cultures. It would be interesting if GATA-3 expression was also affected by the suppression of IL-2 by S4B6.

It has been stated by some studies that IL-2 plays an important role in the generation of IFNγ responses (409). Th1 development is dependent on the triggering of STAT4 by IL-12 present in the microenvironment. This in turns leads to T-bet expression that mediates the production of IFNγ. This process has been described to be enhanced by co-production of IL-2 that drives greater expression of both T-bet and IL-12R (417). However, the data presented in this chapter suggest that changes to IL-2 signalling do not significantly affect IFNγ production. As shown in previous chapters the levels of T-bet and IFNγ are far more resistant to changes in TCR signal strength than IL-4 or IL-17 suggesting that IL-2 is not the primary mediator in this model. If conditions were optimised to Th1 generation (e.g. the addition of exogenous IL-12 or anti IL-4 mAbs) the influence of IL-2 may become more apparent.

Another important aspect of this project was how changes in IL-2 signalling would affect the production of GM-CSF from T cells. Unlike IL-17, GM-CSF did not appear to be regulated by IL-2 despite the significant increase in ROR-γT following addition of S4B6. The data presented in this chapter appear to contradict the findings by Codarri *et* al that suggest a positive role for ROR-γT in GM-CSF expression (239). However, there is a trend towards greater GM-CSF in the absence of anti-IL-2. This is in line with other studies that have claimed that GM-CSF expression is positively regulated by IL-2 (439). It has been suggested that IL-2 enables Th17 cells to co-express IFNγ and IL-12R. It would be interesting to further understand the role of IL-2 in the plasticity of Th17 cells, particularly with regards to the expression of other effector cytokines given the importance of these cytokines to disease development *in vivo*.

In summary, the data presented in this chapter provide a potential mechanism that links changes in TCR signalling to Th17 generation via differential IL-2 signalling. Both production and receptor expression are distinct in those T cells activated with the higher affinity cognate peptide when compared to the weaker citrullinated form.

Together these determine the balance between STAT3 and STAT5 tyrosine phosphorylation, thereby profoundly influencing the development of Th17 responses. Thus, with a higher intensity TCR signal, IL-2-induced STAT5 is able to compete with STAT3, driven by the exogenously supplied IL-6 and IL-23, to inhibit both IL-17 and ROR-γT expression. The data presented in this chapter highlight the cooperation between those signals derived from the cytokine milieu and those intrinsic signals generated by the TCR in the development of the Th17 phenotype.

6 General Discussion

6.1 Summary of findings

Growing evidence has shown the importance of TCR signalling in shaping the type of Th response, the effect which is well characterised with regards to other Th subsets including Th1, Th2, T_{FH} and Tregs but not Th17 (159, 271, 273, 440, 441). This project aimed to assess how changes in TCR signal intensity by alterations in either peptide density or affinity could impact on the murine Th17 response to the candidate autoantigen aggrecan. Essential to addressing this question was the use of the 5/4E8 TCR Tg mouse. This provided a readily available a source of naive TCR Tg T cells of which over 85% were specific for the arthritogenic aggrecan peptide p89-103. Using these tools the major findings of this project are as follows,

- Development of the Th17 phenotype from naive 5/4E8 TCR Tg T cells was enhanced at a lower density of cognate peptide. This was apparent both in the expression and production of IL-17 as well as at the level of ROR-γT induction.
- Citrullination of a putative TCR contact point, R93, produced a partial agonist for the 5/4E8 TCR. The weaker affinity of R93Cit also led to an enhanced induction of the Th17 subset at higher peptide doses when compared to the equivalent concentration of the cognate peptide p89-103.
- A lower TCR signalling intensity also promoted greater production of Th17-associated cytokines IL-22 and GM-CSF, which have been associated with pathology *in vivo* in certain disease models.
- Differences in TCR affinity also regulated both IL-2 cytokine production and IL-2Rα receptor expression.
- The differential IL-2 signalling induced by changes in TCR affinity altered the pSTAT3:pSTAT5 ratio that was enhanced in T cells activated with a weaker affinity APL such as R93Cit.

From the data presented in this thesis, I propose the following model, which is summarised in **Figure 73.** The reduced TCR signal intensity caused by citrullination at the putative TCR contact R93 leads to a weakening in the interaction between the 5/4E8 TCR and R93Cit-MHC presented by LPS mDCs. Given that IL-2 and IL-2Rα are

both the result of TCR signalling, the weaker affinity of R93Cit induces lower levels of expression of these proteins when compared to the cognate peptide. This in turn leads to reduced STAT5 activity in Th cells activated with the citrullinated peptide. While the STAT3 induction is primarily (but not entirely) due to the presence of exogenous IL-6 and IL-23 in the cytokine environment, the relative reduction in pSTAT5 activity has the consequence of less competition between these STATs at the *il17a* locus. Hence, there is greater Th17 induction when the negative influence of IL-2 is reduced in those naive 5/4E8 TCR Tg T cells activated with a lower affinity agonist such as R93Cit.

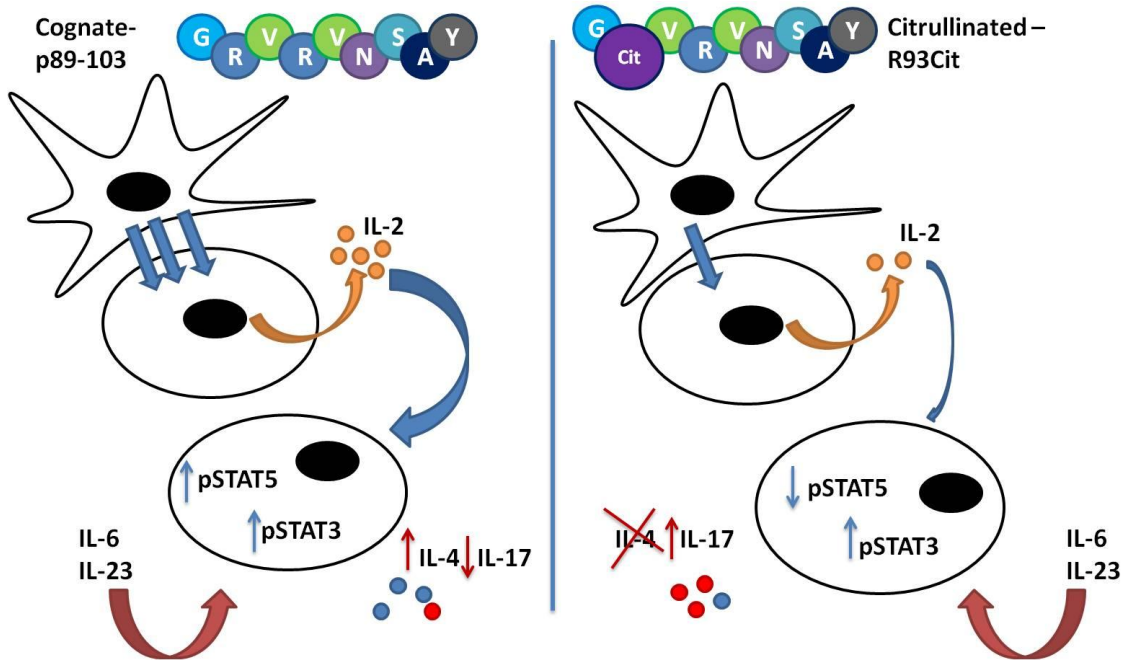

Figure 73 - Model of how peptide affinity promotes Th17 development by alteration to the pSTAT3/5 balance.

6.2 Strengths & Weaknesses

Studying the influence of TCR signalling intensity on Th differentiation remains challenging with polyclonal precursor populations as occurs in WT hosts (273). In order to overcome the diversity in endogenous TCRs, surrogate antigens such as anti-CD3/CD28 have been developed. These bypass the TCR altogether to ensure each Th cell receives the same stimulus. However, the affinity of antibodies is significantly greater than that of a TCR interacting with even high affinity pMHC complexes (273). Furthermore, while modulation in the ratio of anti-CD3 relative to T cells allows for variations in the quantitative aspects of TCR signalling, it is not possible to alter the affinity between the antibody and TCR. The use of the 5/4E8 TCR Tg model permits access to large numbers of naive precursors expressing a known TCR. Thus, both

quantitative and qualitative changes to TCR signal intensity can be made by altering peptide concentration and sequence, respectively. Furthermore, unlike studies that utilise Tg TCRs that recognise model antigens such as OVA or MCC, the antigen, p89-103, is derived from a disease-relevant autoantigen aggrecan. Indeed, Th responses against this epitope and the citrullinated form can be detected in patients with inflammatory arthritis (332, 442). Thus, the 5/4E8 TCR Tg model provides a more physiological context to assess the influence of TCR signalling intensity upon the Th17 phenotype.

While pro-Th17 cytokines were added to cultures of naive 5/4E8 TCR Tg T cells in order to facilitate induction of the Th17 phenotype, no antagonistic mAbs (e.g. anti-IL-4 or anti-IL-12 etc) were included during primary co-culture. Thus, it was also possible to assess how TCR signalling affected the development of other subsets, such as Th2 and Th1 cells, in parallel. Furthermore, as well as assessing the production of IL-17a typically used to define the Th17 phenotype throughout this study, the levels of GM-CSF and IL-22 were also determined. Production of these Th17-associated cytokines suggests that, not only, are Th17 cells induced at a lower signal strength but these co-express cytokines typical of the pathological subset in some *in vivo* disease models (196, 239, 241). It is increasingly recognised that Th17 cells are not a homogenous subset with distinct phenotypes occurring within the IL-17a- expressing population (290, 376, 443).

The use of a TCR Tg system, while an absolute necessity to assess the influence of peptide affinity, does not reflect the complexity of polyclonal T cell responses. Approximately 0.1-1% of T cells in a lymph node are capable of responding to an immunising antigen (273). It cannot be ruled out that the high frequency of responders in these co-cultures (>85% Vβ4+) could have affected the observed outcome. Thus, while the knock-in of the TCR transgene provides an important tool to assess these factors, caution must be applied when extrapolating the same principles to the more complex situation when multiple TCRs of differing affinities compete to bind a particular pMHC.

Furthermore, this project is limited by the lack of *in vivo* data. The relatively short term *in vitro* co-culture of naive Th cells with LPS mDCs in plastic plates lacks the complexity

that occurs in a lymph node. Further confirmation using adoptive transfer of naive 5/4E8 TCR Tg T cells into a congenic host would prove valuable. Moreover, the addition of exogenous cytokines means that further optimisation would be needed in order to generate a substantive Th17 response i*n vivo*. The importance to disease progression remains to be confirmed until both peptide density and affinity are assessed in the context of an inflammatory disease model such as PGIA. Expression of GM-CSF and IL-22 are linked to pathology *in vivo* but confirmation that the Th17 cells induced by R93Cit are more pathogenic than those induced by cognate peptide would be valuable if replicated *in vivo* (239, 444).

Moreover, only LPS mDCs were used during this study to standardise the APC and co-stimulatory signals that naive 5/4E8 TCR Tg T cells received during activation. Other APCs such as B cells and macrophages can interact with and activate T cells leading to distinct Th responses (445-447). The influence of different APC types and co-stimulation pathways is another important influence on Th differentiation and worthy of further study.

Finally, care must always be exercised when extrapolating between murine models and humans. While the influence between a low TCR signalling intensity and induction of the Th17 phenotype in mice agrees with previous human studies in our group, the influence of IL-2 antagonism may differ between species (283). There are no current data using human Th cells to confirm that the mechanism proposed by this project operates in both mouse and man. Moreover, the differences between human and murine *in vitro* Th17 induction makes direct comparisons challenging. Many important factors differ including the source (human - peripheral blood vs. murine - spleen cells) and type of Th population (Th17 induction from naive human T cells is challenging), as well as activating antigen and cytokine milieu (448-450). Also epigenetic markers that are thought to be important in controlling Th17 plasticity may differ between the species with some studies suggesting that human Th17 express IFNγ and T-bet more readily than murine Th17 cells through the expression of IL-12R (449, 451). Indeed, GM-CSF in humans appears to be more directly influenced by T-bet than ROR-γT as is the case in murine studies (452). The precise influences that these variables have on the observed Th response remains to be fully qualified and in some cases are more

reliant on the experimental setup and temporal factors than any species difference (450).

6.3 Future studies

6.3.1 The role of PTPN22 in Th responses

PTPN22 encodes for the enzyme Pep (Lyp in humans), a tyrosine phosphatase that inhibits the activity of Src-family kinases, including ZAP-70, LCK and VAV, required for TCR signal induction (453). Polymorphisms in the *Ptpn22* gene are association with arthritis in a Japanese cohort. Indeed, the R620W SNP is found to be associated with an increased prevalence of type I diabetes, RA, SLE and Grave's disease (454). The functional consequence of the latter remains unclear as to whether it represents a gain-of-function mutation, suppressing TCR signalling, or enhances the degree of signalling due to a loss of phosphatase activity.

Interesting data has arisen from studies of *ptpn22*$^{-/-}$ mice which have shown an enhanced degree of phosphorylation in a number of TCR signalling components including ZAP-70 (455). Moreover, ZAP-70 mutations form the basis of the SKG murine arthritis model (456). These mice develop a spontaneous arthritis that is dependent on Th17 cells. Indeed, *Il17a*$^{-/-}$ T cells from SKG mice are unable to induce pathology if adoptively transferred.

In *Ptpn22*$^{-/-}$ mice, the development of Tregs both in the thymus and in the periphery is enhanced (453). As expected these mice have significantly curtailed EAE compared to WT mice (457). These findings agree with data from the Allison lab highlighting the importance of a high affinity peptide for Treg development. However, the influence of such a mutation on Th17 cells remains to be explored. It would be an interesting validation of the hypothesis that a lower TCR signalling intensity promotes Th17 induction if *Ptpn22*$^{-/-}$ mice show a suppressed Th17 response due to more sustained TCR signalling.

6.3.2 IL-2 in human Th17 response

With growing evidence highlighting the role of IL-2 as a negative regulator of the murine Th17 response, it remains to be assessed if a similar mechanism operates in man. The data remain unclear with the routine use of IL-2 in human Th17 cultures in

many labs. However, others have indicated a suppression of IL-17 release from human memory T cells when exposed to IL-2 (458). Interestingly, Santarlasci *et al* have shown a reduced degree of proliferation and IL-2 release by Th17 cells as compared to other subsets when stimulated with anti-CD3/28 (459). Moreover, these cells upregulate expression of IL4I1, a secreted L-phenylalanine oxidase, under the control of ROR-γT. IL4I1 has been shown to negatively regulate CD3ζ expression limiting the expansion of these cells. A subsequent study has also linked IL4I1 to upregulation of an antiproliferative protein Tob1 (460). Capable of negatively regulating a number of mitogenic factors including SMADs, ERKs and CTNNB, overexpression of IL4I1 can lead to cycle arrest in primary T cells. Indeed, Schulze-Toppohoff *et al* have proposed Tob1 as one of the most promising biomarkers for diagnosis and prognosis of MS with 92% of Tob1+ patients converting into Clinically Defined Multiple Sclerosis (CDMS) (461). This evidence has also been proposed to explain the apparent scarcity of Th17 cells in the periphery of RA patients. An intriguing hypothesis is that humans T cells are not negatively regulated by IL-2 directly but express negative regulators of IL-2 production such as IL4I1. Thus, the impaired ability of human Th17 cells to produce IL-2 may reflect the poor proliferation capacity and scarcity of this subset relative to others such as Th1 cells in the periphery RA patients.

6.3.3 Epigenetic regulation of Th17 cells

Recent advances in technology has allowed a greater appreciation of the complexity of a Th response with greater knowledge of genomics, proteomics, transcriptomics and epigenomics and how these affect Th cell differentiation (462). Studies by Gottschalk *et al* have identified pathways that can be differentially regulated in response to changes in either peptide affinity or avidity (400). Moreover, Yousef *et al* have profiled the Th17 response at 18 time points over 72h showing that Th17 differentiation occurs in three distinct phases of transcription (463). This approach identified novel 12 regulators critical for establishing Th17 cells. Interestingly, a number of these are involved in the negative regulation of IL-2 production. It would be interesting to assess if these play a similar role to Tob1 in human Th17 cells. Many of these targets have since been confirmed by CHIP-seq data from Ciofani *et al,* enabling systematic ranking of key factors and transcriptional regulators in both wild type and knockout cells (252). Thus, it would be interesting to apply techniques such as RNA-seq to profile the

heterogeneity of Th responses with the possibilities of identifying novel subsets within a T cell population. Analysis may help identify important check points in Th17 development and which genes that when expressed can determine whether Th cells become pathogenic at the level of a single cell. Indeed, identification of pathogenic subsets may lead to more targeted therapies and discovery of novel biomarkers for diagnosis.

A further layer of regulation occurs through the differential expression of miRNAs, consisting of short sequences of RNA nucleotides (21-24nt in length) that can affect gene expression through a number of mechanisms thought to be dependent on the affinity for a target mRNA (464). These range from inhibition of mRNA translation to direct targeting of mRNA for degradation. Evidence has been growing as to the role of miRNA in promoting Th17 development with miRNA-326 found to impair the expression of a negative regulator of Th17 cells ETS-1 (465). Haas *et al* have shown that miRNA-133b and miRNA-206 can promote IL-17a/f production from both αβ and γδ T cells in response to IL-23 stimulation (466). miRNA-155 has also been shown to enhance Th17 cells development and EAE possibly through the negative regulation of c-maf induced IL-4 (467). Other miRNAs have been shown to be directly linked to TCR signalling (468). For example, miRNA-181a is known to be expressed during maturation during the thymus. It is thought that miRNA-181a can repress the expression of phosphatases and so can enhance the degree of TCR signalling and T cell sensitivity to antigen. Indeed, an increase in copy number per cell from 25 to 70 lead to a decline of target phosphatases with a corresponding rise in TCR activation. Assessing expression of miRNA-181a in our model would also be of interest.

6.3.4 Further *in vivo* characterisation

It would be of interest to explore to confirm the role of IL-2 through the ablation of critical targets including CD25, IL-2 and STAT5. Given the discrepancies between the CD25 knockout and STAT5 mice found in studies by Fuijmara *et al* and Laurence *et al,* respectively, it would be of interest to determine if a similar result would occur using the TCR Tg system used in this project (421, 427). Furthermore, the 5/4E8 TCR Tg mice are the basis for the PGIA model of inflammatory arthritis. Many characteristics of PGIA reflect aspects of polyarthritis and ankylosing spondylitis shown externally through redness and swelling due to oedema and proliferation of immune cells (339).

The globular domain of aggrecan is highly conserved between species especially between humans and mice. Given the model is reliant on cross reactivity between different species, it would be interesting in future to assess if citrullinated peptides could alter the course of pathology in ongoing PGIA.

Further screening of APLs could identify a peptide capable of negatively regulating either IL-17 release or promotion of the Treg phenotype given the panel used in this study was relatively limited compared to other studies using all 20 amino acids at each position. The next challenge would be to assess the impact of these APLs on ongoing arthritis. However, the use of APLs in patients remains challenging given the heterogeneity of autoantigens within patient immune responses. Moreover, despite promise in modulation of murine EAE, Bielekova *et al* indicated that the APL to MBP83-99 was poorly tolerated resulting in halting on the phase II trial (469). Thus consideration must be given to the complexity of introducing antigenic peptides into an ongoing inflammatory response. It would have to be demonstrated that any APL inhibited pre-existing disease before being carried through to human studies.

6.3.5 The role of co-stimulation on T cell phenotype

With TCR signalling playing a significant role in the shaping of the Th response, an interesting question arises of how co-stimulation can shape this response. CD28 is the primary co-stimulatory molecule involved in the modulation of the TCR signalling pathway playing a critical role in the amplifying signal in a number of shared pathways including PIK, ITK, TEC, Akt and VAV1 (136). Studies by Bouguermouth *et al* have shown that the addition of soluble anti-CD28 negatively regulated Th17 differentiation from anti-CD3 stimulated naive murine T cells (360). Similarly activation of naive T cells with mature DCs suppressed the frequency of Th17 cells relative to those exposed to immature DCs. Blockade of CD28 signaling using CTLA4-Ig also positively regulated development of Th17 in both human and murine cultures (470). Indeed, treatment of EAE mice with CTLA4-Ig enhanced pathology due to increased Th17 activity. Thus, these data agree with the hypothesis that a reduced signal strength is important in Th17 cell differentiation. The maturation conditions used throughout this study were kept constant, meaning the role of co-stimulation could not be assessed. It would be interesting in future studies, given the importance of CD28 signalling to IL-2

production, to assess if the changes to this pathway also impact on the pSTAT3/5 balance.

Engagement of other co-stimulatory pathways can also regulate Th17 function. CD40 has been shown to induce an antigenic Th17 response even under tolerogenic conditions (471). CD40L knockout mice preferentially lack Th17 cells with no alteration in Th1 cells suggesting that CD40 is of particular importance for Th17 development. Others have shown that anti-CD3/CD5 could alter transcription leading to enhanced Th17 generation relative to CD28 stimulation (472). Moreover, OX40 has also been shown to affect Th17 development positively. OX40-activated T cells are capable of inducing pathology following adoptive transfer in the murine model of antigen specific uveitis (473). Paulos *et al* have also shown that ICOS signalling has a preferential role in the development of human Th17 cells (474). However, these data conflict with EAE models in which ICOS knockout mice show exacerbated pathology (475). Interestingly, while ICOS is a member of the CD28 protein family, it fails to induce IL-2 production and so would provide an interesting tool to assess the role of IL-2-driven STAT5 signalling in Th17 cell development (476). Thus, clarification on the role of co-stimulation would be an interesting avenue for future research especially if a hierarchy of importance could be determined if all the molecules were assessed in the same model.

6.3.6 The effect of other PTMs on T cell responses

Citrullination is just one a number of PTMs that proteins can undergo (312). Growing evidence has also highlighted the relevance of carbamylation; the conversion of Lysine to Homocitrulline residues (477). As with citrullination, current knowledge has focused on the autoantibody response with anti-carbamylated protein (CarP) antibodies being the useful prognostic markers particularly in those patients that lack a response to citrullinated antigens (478). Intriguing data has shown an interaction between carbamylated and citrullinated peptides following intra-articular immunisation (479). Alone the citrullinated peptide failed to induce pathology but if mice were initially primed with a carbamylated peptide, the degree of pathology was significantly increased. Thus, it would be of interest to study further how responses to distinct PTMs can interact and if the presence of these facilitates epitope spreading through cross-reactivity between specific forms of PTM (480). Whether the modified antigens

are recognised by the same or distinct Th subpopulations remains to be fully characterised within patients with autoimmune diseases. A growing hypothesis suggests that PTMs such as citrullination are linked to chronic inflammation and defective processes such as apoptosis and autophagy (480). Given that thymic expression of PAD and citrullinated antigen remains unclear it would be possible that PTMs generate a pool of neo-epitopes capable of breaking central tolerence to excerbate and sustain an autoreactive Th response.

While the focus of this study has been on citrullination in the context of autoimmunity, understanding how these modifications can generate neo-epitopes may be of importance in tumour and infectious immunology (312). In the case of the former enhanced CTL responses have been detected to deiminated and cysteinylated epitopes which are greater than to unmodified form (481). In the context of influenza infection, reduction of cysteine-containing epitopes derived from viral nuclear protein (NP) increased the magnitude of the immune response 10-100 fold (482). In contrast, glycosylation of NP reduces its immunogenicity through alteration of epitope processing. Others have shown that modifications such as nitrosylation can change immune function through PTM of chemokines (483). These have been shown *in vivo* to impact on chemotaxis of APCs (484). Thus, further characterisation of the effect that the growing number of PTMs have on T cell responses is an area of increasing interest.

6.4 Conclusions

In summary, the data present in this thesis demonstrate the profound impact of TCR signalling intensity on the development of effector Th cells from naive precursors. Reinforcing data from previous studies, I have shown that low concentrations of cognate peptide favoured Th17 development at the expense of Th2 cells. Moreover, the introduction of a citrulline to a putative TCR contact produced a lower affinity APL that also promoted Th17 differentiation. It is this interaction between IL-2 signalling, a major product of TCR engagement, and the pro-Th17 cytokine milieu that promotes this shift from a Th2 response to one dominated by Th17 cells. The data presented in this project showing antigen- induced alteration in the ratio between STAT3 and STAT5, indicates how products of the TCR pathway can interact with the cytokine environment. Further understanding of the mechanisms that regulate Th17 responses may lead to specific targeting of this pathogenic subset. The data presented here have

implications for IL-2 therapies, which may exert influence through antagonism of Th17 cells. It is a pertinent question whether IL-2-mediated suppression of Th17 cells influences both *in vivo* and human Th17 immunobiology.

7 References

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