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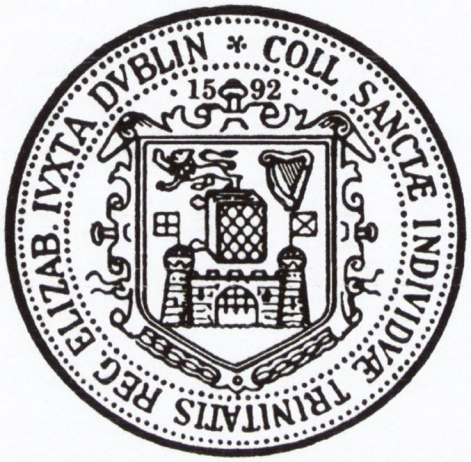
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Novel mediators regulating innate and adaptive type 17 immune responses.



Shane E. Russell BSc (Hons)

2013

Trinity College Dublin

A Thesis submitted as completion of the degree of

Doctor of Philosophy

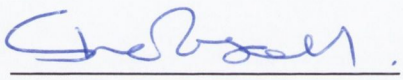
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27th March 2014



Thesis 10399

Abstract

The discovery of a third subset of T helper cells and more recently innate T cell subsets which preferentially express IL-17A has led to intensive investigation into the roles these cells play in both host defence and autoimmune settings As such, understanding the mechanisms related to the of the generation, function and regulation of these cells are crucial to the development of therapeutic interventions to counteract associated inflammation and pathology. In these studies we have focused on two of the more recently described pathways of regulation of IL-17A expressing T cells, namely the IL-2/IL-2 receptor signalling axis and TIR8 a negative regulator of IL-1R1 signalling.

IL-2 has been implicated as a negative regulator of Th17 responses *in vitro* and *in vivo.* Mutations at *CD25*, the α chain of the IL-2 receptor, confer susceptibility to a variety of autoimmune diseases with a number of these susceptibility alleles correlating with elevated levels of the soluble form of the α receptor chain (sCD25). We have demonstrated that elevated levels of sCD25 *in vivo* resulted in enhanced antigen-specific Th17 responses in the periphery and exacerbated experimental autoimmune encephalomyelitis (EAE). We report that sCD25 is capable of enhancing Th17 responses *in vitro* with no impact on Th1 responses or Treg generation. This enhancement of the Th17 response is characterised by decreased STAT5 signalling downstream of the IL-2 receptor, which facilitates an enhancement of IL-6 receptor driven STAT 3 phosphorylation. sCD25 exerted its effects early during the Th17 developmental programme, leading to increased expression of the signature transcription factor RORγT, as well as IL-17A expression after just 48 hours. sCD25 did not directly impact cellular expression of IL-2 or act at the cell surface, but was found to greatly inhibit the detection of secreted levels of IL-2 by ELISA through direct interaction with IL-2 itself, demonstrating its ability to act as an IL-2 'sink' in the T cell microenvironment. These data identify the ability of sCD25 to promote autoimmune disease pathogenesis and enhance Th17 responses through its ability to sequester local IL-2.

Th17 cells have received considerable interest since their discovery; however, recently it has become clear that a large proportion of the IL-17A expression in both host defence and autoimmune settings is accounted for by innate T cell subsets. One such subset is γδ T cells, which have been shown to express IL-17A directly in response to IL-1β and IL-23 stimulation. TIR8, an orphan receptor of the Toll/IL-1 receptor superfamily has been established as a negative regulator of IL-1R1 signalling and in this role has also been implicated as a key intrinsic regulator of Th17 responses and autoimmunity. However, its role in the modulation of IL-1R1 driven IL-17A expression by innate lymphocyte populations or its potential to modulate inflammation in disease associated with these cells has yet to be established. Expression of TIR8/SIGIRR has been reported to be reduced in the peripheral blood of psoriatic arthritis patients. However, whether TIR8/SIGIRR activity plays a specific role in regulating psoriatic inflammation is unknown. We report that *Tir8/Sigirr* deficient mice develop more severe psoriatic inflammation in both the chemical (Aldara™) and cytokine (recombinant IL-23) induced models of psoriasis. Increased disease severity was associated with enhanced infiltration of $V\gamma4^+$ γδ T cells which express significantly elevated levels of IL-17A. Critically, we also demonstrate that TIR8/SIGIRR activity directly suppressed innate IL-17A expression by γδ T cells *in vitro* and *in vivo*. Importantly, treatment of *Tir8/Sigirr-/-* mice with an IL-17A neutralisation antibody reversed the enhanced disease severity observed in these mice. This study

identifies TIR8/SIGIRR as a novel intrinsic negative regulator of innate IL-17A expression and characterises a novel mechanism involved in the regulation of psoriatic inflammation.

Taken together these data further highlights the central role IL-17A plays in inflammatory states mediated by both innate and adaptive T cell and identifies both sCD25 and TIR8/SIGIRR as signalling mediators acting to directly modulate these events *in vivo.*

List of publications

Russell, S. E., A. M. Stefanska, M. Kubica, R. M. Horan, A. Mantovani, C. Garlanda, P. G. Fallon, and P. T. Walsh. 2013. Toll IL-1R8/Single Ig IL-1-Related Receptor Regulates Psoriasiform Inflammation through Direct Inhibition of Innate IL-17A Expression by gammadelta T Cells. *J Immunol* **191**: 3337-3346.

Russell, S. E., A. C. Moore, P. G. Fallon, and P. T. Walsh. 2012. Soluble IL-2Ralpha (sCD25) exacerbates autoimmunity and enhances the development of Th17 responses in mice. PLoS One **7**: e47748.

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For Mam and Dad

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I wish to dedicate this thesis to my parents; nothing I have ever done in life would have been possible without their help and support and I'm indebted to them every day for the chances they have given me and the sacrifices they have made to allow me these opportunities. I count myself extremely lucky to have parents like you and hope that one day I can pay you both back somehow.

List of abbreviations:

7-AAD	7-Amino-Actinomycin D
APC	Antigen presenting cell
BCR	B cell receptor
BSA	Bovine serum albmin
CC	Chemokine (C-C) motif
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine synthetic oligonucleotide motif
CXC	Chemokine (C-X-C) motif
D	Diversity
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DN	Double Negative
DMEM	Dulbecco's Modified Eagle Medium
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EtOH	Ethanol
FACS	Fluorescence activated cellular sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead winged box transcription factor 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hanks balanced salt solution

HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
IL-R1	Interleukin receptor 1
IPEX	Immune dysregulation polyendocrinopathy X-linked syndrome
IRAK	IL-1R-associated kinase
iTreg	Inducible regulatory T cell
J	Joining
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MMP	Matrix metallo protease
mRNA	Messenger RNA
MS	Multiple sclerosis
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor κB
ng	Nanogram
NK	Natural killer
nTreg	Natural regulatory T cell
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
ROR	Retenoic acid receptor related orphan receptor
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCID	Severe combined immune deficiency

SIGIRR	Single immunoglobulin interleukin 1 receptor related molecule
STAT	Signal transducer and activator of transcription
T-Bet	T-box expressed in T cells
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper cell
TIR	Toll/Interelukin -1 receptor
TIR8	Toll Interleukin-1 receptor 8
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor accosiated factor
Treg	Regulatory T cell
BSA	Bovine serum albumin
H&E	Haematoxylin and eosin
PBMC	Peripheral blood mononuclear cells
ION	ionomycin
PMA	Phorbol 12-myristate 13-acetate
ROR	Retinoic Acid Orphan receptor
V	Variable
WT	Wild Type

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Chapter 1: General Introduction

1. Introduction

The mammalian immune system is a highly sophisticated network of organs and complimentary cells whose primary function is protection of the host from infection by parasitic bacteria, viruses and protozoa, whilst maintaining tolerance to endogenous healthy tissues. This essential ability to discriminate between "self" and "non-self" has evolved in conjunction with equally important and powerful regulatory mechanisms which maintain immune homeostasis whilst facilitating the generation of appropriate immune responses to combat against foreign pathogens and cancer (1).

The immune response can be generally classified into the innate and the adaptive arms. The innate immune system provides an extremely rapid, short lived response but with broad specificity. It acts as the first line of defence against foreign pathogens recognising microbial components, conserved across many microbial pathogens through its system of intracellular and extracellular pattern recognition receptors (PRR). Effector cells of the innate immune system such as macrophages and neutrophils act to efficiently phagocytose and destroy these pathogens, with cytokines and chemokines expressed directly in response to such exposure acting to shape the nature of the subsequent adaptive response (2, 3).

A direct link between the innate and adaptive systems is provided by antigen presenting cells (APC) which include both macrophages (2) and a type of professional APC known as the dendritic cell (DC) (3). These cells process and display fragments of antigen bound to major histocompatibility (MHC) molecules which are subsequently presented to cells of the adaptive immune system to initiate an antigen specific response. Furthermore, the specific cytokine milieu expressed by the APC at this time will serve to directly modulate the nature of the subsequent antigen specific immune response (4).

The adaptive immune system is primarily responsible for resolving infections which the innate immune system has failed to efficiently clear and whilst it is slower to react, adaptive immune responses are highly specific to the pathogen of interest and repeated exposure will serve to provide lifelong antigen specific immunological memory against these pathogens.

The key effector cells of the adaptive system immune system are B and T cells which recognise the antigen derived peptide fragments processed and presented by APCs through their activating cell surface receptors, termed the B (BCR) and T cell receptor (TCR) respectively. Activation of these cells generates an immune response which is specifically tailored to eliminate the pathogen in question or pathogen infected cells. This acquisition of specificity and memory, through the generation of antigen specific cellular receptors, contrasts with PRRs on innate immune cells which are germline encoded and one of the key distinctions between innate immunity and adaptive immunity.

It has become clear that a gap exists between the innate immune response which can take a matter of hours to resolve and the induction of a robust adaptive response which can take up to 5 days to initiate. In recent times, cells which possess some characteristics of both innate immune cells and also adaptive immune cells have been described (5). These cells, one particular example of which are known as γδ T cells, possess the ability to respond rapidly to environmental signals such as microbial insult or tissue necrosis to provide an important source of T cell associated cytokines early in the response before the induction of adaptive immunity (6, 7).

Despite fundamentally different effector cells and kinetics of induction it is increasingly clear that both arms of the immune system act in a complimentary fashion with the nature of the adaptive immune response directly influenced and shaped by the preceding innate response.

1.1 T cells

T cells develop from haematopoietic progenitor cells in the thymus. They do not have phagocytic abilities but rather act as orchestrators of cell mediated immunity. Naïve T cells recognise antigen through a clonally distributed heterodimeric surface receptor composed of transmembrane glycoprotein α and β chains, known as the TCR. The vast majority of T cells express αβ TCR chains however there are also subsets of cells which express alternative receptor chains consisting of γ and δ chains (5).

T cells can be further categorised into two subsets based on their functions and expression of surface co-receptors. Cluster of differentiation (CD)8+ T cells also known as cytotoxic T cells are responsible for direct killing of bacterially or virally infected cells through the secretion of a variety of cytolytic proteins. $CD4^+$ T cells, commonly termed T helper (Th) cells, upon activation, are responsible for secretion of cytokines which orchestrate the actions of other immune cells and relevant tissues throughout the immune response.

The TCR is incapable of recognising antigens in their native form, instead antigen is degraded and processed to peptide complexes and presented to T cells on surface MHC molecules. Antigen from intracellular bacteria or virus is degraded to peptide fragments in the proteosome before trafficking to the cell surface as part of a complex formed with MHC class I molecules for presentation to $CD8^+$ cytotoxic T cells leading to the killing of the infected cell.

In the case of extracellular pathogens, antigen is phagocytosed and endosomally degraded to peptide fragments by the APC. These peptide fragments bind to the assembled MHC class II complex which trafficks to the cell surface for presentation to $CD4^+$ T helper cells and initiates a cell mediated adaptive response and contributes to a humoral response. MHC class

I is ubiquitously expressed across all nucleated cells in the body providing the cell in question with the ability to indicate its infected state to the immune system. In contrast expression of MHC II is more restricted with expression limited to APCs including DCs, macrophages and B cells.

1.1.1 Activation of Th Cells

For Th cell activation to take place, a naïve Th cell must encounter antigen presented on MHC class II by a pathogen primed APC such as a DC, macrophage or B cell. This presentation is commonly referred to as signal 1, and is a tightly restricted process whereby each TCR possesses sole specificity for a single antigen peptide. Signal 2 involves initiation of a co-stimulatory signalling cascade through engagement of T cell surface markers such as CD28 with the ligands CD80 and CD86 expressed by mature DC. This interaction is referred to as co-stimulation as it reinforces the previous TCR/MHCII interaction. Signal 3 is provided by the cytokine milieu expressed by the activated APC or adjacent accessory cells. Initially, activated Th cells are referred to as Th0 cells. However the Th0 cell is skewed towards a preordained lineage dependant on the required nature of the cell mediated response as indicated by the local cytokine environment. Effector Th cells are classified into three distinct subsets, Th1, Th2 and Th17. These classifications are broadly based upon their function and interleukin (IL) expression profile (1, 8) (Fig 1.1). In recent times the number of T cell subsets has further expanded beyond the lineages described above to include regulatory T cells (9, 10) Tr1 regulatory cells (11), T follicular helper cells (12), Th9 cells (13, 14). Th1, Th2, Th17 and regulatory T cells will be discussed in more detail below.

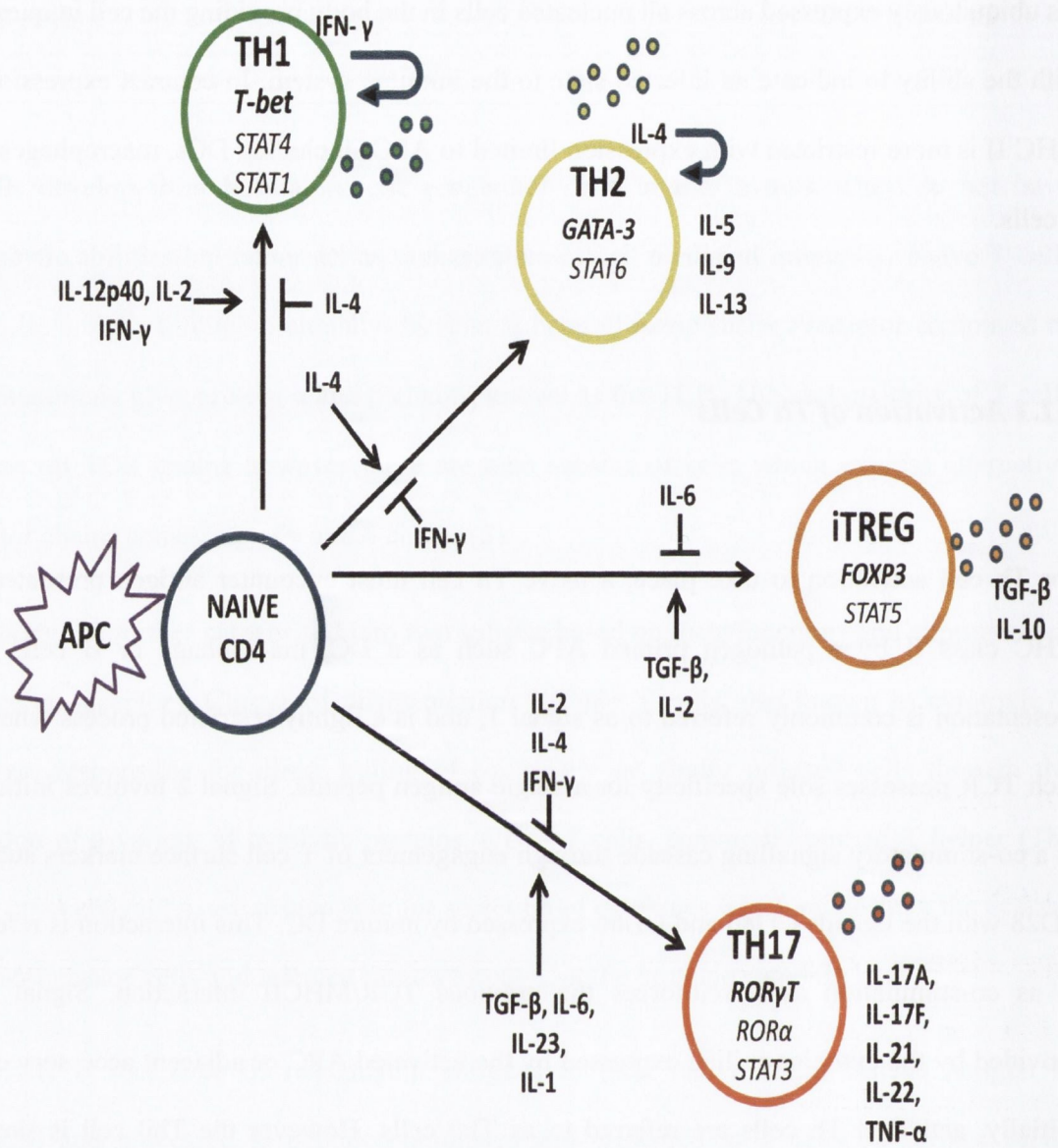


Fig. 1.1 Overview of T helper cell differentiation.
Overview of the differentiation of naïve T cells in the periphery to specific lineages as a consequence of the local cytokine milieu. For example TH1 cells differentiate from naïve T cells in the presence of of instructive signals which can include IL-12p40, IL-2 and IFN-γ. This differentiation can be inhibited by mediators such as IL-4. The activites of Th1 cells are under the governance of the lineage specific transcription factor T-bet as well as STAT4 and STAT1. These cells are capable of expressing a cytokine repertoire which can include IFN-γ which is also capable of acting in an autorcine manner

1.1.2 Th1 and Th2 cells

When the heterogeneity of T helper cell subsets was first proposed by Mossmann and Coffman, Th cells were initially divided into Th1 and Th2 subsets based on their cytokine expression profile and involvement in distinct immunological processes (8) (Fig. 1.1) .

Th1 cells, which produce IFN (interferon)-γ as their lineage signature cytokine, are involved in macrophage activation and defence against intracellular pathogens (15). Their differentiation is reliant on IL-18, IL-12 and IFN-γ. Innate and autocrine IFN-γ leads to the activation of signal transducer and activator of transcription (STAT)-1 which induces expression of the Th1 lineage specific transcription factor, T-box expressed in T cells (T-bet). This expression results in enhanced Th cell expression of IFN-γ, upregulation of IL-12 receptor and subsequent activation of STAT-4 by IL-12. These steps also promote expression of the IL-18 receptor and further enhance production of IFN-γ (16, 17). Expression of T-bet is essential for IFN-γ expression (18) and T-bet deficient mice display severely impaired Th1 responses both *in vitro and in vivo* (19). Differentiated Th1 cells can also selectively produce IL-2, IL-3 and Tumour Necrosis factor (TNF) α and β which are also capable of acting in an autocrine manner to enhance Th1 differentiation.

Cytokines expressed by Th1 cells can activate the phagocytosis activity of macrophages and contribute to the production of complement components and antibodies by B cells. Furthermore Th1 cells are important for the expression of adhesion molecules on endothelial cells and the induction of chemokines for the attraction of mononuclear cells to sites of inflammation.

Cytokines produced by Th1 cells also participate in the generation of cytoxic lymphocyte (CTL) $CD8^+$ t cell responses. $CD8^+$ t cells cells require activation through their TCR and co-

stimulation in a similar manner to $CD4^+$ T cells and in the case of $CD8^+$ T cells, early exposure to distinct cytokines most commonly influences the balance between the development of short-lived, terminally differentiated effector cells and memory precursors (20) (21). In many cases these priming signals eminate from Th 1 cells, for example IL-2 and IFN-γ induces the release cytolitic particles from $CD8^+$ effector t cells acts to kill virally or microbailly infected cells (22). Furthermore, the expression of IL-2 from $CD8^+$ T cells is more transient in nature compared to $CD4^+$ T cells (23), where after the inital period post activation $CD8^+$ T cells require extrinsic sources of IL-2, likely from $CD4^+$ T cells to maintain proper expansion, function and the generation of the shorter lived effector response (24). The influence of $CD4^+$ t cells on $CD8^+$ t cell responses is further underscored by the profound deficiencies in $CD8^+$ memory T cell populations when generated in the absence of $CD4^+$ T cells (24, 25). Other T helper cell derived cytokines such as IL-21 have also been shown to influence the nature of the $CD8^+$ t cell response with IL-21 acting to counterbalance the impact of IL-2 by driving the expansion of $CD8^+$ memory poplatons (26)

Th2 cells produce IL-4, IL-5, IL-6, IL-10, IL-13 and IL-25 (27). These cells provide assistance to many facets of the humoral immune response including B cell activation, IgG class switching and eosinophil recruitment. Their differentiation is driven by IL-4 which leads to the activation of STAT6 and expression of the Th2 lineage governing transcription factor GATA3 (28). Other cytokines including IL-13 and IL-25 have also been implicated as drivers of Th2 differentiation. IL-4 activates mast cell sensitisation and is capable of enhancing Th2 differentiation in an autocrine loop. IL-13 acting in conjunction with IL-4 acts to drive the maturation of plasma cells leading to the expression of immunoglobulins (29).

Cytokines produced by Th1 and Th2 cells serve to enhance to their own differentiation whilst displaying an ability to inhibit other lineages. IFN-γ, crucial for Th1 lineage commitment, is capable of inhibiting expression of GATA3 and conversely IL-4 can restrain Th1

development by upregulation of GATA3 whilst inhibiting expression of IL-12 receptor (28) (Figure 1.1). This reciprocal regulation between Th1 and Th2 cells provided a useful model system for immunologists to investigate the cellular immune response. However, as the knowledge of Th helper cell biology developed it became increasingly clear that the Th1/Th2 model was an overly simplistic paradigm to explain the events at play in many settings, due in large part due to anomalies observed in studies into T cell driven autoimmune disease. Consequently the Th1/Th2 paradigm has now been expanded to include a third distinct subset of Th cells termed Th17 which are discussed below. (Fig. 1.1) (30).

1.1.3 Th17 cells

Th17 cells are a subset of $CD4^+$ T helper cells characterised by expression of IL-17A, IL-17F, IL-22, IL-21, GM-CSF and the lineage specific transcription factor retinoid orphan nuclear receptor (ROR)γT (31). These cells have important functions as part of the cellular immune response against various extracellular pathogens such *Klebsiella pneumonia* (32) and fungi such as *Candida albicans* (33), with mice deficient in IL-17A displaying significantly enhanced morbidity in response to infection by these pathogens. Furthermore, their discovery has greatly enhanced the understanding of the organ specific autoimmune disorders which were imperfectly explained utilising the Th1/Th2 model.

1.1.3.1 Differentiation of Th17 cells

Passive transfer experiments had established the role of Th1 cells in auto antigen initiated inflammatory disorders; however results in these models were at times somewhat counter intuitive. For example, treatment with IL-12 blocked initiation of experimental autoimmune encephalomyelitis (EAE) (34), whilst depletion of IFN-γ enhanced disease pathogenesis (35). These findings raised the possibility that Th1 cells were in fact acting in a suppressive role through IFN-γ expression and that the predominant cell type mediating chronic inflammation in these settings was undefined.

Interest in IL-17A was heightened by a number of studies, including work which demonstrated an IL-23 driven population of IL-17A producing $CD4^+$ T cells which were capable of inducing EAE upon adoptive transfer to naïve mice (36). Coupled with observations that IFN-γ and IL-4 were capable of inhibiting IL-23 driven IL-17A production from T-cells (37), the hypothesis that a subset of $CD4^+$ T cells characteristically producing IL-17A was formed.

Naïve T cells do not express IL-23 receptor so mechanisms involved in Th17 lineage commitment remained unclear until a number of studies demonstrated that transforming growth factor beta (TGF-β) in combination with IL-6 was capable of driving IL-17A expression in naïve activated T cells. Importantly, this cytokine combination also served to upregulate the expression of the newly identified Th17 lineage governing transcription factor RORγt (38, 39).

IL-6 deficient mice were shown to be protected from EAE (40). Furthermore, IL-6 was already established as an inhibitor of a subset of T cells which regulate autoimmune inflammation known as regulatory T cells (Treg) (41). In the setting of Th17 differentiation, IL-6 became implicated as driving factors important to Th17 generation such as IL-23

receptor and IL-21 expression whilst simultaneously inhibiting the potential development of T cell suppressive function (31, 42).

TGF-β was widely recognised for its anti-inflammatory effects and role in the induction of Treg cells but it had been reported that mice lacking in TGF-β were also deficient in Th17 cells (39). It also became clear that TGF-β was capable of indirectly driving Th17 lineage commitment through its ability to block STAT4 induced GATA-3 expression (43). Thus, the model for Th17 differentiation describes a mechanism whereby IL-6 and TGF-β synergise to drive upregulation of crucial Th17 transcription factors and cytokines whilst suppressing factors required for differentiation toward alternative lineages. It is noteworthy that TGF-β and IL-6 alone are insufficient to drive pathogenic T cells until IL-23 is present (44). The role of IL-23 whilst still incompletely understood is thought to extend to expansion (36) and stabilisation of the Th17 phenotype (45). However some debate still persists as to its exact role and at what point in the Th17 differentiation programme it mediates these effects.

Th17 cells function predominantly at mucosal surfaces where IL-17A expression acts to upregulate expression of G-CSF and CXC chemokines in mucosal epithelial cells as well as the local stromal cells. These actions predominantly serve to mobilise and activate neutrophils (46) as well as inducing the expression of anti-microbial peptides (47). Furthermore IL-17A is capable of driving the expression of a range of cytokines including IL-6, IL-8 and GM-CSF from macrophages, which act to further enhance the recruitment of neutrophils (48).

IL-17A signals through its receptor IL-17 receptor A (IL-17RA), the most comprehensively studied receptor of the newly defined IL-17 receptor family (49). Despite the expression of IL-17A being limited to a tightly restricted selection of immune cells, IL-17RA is constitutively expressed across a wide range of cell types and tissues enabling IL-17A to

exhibit widespread proinflammatory effects (50). Upon engagement with IL-17A, IL-17RA forms a heterotrimeric complex with IL-17 receptor C (IL-17RC) to initiate downstream signalling events mediated by Act1 (51) and TNF receptor associated factor 6 (TRAF6) (52) which leads to activation of nuclear factor kappa-B (NF-κB) (53).

IL-17F is a cytokine also commonly expressed by Th17 cells which studies have demonstrated as playing a role in autoimmunity in its own right (54). IL-17F also utilises IL-17RA to form its receptor signalling complex, however the strength of its signalling ability is not considered as strong as IL-17A (49). Interestingly IL-17F readily forms heterodimers with IL-17A which also signal through the IL-17RA complex (55), however the functional role of this heterodimer *in vivo* has proven difficult to define.

IL-22 is another cytokine expressed by Th17 cells which plays an important role in host defence at mucosal surfaces through its ability to induce the expression of antimicrobial peptides (56). It can also act as a pro-inflammatory and a pro-resolving mediator in autoimmune disease depending on the specific disorder. The IL-22 receptor (IL-22R) is not expressed on hematopoietic cells but rather is highly expressed on epithelial and stromal cells of various tissues (57, 58). IL-22R signalling is important for the cellular differentiation and proliferation of keratinocytes and as a consequence its dysregulated expression in psoriatic lesions is thought to contribute to the pathogenesis of psoriasis (59). Conversely its ability to maintain the barrier integrity of epithelial cells is seen as important for mucosal wound healing in settings of dextran sodium sulfate (DSS) induced colitis (60).

IL-21 acts in an autocrine loop, signalling through STAT3 to further enhance the early induction of Th17 cells. IL-21 signalling is capable of inducing RORγT and IL-23 expression however this is a less potent differentiation signal than IL-6 (61). GM-CSF is an emerging player in inflammatory conditions mediated by both Th17 and Th1 cells. It promotes the

differentiation of progenitor cells into granulocytes and macrophages and is also important for sustaining an inflammatory state in settings of neuroinflammation (62).

1.2 Innate IL-17A.

The characterisation of a distinct subset of $CD4^{+}$T cells which preferentially express IL-17A has led to intensive investigation into the regulation and function of such cells in the context of chronic inflammation and autoimmunity (36, 45). However, development of an adaptive response leading to Th17 cell expression of IL-17A and associated cytokines takes up to 5 days. As a consequence it has become clear that cell subsets from the innate arm account for a significant proportion of the IL-17A and other Th cell associated cytokines expressed early on and indeed throughout the immune response (6). Examples of such subsets include invariant natural killer (NK) T cells which are innate T cells acting in defence against microbial infections and have a role in tumour immunity (63). Innate lymphoid cells (ILC) are a newly characterised subset of immune cells which are capable of expressing large amounts of Th cell associated cytokines, including IL-17A but they lack the expression of cell surface markers which are classically associated with more established immune cell lineages. Furthermore, these lineage marker negative ILC subsets do not express a T cell receptor and thus do not respond in an antigen-specific manner (64). An interesting subset of innate IL-17A expressing T cells are γδ T cells, which have also been shown to express IL-17A in a non antigen specific manner in some settings of infection (65) and autoimmunity (66).

1.2.1 γδ T cells

Arguably the most extensively described innate lymphoid cell subset are γδ T cells, which comprise about 5% of the overall T cell population. They differ from conventional αβ T cells in that they express γ and δ chains as part of their T cell receptor and are resident predominantly at mucosal sites (6). γδ T cells appear to lack the requirement for conventional antigen presentation and this has contributed to the hypothesis that these cells act as tissue resident immune sentinel cells. Recently it has become clear that γδ T cells respond to IL-1 and IL-23 stimulation by expressing significant levels of IL-17A (66). These cells which constitutively express CD44, CCR6 and the transcription factor RORγT may act as an important instructive signal to promote the generation of adaptive Th17 type responses but have also been implicated as important mediators of disease in their own right (66, 67).

RORγT $^{+}$ γδ T cells are CD27 negative but constitutively express CD44 and CCR6, (68) and are capable of expressing IL-17A directly in response to stimulation with IL-1β and IL-23. They have been shown to enhance IL-17A expression from $CD4^{+}$ T cells and are present in the brains of mice with EAE (66). In particular, γδ T cells which express IL-17A play an important role in driving neutrophilia and dermal inflammation in psoriasis (69, 70). IL-17A expressing γδ T cells are CD25 positive in the thymus but this is downregulated upon trafficking to the periphery, possibly to protect the cells from the inhibitory effect of IL-2 on IL-17A expression (6). A number of specific subsets of γδ T cells have been described in both humans and mice based on their TCR useage with prominent roles in cancer protection and autoimmune inflammation. In mice a subsets of γδ T cells which display prominent dendritic morphology had been implicated as important players in tumour surveillance and barrier integrity surveillance in the skin displaying usage of the Vγ5 TCR (71). Murine γδ T

cells have also been identified as important sources of IL-17A at sites of inflammation in murine models of autoimmune inflammation such as experimental autoimmune encephalomyelitis (66) and induced psoriasiform like inflammation (72, 73). These cells which express the Vγ4 TCR, IL-23 receptor and CCR6 are capable of expansion in response to pathogen derived and environmental signals (67). Murine γδ T cells are also capable of expressing abundant amounts of IFN-γ (74) and these cells are expanded in *Plasmodium*-infected C57BL/6 (68, 75). CD27 positive γδ T cells are thought to emerge from the thymus preactivated possessing the ability to express IFN-γ (68). They also express CD122, IL-18 receptor and IL-12 receptor (6)

γδ T cells are the most abundant innate T cells in humans and a number of distinct subsets have also been described based on their T cell receptor chain usage. The Vγ9 Vδ2 subset predominates in the peripheral blood and recognising small non-peptide phosphorylated compounds derived from microbes and tumours. These antigens are capable of rapidly and selectively activating these cells to adopt a Th1 like phenotype (76, 77). Furthermore, these cells upon activation induce maturation of DCs and B cells (78, 79). These cells also display potent anti tumour properties *in vitro* and the possibility of using these cells as cell based cancer therapies is being investigated (80). Furthermore, it is also possible to skew these cells towards a phenotype which is permissive of IL-17A expression with Vγ9Vδ2 IL-17A positive cells detectable in the peripheral blood and at the site of disease in children with bacterial meningitis (81). Vγ3Vδ1 cells are a less abundant subset of human γδ T cells and are primarily resident in mucosal tissues and recognise antigens induced through the stress response in the epithelium (82). Upon activation, these cells are capable of killing CD1d+ target cells, as well as the released Th1, Th2, and Th17 associated cytokines as well as inducing dendritic cell maturation (83) *in vitro.* Expansion of Vγ3Vδ1 cells is observed in inflamed tissues in rheumatoid arthritis (84, 85). Furthermore, these cells have been attributed

immunosuppressive properties where stimulatory antibody-activated circulating Vδ1 T cells from healthy donors express FoxP3 and share characteristics with Tregs (86)

γδ T cells play a prominent role in bridging the gap between innate and adaptive immunity providing abundant amounts of Th cell associated cytokines before the establishment of acquired immunity (66, 67). In particular, IL-17A expression by γδ T cells activates signalling pathways mediated by TNF receptor associated factor (TRAF)6, ACT1 and NF-κB, signals which are similar to early innate signalling events associated with members of the Toll/ILR family. In contrast cytokines expressed by γδ T cells which are more commonly associated with alternative Th lineages such as IFN-γ induce the activation of signalling pathways mediated by JAK/STAT which are associated with the adaptive immune system (49). Furthermore, it has recently been demonstrated that innate responding γδ T cells mount an immune response to oral *Listeria monocytogenes* infection which leads to the development of multifunctional memory γδ T cells capable of simultaneously producing interferon-γ and interleukin-17A in the murine intestinal mucosa (87). This intriguing role for γδ T cells as central players at the interface between innate and adaptive immunity highlights their potential as the cellular targets for the development of novel vaccine therapeutics.

1.3 T cell Tolerance

The ability of the immune system to discriminate between foreign pathogens and endogenous tissues and commensal bacteria is of critical importance for the maintenance of immune homeostasis (88). The breakdown of tolerance invariably leads to the development of autoimmune disease; however the immune system has developed a number of mechanisms to ensure that this state of homeostasis is maintained

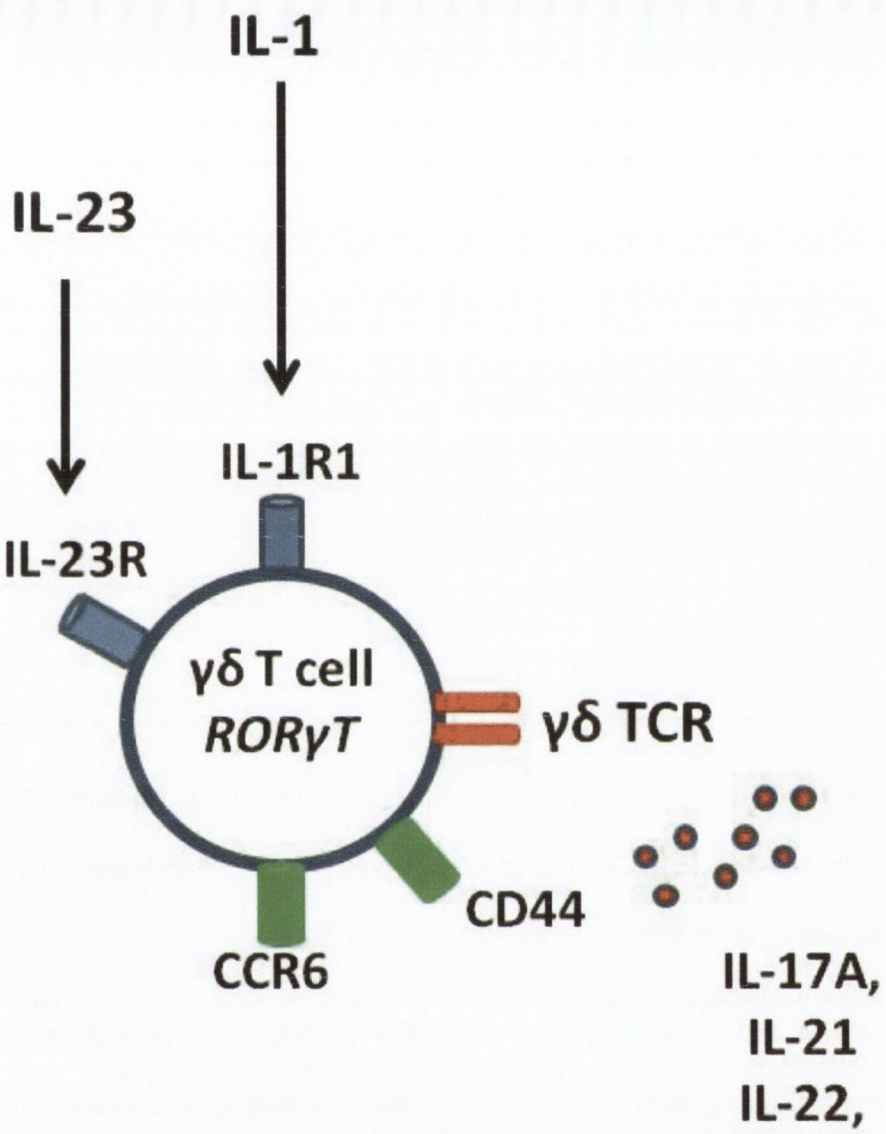


Fig 1.2 Overview of IL-17 expressing γδ T cells.
Surface marker expression, cytokine profile and example of stimulation requirements for IL-17A expressing γδ T cells

1.3.1 Central Tolerance

Central tolerance occurs in the generative lymphatic organs of the immune system and is the process whereby newly developing lymphocytes develop reactivity to MHC molecules and non reactivity to self antigens. Initially, haematopoietic progenitors from the bone marrow

traffic to the thymic cortex to undergo a process known as thymopoeisis, which results in the development of a population of mature T lymphocytes (89).

Initially progenitor cells are double negative (DN) for CD4 and CD8. This double negative stage can be further divided into 4 stages (DN1-4) based on cell surface molecule expression. DN1 cells are not yet committed to a T-cell lineage and can still differentiate into other thymically derived cells such as dendritic cells (DC). Upon expression of CD25, precursors are committed to a T cell lineage and enter the DN2 phase. DN2 phase precursors initiate expression of a diverse T cell receptor (TCR) repertoire. TCR β chain generation is a result of an almost random joining of variable (V), diversity (D) and joining (J) gene segments. In DN2 phase subsets undergo joining of V and D fragments with DN3 phase initiated upon addition of the J segments. In order for DN3 subsets to enter DN4 phase and avoid apoptosis they must co-express functional TCRβ and a pre TCRα. During DN4 phase TCRα rearrangement occurs by V J recombination resulting in display of a fully developed TCR and co-expression of CD4 and CD8 (89).

At this point developing T cells are subjected to rounds of both positive and negative selection in the thymus rendering them reactive to major histocompability molecules (MHC) and non reactive to self antigen respectively.

Positive selection occurs in the thymic cortex and is mediated by epithelial cells expressing MHC molecules. $CD4^+CD8^+$ double positive precursors which react to MHC class I will develop into $CD8^+$ lymphocytes whilst cells reactive to MHC class II will differentiate into $CD4^+$ lymphocytes. If precursors are unable to recognise presented peptide with sufficient affinity they will not receive a survival signal leading to initiation of apoptosis or phagocytosis by macrophages.

Cells surviving positive selection migrate to peripheral areas of the thymic cortex and the thymic medulla. Antigen presenting cells such as DC and medullary thymic epithelial cells present self peptides on MHC molecules. Precursor cells recognising presented peptide at this point undergo apoptosis resulting in the deletion of self reactive T cells. Cells that display weak or no affinity to these self antigens migrate to the peripheral lymphoid organs (90).

1.3.2 Peripheral Tolerance

Despite tight controls on the generation and release of T cells expressing self reactive TCRs in the thymus it is still possible that precursors can evade the mechanisms of central tolerance and migrate to the periphery. In such scenarios it is likely that cells expressing self reactive TCRs did not encounter one of the limited number of APCs expressing their specific cognate ligand in the thymus, alternatively it is possible that there is a low affinity between the peptide and MHC leading to an unstable complex on the surface of the APC. Another possibility is that the antigen in question is expressed developmentally and might not be present in the thymus at the time precursors are undergoing selection, therefore TCRs with affinity to these antigens can escape deletion.

Central tolerance is not a completely efficient process and consequently it is essential to have tolerating processes active in the periphery. They include suppression of auto reactive T cells by regulatory cells (Treg), the induction of anergy, a hyporesponsiveness to antigen owing to absence or inhibition of costimulatory signals and the endogenous expression of suppressive signalling mediators by T cells themselves which can act as 'brakes' on the generation of an inappropriate T cell response .

1.3.3 Regulatory T cells

Tregs are a population of $CD4^+$ lymphocytes possessing the ability to suppress and regulate autoimmune inflammation and associated pathology (10). A number of subsets of Treg cells have been described including $CD4^+CD25^+$ naturally occurring Tregs (nTreg) which develop in the thymus and Treg subsets which are induced in the periphery including Th3 type cells expressing TGFβ and Tr1 cells which express IL-10 (91).

The exact mechanism of nTreg development in the thymus is incompletely understood. However, it is thought that they differentiate from progenitors with a higher than normal affinity to self antigens but not an affinity sufficient to induce cell death (1). nTregs or inducible (iTreg) Th3 type cells characteristically express the forkhead winged helix transcription factor gene, FoxP3, and mice deficient in FoxP3 lack Treg cells and succumb to autoimmune disease characterised by severe lymphocyte mediated organ failure (92). FoxP3 has been demonstrated as essential for development of Treg cells and adoptive transfer of $FoxP3^+$ Treg cells to FoxP3 deficient mice rescues them from fatal autoimmunity (93). Associations have also been demonstrated between mutations in human homolog of FOXP3 and the fatal autoimmune condition immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (94). Close associations in the developmental programs of another pathogenic Th subset such as Th17 cells and iTreg cells have been demonstrated *in vitro.* Activated T cells can be induced to express FOXP3 in the presence of TGF-β (95) (Figure. 1.1), however as noted previously, addition of IL-6 or IL-21 will suppress FOXP3 expression and drive differentiation to a Th17 phenotype (10).

Treg cells express high levels of IL-2R yet in contrast to activated T cell subsets display a hypoproliferative phenotype *in vitro* upon IL-2R stimulation. Numerous studies have demonstrated their ability to suppress the proliferation of activated T cells *in vitro* (96) and control autoimmune inflammation *in vivo* (97).

In vitro, Treg cells suppress through a number of mechanisms, acting directly on FoxP3-ve responder T cells or indirectly through suppression of the APC. Direct methods include the secretion of suppressor cytokines such as TGF-β, IL-10 or IL-35 which inhibit the action of responder cells. Alternatively, Treg cells can also suppress effector T cell responses by consumption of IL-2 leading to growth factor deprivation of the effector T cell population and consequently Bim mediated apoptosis. Cell-cell contact is another mechanism whereby activated Treg cells can act as cytotoxic cells directly killing the target responder cell (98). Tregs can suppress expression of co stimulatory molecules on DCs through CTLA-4, a receptor constitutively expressed on Treg cells (99).

In vivo mechanisms of suppression are incompletely understood, with anti-inflammatory cytokines and bystander cells thought to play a role. Activation of Treg cells appears to be antigen specific (96) however the question of whether *in vivo* suppression is antigen specific is currently unclear (100).

1.4 Autoimmune disease

Loss of immune tolerance to self, environmental or commensal antigens can initiate inappropriate immune responses causing autoimmune disease in the host. These responses can be specifically directed at particular organs in conditions such as multiple sclerosis and

type-1 diabetes. Inflammatory responses can also be systemic, targeting multiple organs as is the case in systemic lupus erythematosus and rheumatoid arthritis. In addition disorders such as Crohn's disease and colitis are mediated in part through loss of tolerance to commensal bacteria in the gut.

In recent times Th17 cells have been implicated as important drivers of inflammation in such settings (101, 102) with intensive research focussed on the development of therapeutic interventions focussing on this area (102). Two autoimmune disorders, the pathogenesis of which are considered to be highly dependent on IL-17A and are the focus of the studies described in this thesis, are discussed in more detail below.

1.4.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that causes significant disability through impaired motor, cognitive and sensory function (103). The disease commonly manifests itself in early adulthood (ages 20-40) with an average survival of up to 30 years post diagnosis (104). A combination of genetic and environmental factors as well as possible microbial infection are thought to contribute to the pathogenesis of the condition, however the specific aetiology remains undefined (105).

In MS patients lesions are formed in the CNS through immune mediated attack of neurons, resulting in the destruction of the myelin sheath which encases the axon of the neuron. Myelin acts as an insulatory material serving to potentiate neurotransmission along the axons and is indispensible for efficient neurotransmission. In the early stages of autoimmune inflammation in MS it is possible for remyelination to occur through the action of

oligodendrocytes. However, sustained demyelination leads to ineffecient repair, the formation of scars on the neuron and sustained loss of function resulting in neuro impairment in the patient (103, 104). The majority of MS patients suffer a disease phenotype characterised by long periods of disease remission followed by periods of exacerbation, known as relapsing remitting MS. Relapse periods are associated with acute inflammatory episodes which result in severely impaired neurological function. In between relapses it is possible for patients to experience some recovery, however sustained bouts of relapse invariably results in the development of a more progressive disease, termed secondary progressive MS, in the majority of cases. This disease phase is associated with increasing loss of neurological function often leading to paralysis and other associated complications including blindness and impaired respiratory function (106).

MS is considered as classic T cell mediated autoimmune disease with self reactive Th1 and Th17 cells initiating immune responses to myelin derived antigens (107, 108). Numerous studies have identified IL-17A and IFN-γ as central mediators of disease, orchestrating the recruitment of phagocytic cells such as macrophages and neutrophils to the CNS (107, 109). These recruited macrophages exhibit increased phagocytic activity and release nitric oxide metabolites, matrix metallo-proteases (MMP) and complement components which contribute to demyelination (110, 111). Furthermore, genetic studies have identified a number of T cell associated genes including *Mhc* along with both *CD25, Il-2r* and *Il-7r* as risk alleles associated with MS, further highlighting the role for T cells in the pathogenesis of this disorder (112). Interestingly, specific susceptibility alleles at the *CD25* gene locus correlate with elevated levels of the soluble form of CD25 in patients plasma although the functional significance of these observations has not been explored (113). This is a specific question which we will begin to address in the first part of this thesis using a relevant preclinical model of MS known as experimental autoimmune encephalomyelitis (113).

1.4.1.1 Autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a demyelinating disease of the CNS which displays clinical and pathological manifestations similar to human MS allowing its use as a animal model for this disorder. EAE is a predominantly Th17 cell driven model of neuro-inflammation through which the immunological events at play in MS have largely come to be understood leading to the development of a number of therapeutics for this disease.

EAE can be induced in several strains of non human primates and rodents through immunisation with CNS extracts, myelin component peptide and synthetic peptides commonly emulsified in complete freund's adjuvant (CFA) (114). The resulting disease phentoype can take a number of forms. For example C57BL/6 mice immunised with the myelin component peptide, myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 develop a chronic progressive disease whereas SJL mice immunised with proteolipid protein (PLP) 139-151 develop a relapsing remitting form of EAE (115). Autoreactive T cells are the predominant immune cell driving the pathogenesis of EAE. These cells are capable of transversing a premeabilised blood brain barrier to the CNS where myelin specific T cells are activated by local and trafficking DCs leading to the induction and persistence of neuroinflammation (114) (Fig 1.3) .

Initially EAE was described as a predominantly Th1 dominated disease as adoptive transfer of Th1 skewed myelin Ag-specific T cells was shown to be sufficient to induce disease in susceptible mice (116). Furthermore, mice deficient in the Th1 lineage specific transcription factor T-bet were protected from EAE (117) and mice deficient in the p40 subunit of the Th1 instructive cytokine IL-12 were also shown to be resistant (118). However the precise role of

IFN-γ has proven controversial as mice deficient in IL-12p35, STAT1 or IFN-γR are more susceptible to EAE (119).

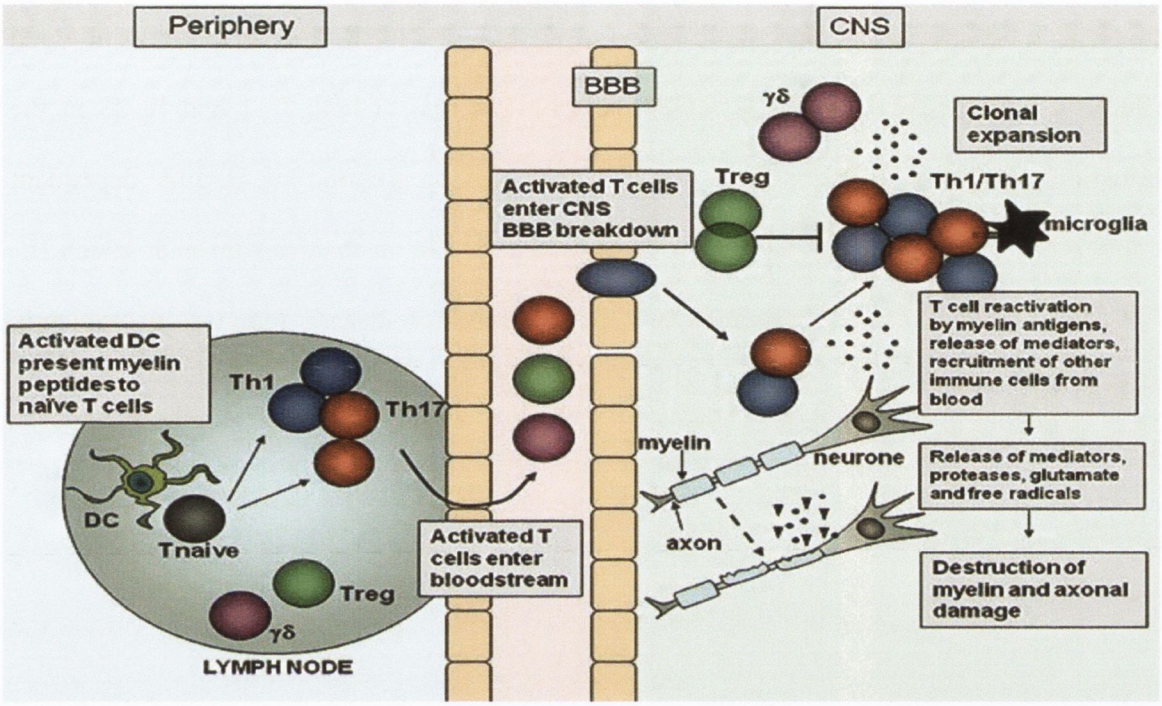


Fig 1.3 Migration and effector function of T cells in the CNS during experimental autoimmune encephalomyelitis (EAE).
Figure modified from Fletcher et.al 2010 (106).

These controversies were resolved somewhat by the report by Cua *et* al. which demonstrated that IL-23 rather than IL-12 was essential for the induction of disease through the expansion of pathogenic Th17 cells. Previous studies investigating the role of IL-12 had utilised animals deficient in IL-12p40, but these mice are also deficient in IL-23 as both IL-12 and IL-23 share the p40 subunit (119). Furthermore IL-23 deficient mice display defects in the initiation of EAE which is associated with the loss of IL-17-producing, but not IFN-γ-producing T cells (36). These observations are similar to those reported in IL-1R1$^{-/-}$ mice which display a similar protection associated with a lack of IL-17A expressing cells (120). Additionally, Th17

cells were found to induce significantly greater encephalitogenic responses on a cell-to-cell basis when compared with Th1 cells in the same experiment (36). EAE is considerably suppressed in mice deficient in IL-17A or its receptor and neutralisation of IL-17A attenuates the observed disease phenotype which is indicative of a role for IL-17A in the induction and effector phases of EAE (121, 122). This indispensible role of both IL-1 and IL-23 in the induction of EAE highlights their importance in the progression of IL-17A dependant autoimmune inflammation in the CNS. However the precise mechanisms through which IL-17A participates in the development and pathogenesis of disease remains incompletely understood.

1.4.2 Psoriasis

Psoriasis is a common chronic immune driven disease of the skin with incidences of between 1 and 3% in the population of western countries. Although the disease rarely proves fatal, patients suffer significant physical discomfort from the appearance of inflamed psoriatic skin lesions which can also be associated with an added psychological burden. The pathogenesis of psoriasis involves a complex interplay of immunological, genetic and environmental factors all of which can contribute in varying degrees to the specific disease phenotype in humans (123, 124). The condition is commonly characterised by rapid and dysregulated keratinocyte proliferation leading to a thickened epidermis (acantosis). This aberrant keratinocyte behaviour often leads to significant retention of nucleated cells within the granular layer (parakeratosis) and a very apparent erythema (redness) due to increasingly dilated blood vessels in the dermis. These manifestations occur in conjunction with a marked

immune cell infiltrate composed primarily of dense concentrations of T cells along with dendritic cells, neutrophils and macrophages (125).

Classically, psoriasis has come to be viewed as a T cell driven autoimmune disorder due at least in part to its responsiveness to cyclosporine treatment (126) and also as Th1 cells, expressing IFN-γ, were seen as the key drivers of disease pathogenesis (127, 128). However, more recently a deeper characterisation of both psoriatic lesional infiltrates and the peripheral blood of patients have uncovered a prominent role for inflammatory cells driven by the IL-23/IL-17 signalling axis as central to disease progression (129, 130). In the setting of psoriatic inflammation IL-17A and its family members can act directly on keratinocytes to stimulate the production of a number of molecules known to be elevated in psoriatic lesions including cytokines, β-defensins, antimicrobial peptides and a selection of chemokines including IL-8, CCL20, CCL2 which act to attract monocytes, neutrophils and macrophages (131, 132).

Critical cells in the context of psoriatic inflammation include subsets of innate γδ T cells and lymphoid cells which express abundant amounts of proinflammatory IL-17A, IL-17F and IL-22 at key skin barrier sites (Fig 1.3) (54, 72). The importance of such cells, along with $CD4^+$ Th17 cells which express a similar cytokine repertoire, in psoriasis pathogenesis, has underscored the development of a range of monoclonal antibodies targeting the IL-23/IL-17 signalling axis and, more specifically, IL-17 receptor A (Brodalumab) and one of its ligands, IL-17A (Ixekizumab) (133, 134). These targeted therapies have met with considerable early success in the clinic and will most likely form the next range of front line therapies for psoriasis treatment. However, despite these exciting advances in drug development, significant gaps remain in our understanding of the regulatory mechanisms which underpin the IL-23/IL-17 signalling axis and the cellular and molecular interactions at play in the context of dermal inflammation in psoriasis.

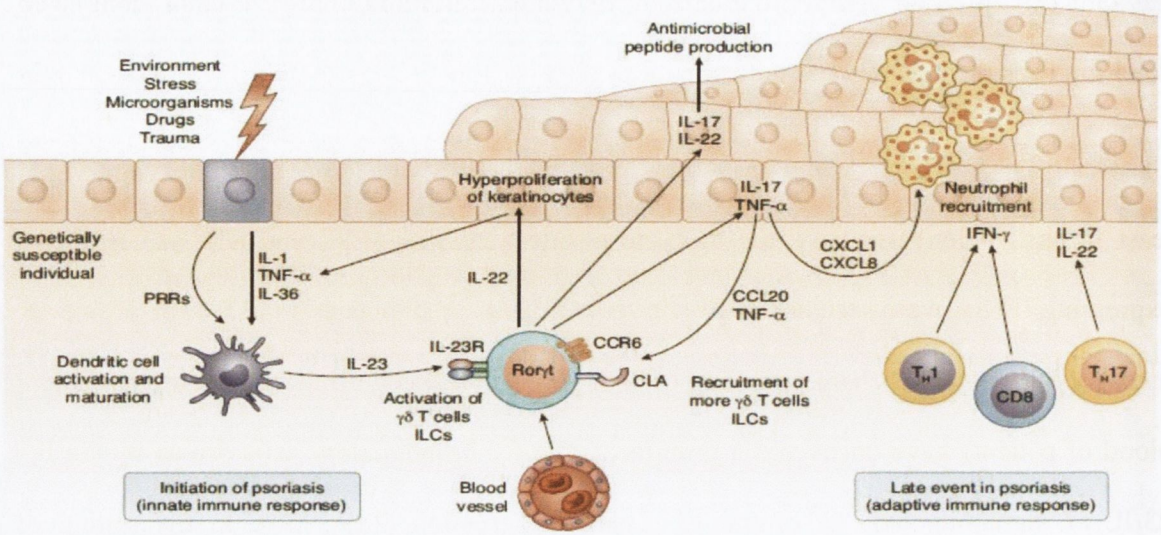


Figure 1.3 Initiation of psoriasis by γδ T cells.
In certain genetically susceptible individuals environmental stresses, immune insult or trauma to the skin can lead to the release of a variety of danger signal whichactivate skin resident antigen presenting cells. As a consequenc of this activation innate immune cells including γδ T cells traffick to the dermal and epidermal layers. In response to the local pro inflammatory signals such as IL-1 and IL-23 thes cells express several pro inflammatory mediators including IL-17A and IL-22 which recruit neutrophils act on the epidermal laters to promote dysregualted proliferation of keratinocytes respectively. This heightened inflammatory environment results in widespresd tissue necrosis which in conjunction with currently poorly defined skin auto antigens leads to the initiation of adaptive immune responses and the persistence of immune mediated inflammation in thses tissues. Figure modified from Becher et.al 2012 (135)

1.4.3 Animal models of Psoriasis

For many years research efforts into the cellular and molecular mechanisms at play in settings of psoriasis were greatly restricted by the lack of a faithful animal model of disease. Early studies have formed much of the understanding of the mechanisms at play in psoriatic inflammation however the model systems utilised proved technically challenging and expensive.

Initial studies focussed on a model utilising mice homozygous for the severe combined immunodeficiency (SCID) mutation which are severely deficient in functional B and T lymphocytes. The mutation appears to impair the recombination of antigen receptor genes

and thereby causes an arrest in the early development of B and T lineage-committed cells; other hematopoietic cell types appear to develop and function normally (136). Human skin was xenografted onto these immune compromised mice with activated PBMCs injected in the presence of absence or superantigen in order to induce psoriatic like symptoms on the grafts (137, 138). This model proved an imperfect system as SCID mice still possess a functional repertoire of natural killer (NK) cells which play important roles in transplant rejection. The activity of both IFN I and IFN II receptors have been shown to be essential for NK cell cytolitic activity *in vitro* and *in vivo* (139), so in an effort to improve the model system mice which are deficient in IFN I and IFN II receptors as well as T and B cells (Rag 2 $^{-/-}$) were generated. Upon grafting of human prepsoriatic skin, these mice display a spontaneous psoriasis phenotype which is mediated by TNFα and proliferation of resident T cells (140). Whilst these model systems have proven valuable in elucidating certain mechanisms at play the technical challenges faced through utilising such systems are compounded by the lack of success in translating these findings into effective antipsoriatic therapies (135, 141).

The requirement for a rapid, cheap and reproducible murine model of psoriasis was met with the development of a system where psoriasiform inflammation was induced through topical application of the TLR7 agonist imiquimod. This model, which is IL-23 dependant, involves daily topical application of imiquimod formulated in a commercially available cream, Aldara™ (142). Aldara™ has been used for some time in the treatment of a variety of dermal malignancies such as actinic keratoses and superficial basal cell carcinomas (143, 144); however a side effect of such treatment is the exacerbation of psoriatic lesions in some patients, even those with well controlled psoriasis (145, 146). Based on this observation, utilisation of Aldara™ in murine studies as an inducer of psoriasiform inflammation has come to be accepted as a clinically faithful model of psoriasis, particularly as the pathological manifestations induced bear striking similarities to human psoriasis, but also because it is

responsive to a range of the current frontline psoriasis therapies (54, 72, 142). Another widely utilised model of psoriasis in mice involves intradermal injection of IL-23 over a number of days resulting in the development of similar immunopathology to that observed in the Aldara™ model and human psoriasis. However this model fails to reproduce certain characteristics of human disease such as infiltration of $CD8^+$ T cells to the inflamed epidermis. This model which is CCR6 dependant has provided useful insight into a number of the components of human disease (147, 148).

1.5 Regulation of type 17 responses

Since the associations were made between the dysregulated activity of IL-17A and the development of autoimmune disease, intensive research has focussed on elucidating the mechanisms through which these responses are regulated. A number of different signalling mediators and pathways have been described to both positively and negatively regulate the generation of Th17 type responses. For example, IL-10 is a classic immunosuppressive cytokine which has long been described as in inhibitor of both Th1 and Th2 responses (149). Recently it has also become clear that IL-10 regulates Th17 activity in settings of autoimmunity with mice deficient in IL-10 developing enhanced Th17 responses through increased IL-6 and IL-23 expression. Furthermore, regulatory T cells expressing IL-10 have been shown as suppressive in autoimmune disease (150, 151). Interestingly, the IL-6 related cytokine IL-27 has also been described as a negative regulator of Th17 cells in settings of autoimmunity with IL-27 receptor alpha deficient mice developing a more severe neuro-inflammatory phenotype in EAE through local increases in Th17 activity (152). The activities of IL-17A can also be regulated through other members of the IL-17 family. The Th2 derived cytokine IL-17E suppresses Th17 cell activity in EAE, where mice deficient in this cytokine

develop enhanced disease which is associated with elevated levels of IL-23 in the periphery and increased numbers of T cells producing IL-17A, TNF-α and IFNγ in the central nervous system (153).

As outlined above, the roles of IL-6, TGFβ, IL-23 and IL-1β, in the generation of Th17 responses are firmly established. This study will focus on the roles of two more recently defined pathways which regulate the development of type 17 resonses. The IL-2/IL-2R signalling axis, which actively inhibits Th17 development in a STAT5 dependent manner (154), and the activation of TIR8/SIGIRR, an endogenous negative regulator of TLR/IL-1R family members and of Th17 responses (155). These pathways are discussed in more detail below.

1.5.1 Interleukin 2:

Interleukin 2 (IL-2), a member of the common gamma chain cytokine family (γc), is a 4-bundle α-helical protein of 15 kDa predominately produced by activated $CD4^+$ and $CD8^+$ T cells. Initially upon its discovery IL-2 was thought of predominantly as a pro-inflammatory cytokine due to its ability to enhance the proliferation, differentiation and survival of already activated T cells (156). However with the generation of the IL-2 and CD25 deficient mice and the uncontrolled lympho proliferation and fatal autoimmunity observed in these mice, IL-2's primary non-redundant role in maintenance of T-cell tolerance came to be appreciated (157).

IL-2 signals through its receptor, a heterotrimeric complex consisting of the α chain (CD25), β chain (CD122) and the γ chain (CD132) (158). Upon engagement of IL-2 with the ligand

specific α chain, a high affinity quaternary complex is formed which mediates IL-2R downstream signalling (158).

The IL-2 /IL-2R complex induces downstream signalling through IL-2Rβ and γc due to their association with the tyrosine kinases Janus kinase 1 (JAK1) and JAK3 leading to phosphorylation of the JAKs as well as the three key tyrosine residues within the cytoplasmic tail of IL-2Rβ. These downstream signalling events lead to activation of three main intracellular signalling pathways mediated by mitogen-activated protein kinase (MAPK) , signal transducer and activator of transcription (STAT) and phosphoinositide 3-kinase (PI3K) activation (159).

STAT5, a transcription factor activated by IL-2R signalling is established as essential for cell cycle progression (160), and mice displaying constitutively active STAT5 possess expanded populations of memory $CD8^+$ T cells and also Treg cells (161). STAT5 activation through IL-2R signalling has also been shown to directly drive FOXP3 expression in peripheral naïve $CD4^+$ T cells. (162).

IL-2R signalling also plays a key role in Th17 cell generation where the absence of IL-2R signalling, or disruption of the signalling pathways through deletion of STAT5, leads to enhancement of Th17 cell development *in vitro* and *in vivo* (154). STAT5 and STAT3, activated by IL-6 receptor signalling, have recently been shown to compete for binding to the *IL-17A* locus at the same position, indicating that the level of Th17 development is in fact a balancing act between the relative levels of IL-2R and IL-6R signalling i.e. maximal Th17 development can be achieved with minimal IL-6R signalling provided IL-2 is neutralised and vice versa (163). The role of IL-2 in the development of Th17 cells was further expanded with reports that Treg cells could enhance the early generation of Th17 responses through their ability to regulate the bioavailability of IL-2. This consumption of IL-2 served to protect

the developing Th17 cells from the inhibitory effects exhibited by IL-2 (164, 165). These observations are underscored by the strong genetic linkage between mutations at both the *IL-2* and *IL2RA/CD25* gene loci and several T cell mediated autoimmune diseases in humans (113). However, at this time, the functional mechanisms of how such mutations confer susceptibility to autoimmune disease are not yet understood.

1.5.1.1 Soluble IL-2 Receptor Alpha (sCD25)

Whilst the α chain of the IL-2 receptor is commonly thought of as a low affinity receptor for IL-2, it noteworthy that this description is a consequence of its binding affinity relative to that of the IL-2 receptor complex as a whole. It has been demonstrated that whilst IL-2Rα does not display any signal transduction capabilities, its binding to IL-2 is absolutely required as an initial step for subsequent efficient formation of the quaternary IL-2 receptor complex and optimum signalling (158, 166).

A soluble form of the IL-2Rα chain, (sCD25), is generated through proteolytic cleavage at the surface of activated T cells with the level of shedding correlating with the extent of T cell activation (167). A number of other soluble cytokine receptors have been identified as both having antagonistic properties, IL-6R (168) or agonistic properties, IL-1RI (169) but the role of sCD25 remains controversial.

Interestingly, elevated serum levels of sCD25 correlate with a number of specific *CD25* alleles associated with autoimmune susceptibility (113, 170). Despite such observations, suggesting a role for sCD25 in disease pathogenesis, its precise role in directing pathogenic T cell subsets, if any, is currently unclear.

Elevated levels of sCD25 have been correlated with poor prognosis from patients presenting with follicular B-cell non Hodgkins lymphoma. In these patients, enhancement of IL-2 signalling caused by its interaction with sCD25, promoted differentiation towards Treg cells rather than Th17 or Th1 cells (171), this agonistic observation contrasts with reports that elevated levels of sCD25 can inhibit IL-2R signalling in patients presenting with multiple sclerosis (113). Given the clear association between sCD25 levels and T cell mediated inflammatory disease we were interested in investigating whether sCD25 plays any functional role in modulating proinflammatory T cell responses.

1.5.2 Influence of the Toll/Interleukin-1 (IL-1) Superfamily on type 17 responses:

The Toll/IL-1 super family is a large family of cell receptors which are recognised as key players in bridging the gap between innate and adaptive immunity. Toll like receptors (TLR)s are highly conserved PRRs and are established as important drivers of the initial innate response, the manner of which greatly influences the nature of the subsequent adaptive response (172). They are expressed across a wide variety of immune cells including macrophages and certain T and B cells. In contrast, IL-1 family related receptors are more ubiquitously expressed and the family boasts both pro and anti-inflammatory mediators that are capable of controlling inflammation at both the receptor and the nuclear level. They function mainly through recognition of soluble cytokines which can act in both a paracrine and autocrine fashion (173).

Structural homology between TLR and IL-1 family receptors includes the sharing of an intracellular Toll/IL-1R signalling domain (TIR). TIR domain adaptor proteins such as

myeloid differentiating factor 88 (MYD88), amongst others, contribute to a signalling cascade that activates a number of downstream signalling pathways including NF-κB. TLRs express extra cellular domains which have leucine rich repeats, in contrast IL-1 family receptors express extra cellular domains which possess immunoglobulin (Ig) folds (172).

A number of Toll/IL-1R family members have been implicated as key players in the progression of Th17 driven autoimmune disease. IL-1β signalling through IL-1 receptor 1 (IL-1R1) has been demonstrated as capable of enhancing RORγt expression and early differentiation of Th17 cells and is crucial for driving pathogenic T cell responses *in vivo* (120, 174). Recently an appreciation for the role TLR signalling plays in the direct modulation of adaptive T cell responses has come to be appreciated (175), with such observations now extending to the regulation of Th17 responses. Stimulation of Th17 cells with the synthetic TLR2 ligand Pam3Cys-Ser-(Lys)4-trihydrochloride (Pam3Cys) led to enhanced Th17 cytokine expression *in vitro* where TLR2 deficient mice display ameliorated EAE compared to wild type mice (176).

Whilst a number of Toll/IL-1R family members have demonstrated an ability to enhance the progression of Th17 driven autoimmunity there are also members which play a regulatory role in these settings. One such receptor is single immunoglobulin IL-1 receptor related molecule (TIR8/SIGIRR). TIR8/SIGIRR is established as a negative regulator of both IL-R1 signalling and TLR signalling (177) with TIR8 deficient mice more susceptible to lethal endotoxin challenge and enhanced *in vivo* inflammation in response to IL-1β injection (178) .

1.5.2.1 Single Immunoglobulin IL-1 Receptor Related (TIR8/ SIGIRR)

(TIR8/ SIGIRR) is a distinctive member of the IL-1R family which also plays a regulatory role in settings of inflammation dependant on TLR and IL-1R signalling. (179). The *Tir8/Sigirr* transcript encodes a protein of 410 amino acids in humans and 409 amino acids in mice, however interestingly the real size of the protein is much greater and varies among tissues due to differing glycosylation patterns. TIR8/SIGIRR is as an orphan receptor within the Toll/IL-1R superfamily possessing only a single extracellular Ig like domain which, due to its small size of 118 amino acids, is thought to make binding of an extracellular ligand impossible (Fig 1.4). Furthermore, the intracellular TIR domain of TIR8/ SIGIRR is currently seen as incompatible with conventional signalling activities due to two crucial amino acid substitutions, Ser 447 and Tyr536 replaced with Cys222 and Leu205. However the lack of known ligands mean the implications of this observation, if any, are currently unknown. Expression of TIR8/SIGIRR is highly detectable in the epithelium of the gut and kidney as well as in the lung and among T cell populations with low or undetectable levels present in fibroblast or endothelial cell lines and B cells (178, 180).

A number of studies have identified TIR8/SIGIRR a negative regulator of IL-1R1 signalling (178, 181). Furthermore roles for TIR8/SIGIRR as a negative regulator of IL-18, ST-2, TLR4 and TLR9 signalling have also been demonstrated (178, 182). Mechanistically, TIR8/SIGIRR is thought to inhibit Toll/IL-1R1 signalling through its ability to interfere with efficient heterodimerisation between IL-1R1 and its associated receptor accessory protein. TIR8/SIGIRR also exerts its inhibitory effects through competitive recruitment of TIR domain adaptor proteins including MYD88, IRAK and TRAF6 (Fig 1.5). It is interesting to note that the extracellular Ig domain is dispensable for inhibition of TLR4 signalling but both domains appear to be required for inhibition of IL-1R1 signalling (183). In contrast ST2, the ILR

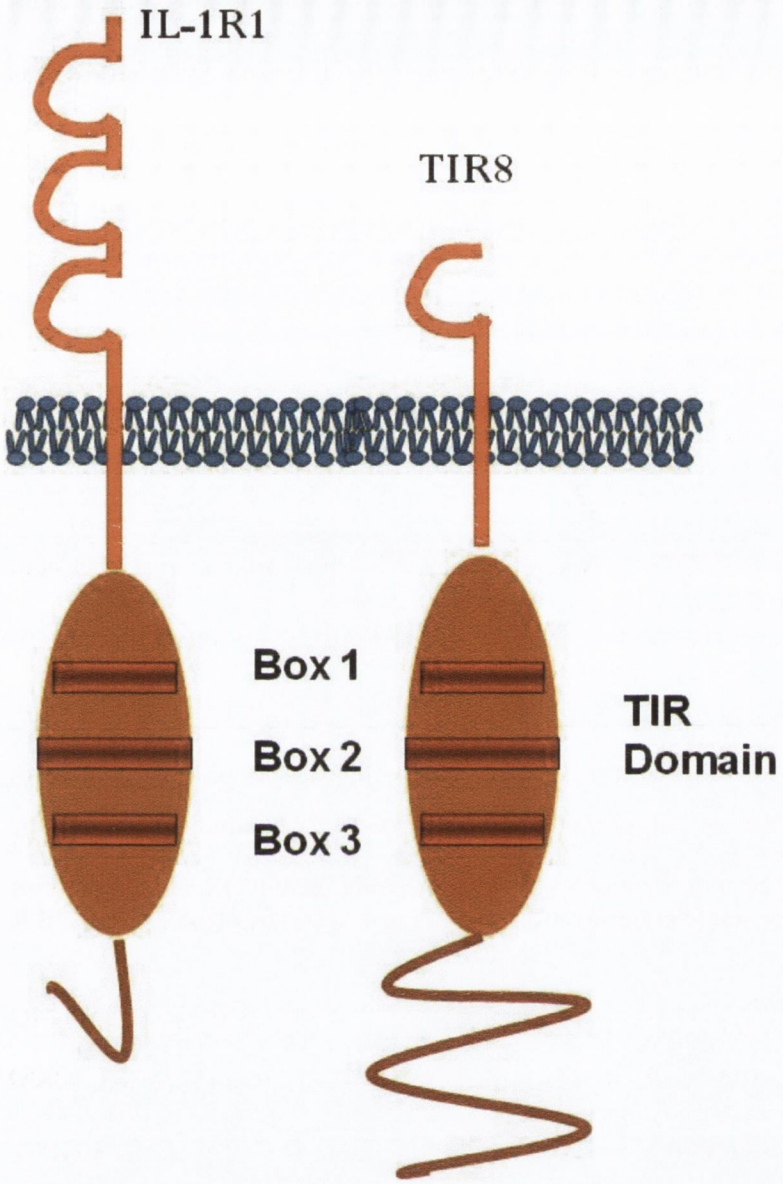


Figure 1.4 Structure of TIR8/SIGIRR
Structuarl schematic of TIR8/SIGIRR demonstrating the single extracellular Ig domain and long C-terminal domain

family member receptor for IL-33 interacts with both the TIR and extracellular domains but it appears that it is the TIR domain which is crucial for TIR8/SIGIRR activity in this setting (182). Despite these observations the mechanisms through which TIR8/SIGIRR exerts its regulatory activities have yet to be clearly defined. It is possible that activity occurs in a highly tissue specific manner as a consequence of the differential glycosylation patterns

observed. However succinct observations relating to such mechanisms have not been investigated. In spite of these issues a number of studies have characterised TIR8/SIGIRR as an important non redundant negative regulator in settings of inflammation and autoimmunity(177, 184).

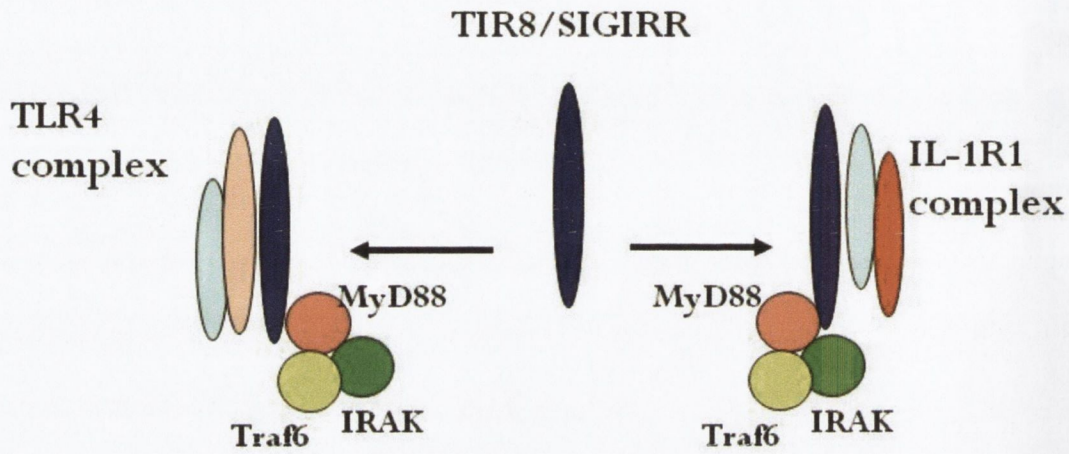


Figure 1.5 Proposed mechanism of suppression of TLR and ILR family response by TIR8/SIGIRR
TIR8/SIGIRR is speculated to act as a decoy receptor to competetively recruit TIR domain proteins such as MyD88 and IRAK family members away from signalling complexes of other TOLL/ILR family members.

1.5.2.2 Role of TIR8/SIGIRR in infection

Tir8/Sigirr deficient mice display enhanced susceptibility to LPS induced septic shock (178) and also succumb more readily to *Mycobacterium tuberculosis* infection in the lung (185). Furthermore these mice also display enhanced susceptibility to *Candida albicans* and *Aspergillus fumigates* infection (155). These fungal infections occur in association with enhanced IL-1 signalling and elevated Th17 responses. Despite the elevated inflammatory response in such settings of infection, *Tir8/Sigirr* $^{-/-}$ mice display an impaired ability to clear the pathogen of interest and eventually succumb to overwhelming systemic inflammation.

Tir8/Sigirr $^{-/-}$ mice display a similar enhanced susceptibility to *P.aeruginosa* lung infection, a phenotype which is rescued when these mice are crossed with IL-1R1 -/- mice, *Tir8*$^{-/-}$ *IL-1RI* $^{-/-}$ double knockout mice were observed to have higher survival rates, enhanced bacterial clearance, and reduced levels of local and systemic cytokine and chemokine expression compared to *Tir/Sigirr*$^{-/-}$ *mice* (186). Moreover, *Tir8/Sigirr*$^{-/-}$ mice exhibit dysregulated signalling in the gut epithelium in response to commensal bacteria in inflammatory settings as a result of *Salmonella typhimurium* colonisation (187), leading to disruption of gut epithelium homeostasis and hypersensitivity in these mice to dextran sodium sulphate (DSS) induced colitis (188). These studies clearly identify TIR8/SIGIRR as a signalling mediator of critical importance in the modulation of inflammation and tissue pathology associated with the signalling of members of the Toll/IL-1R family in response to exogenous and endogenous micro-organisms. Furthermore, the nature of this regulatory activity also suggests TIR8/SIGIRR as a potential regulator of autoantigen driven inflammation which is dependent on IL-1 and IL-17A.

1.5.2.3 Role of TIR8/SIGIRR in autoimmunity

Uncontrolled or dysregulated signalling by IL-1R family members can lead to the development of autoimmune diease (173). In particular IL-1 has been identified as a key cytokine involved in the development of Th17 cells and associated autoimmune inflammation (174). TIR8/SIGIRR, whose expression is induced upon the initiation of the Th17 development program *in vitro,* has been shown to suppress IL-1 driven proliferation and proinflammatory cytokine secretion in these cells. This occurs in association with enhanced IL-1-induced phosphorylation of JNK and mammalian target of rapamycin

(mTOR) kinase in *Tir8/Sigirr*$^{-/-}$ Th17 cells compared to wild-type controls. Mice deficient in *Tir8/Sigirr* also display exacerbated EAE with enhanced CNS pathology and infiltration of proinflammatory T cells (181). Increasing evidence also suggests a role for Toll/IL-1R family members in the pathogenisis of rheumatoid arthritis (RA) (189, 190), with recent studies identifying TIR8/SIGIRR as a potential therapeutic target in such disorders. A study by Drexler et. al. has demonstrated that *Tir8/Sigir*$^{-/-}$mice display enhanced disease in both the collagen antibody and zymosan induced models of arthritis. There are likely dual roles for TIR8/SIGIRR in the observed phenotype as *Tir8/Sigirr*$^{-/-}$ mice showed a clear reduction in disease severity when treated with IL-1Ra however significant inflammation was still clearly evident, likely due at least in part to dysregulated TLR induced inflammation in the absence of TIR8/SIGRR. Importantly, TIR8/SIGIRR overexpression in human primary myeloid cells inhibited TLR-induced cytokine production, whilst *Tir8/Sigirr* knockdown resulted in increased cytokine production following TLR stimulation. Moreover, SIGIRR overexpression inhibited the spontaneous release of cytokines by human RA synovial cells (191) .

Data implicating TIR8/SIGIRR in the pathogenesis of human disease is limited. However the observations from the murine RA studies are broadly in line with a psoriatic arthritis (PsA) study identifying *Tir8/Sigirr* as significantly downregulated amongst a cohort of genes involved in the inhibition of innate and acquired immune responses, suggesting that its reduced expression in PsA could also be influencing disease susceptibility or severity (192).

Based on these observations we were interested to assess a direct role for TIR8/SIGIRR in the regulation of dermal inflammation in psoriasis and more specifically investigate its potential role in the regulation of innate IL-17A and proinflammatory cell subsets in such settings

1.6 Aims

The overall aim of this study was to investigate novel mediators of type 17 responses and the mechanisms of action whereby sCD25 and TIR8/SIGIRR regulate the expression of IL-17A from $CD4^+$ and innate T cells respectively. Furthermore we will investigate the impact of such regulatory activity on the loss of self tolerance in autoimmune disease. Specific aims are as follows:

- To assess the role of sCD25 in the progression of autoimmune disease and provide novel insights on its impact on both pathogenic and regulatory T cell subsets *in vivo*.

- To investigate the impact of sCD25 on Th17 differentiation, the lineage specific characteristics of Th17 cells and the pathways implicated in their regulation.

- To investigate the role TIR8 plays in the regulation of IL-1R1 signalling and associated IL-17A expression in γδ T cells and other innate lymphoid subsets.

- To investigate a possible role for TIR8/SIGIRR in the regulation of innate IL-17A expression in settings of psoriatic inflammation and to define and characterise the cell types subject to this regulatory activity.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Ammonium chloride lysis solution

0.87% Ammonium chloride (NH_4Cl)

Dissolved in 100 ml ddH_2O and filter sterilised

2.1.2 Cell culture medium

Roswell Park Memorial Institute-1640 medium (RPMI) or Dulbecco's Modified Eagle Medium (DMEM) (both from Sigma) were supplemented with 10% heat inactivated foetal calf serum (FCS; Biosera), 100 mM L-Glutamine (Gibco) and 100 μg/ml penicillin/streptomycin (Biowest). cRPMI supplemented with β-mercaptoethanol (2-ME 50 μM) was used to culture T cells, splenocytes and lymph node cells *ex vivo*.

2.1.3 ELISA stopping solution

1 M H_2SO_4

2.1.4 ELISA washing buffer

1x PBS 0.5% Tween

Table 2.1 ELISA kits used in the study

Cytokine	Supplier	Top Working Standard
IL-2	eBioscience	4-500 pg/ml
IL-17A	eBioscience	8-1000 pg/ml
IL-1β	eBioscience	8-1000 pg/ml
IL-23	eBioscience	8-1000 pg/ml
IFN-γ	eBioscience	15-2000 pg/ml
IL-17 F	eBioscience	15-2000 pg/ml
IL-17A/F	eBioscience	15-2000 pg/ml

2.1.5 MACS buffer

2% FCS

2 mM EDTA

Made up in sterile PBS

2.1.6 Phosphate-buffered saline (PBS) 20X

800 g NaCl

92 g Na_2HPO_4

20 g KH_2PO_4

20 g KCL

Dissolved in 5L of dH20, pH 7 5

2.1.7 Formalin Solution (10%, unbuffered):

10 ml Formaldehyde (37-40%)

90 ml Distilled water

2.1.8 Isotonic percoll solution

Isotonic percoll solution was prepared by diluting Percoll reagent (GE Healthcare) 9:1 with sterile 10X PBS (Sigma-Aldrich). Isotonic percoll solution prepared this way had density of 1.123 g/ml. 40% and 70% isotonic percoll solution (made in sterile PBS) was used for isolation of mononuclear cells from brain tissue.

Table 2.2 Recombinant cytokines used in these studies

Cytokine	Supplier	Conc. *In vitro*	Dose *In vivo*
TGF-β	Miltenyi Biotech	5 ng/ml	N/A
IL-6	eBioscience	10 ng/ml	N/A
IL-1β	eBioscience	10 ng/ml	100 ng/mouse
IL-23	eBioscience	10 - 50 ng/ml	100 ng/mouse
IL-2	Miltenyi-Biotech	200 U/ml	N/A
sCD25	SINO Biological	1 µg/ml-20 µg/ml	25 µg/dose

Table 2.3 TLR ligands used in the study

TLR Ligand	Supplier	Conc. *In vitro*	Dose *In vivo*
LPS	Invivogen	N/A	500ng
CpG9	Sigma-Genosys	N/A	10µg
Imiquimod	Invivogen	2 µg/ml	N/A

Table 2.4 Inhibitors used in the study

Inhibitor	Supplier	Conc. *In vitro*
SP600125	Sigma	2μM - 10μM
Temsirolimus	Sigma	0.4nM – 2 nM

Table 2.6 Antibodies used in cell cultures and *in vivo*

Antibody	Supplier	Conc. *In vitro*	Dose *In vivo*
Anti IFN-γ	BioXcell	10 μg/ml	N/A
Anti IL-2	eBioscience	10 μg/ml	N/A
Anti IL-17A	BioXcell	N/A	100 ng/mouse
Unspecified IgG1	BioXcell	N/A	100 ng/mouse
Anti-γδ TCR	BioXcell	N/A	125-250 μg

Table 2.7 Antibodies used for FACS and flow cytometric analysis

Antibody	Flurochrome	Clone	Supplier
CD4	PE	GK1.5	eBioscience
CD4	APC	RM4-5	eBioscience
CD4	PE-CY7	GK1.5	eBioscience
CD3	FITC	145-2C11	eBioscience
CD3	PE	145-2C11	eBioscience
CD3	APC	145-2C11	eBioscience
CD25	PE	PC61.6	eBioscience
CCR6	Alexa F647	236A/E7	BD
pStat3	PE	pY705	BD

pStat5	PE	pY694	BD
RORγT	PE	AFKJS-9	eBioscience
FOXP3	APC	FJK-16S	eBioscience
CD45	e450	30-F11	eBioscience
γδ TCR	APC	eBio GL3	eBioscience
γδ TCR	FITC	eBio GL3	eBioscience
Vγ4	PE	UC3-10A6	Biolegend
IL-17A	PE	eBio 17B7	eBioscience
IL-17A	FITC	TC1118H-10.1	Biolegend
IL-17A	APC	eBio 17B7	eBioscience
IFN-γ	APC	XMG1.2	eBioscience
IL-2	PE	JES6-5H4	eBioscience
IL-22	APC	IL-22JOP	eBioscience
BRDU	FITC	B44	BD

Table 2.8 Other reagents used in FACS and flow cytometric analysis

Chemical	Description	Supplier	Concentration
CFSE	Cell membrane dye (FL-1)	Invitrogen	2.5 μg/ml
7-AAD	Viability dye (FL-3)	eBioscience	1:100
PMA	Mitogen	Sigma	10 ng/ml
Ionomycin	Ionophore	Sigma	1 μg/ml
Brefeldin-A	Golgi inhibitor	Sigma	5 μg/ml

Table 2.9 Primers used in quantitative real time PCR

Gene	Protein Product	Code	Supplier
18S rRNA	18S rRNA	4319413E	ABI
Il-7A	IL-17A	Mm00439619_m1	ABI
Il-23	IL-23	Mm00518984_m1	ABI
Il-1β	IL-1β	Mm00434228_m1	ABI
Il-22	IL-22	Mm00444241_m1	ABI

2.1.9 Animals

Wild type C57BL/6 mice were purchased from Harlan UK. *Tir8/Sigirr*$^{-/-}$ mice on a C57BL/6 background were generated as previously described (193). IL-17A-eGFP transgenic reporter mice were provided by Prof. P. Fallon (TCD). All mice were housed under specific pathogen free conditions at Institute for Molecular Medicine, St. James Hospital Dublin. All animal experiments were performed in compliance with Irish Department of Health regulations (license number B100/4272) and approved by the institutional ethical review board. Mice were age matched for every experiment and were 12-20 weeks old at the initiation of the experiments.

2.2 Methods

2.2.1 Cell counting

Cell counts were performed by diluting cells (routinely 1/10 dilution) with trypan blue (Sigma). A 10 µl volume of the cell suspension was then loaded onto a disposable hemocytometer (Hycor Biomedical, UK). The number of viable cells (appearing as white in contrast to dead cells, which stain blue) was counted using a light microscope. The number of cells per ml, was calculated using the following formula: number of cells per ml = cell number x 10^4 x dilution factor.

2.2.2 CFSE labelling of cells

Carboxyfluorescein succinimidyl ester (CFSE) is a lipid-based fluorescent dye that binds to cell membrane without affecting cell function and is used to assess cell proliferation. When cells divide, CFSE labelling is distributed equally between the daughter cells, which therefore have half of the fluorescence of the parent cell. Proliferation can be monitored by flow cytometry (FL-1) and is seen as a decrease in fluorescence intensity. A single cell suspension was prepared from spleens of naïve mice. Red blood cells were lysed with 1 ml of heated 0.87% ammonium chloride for 2 min, washed and pelleted by centrifugation at 1200 rpm for 5 min. Cells were resuspended in 5 ml of warm PBS and added to previously prepared 5 ml of 5 µg/ml CFSE in PBS. Cells were incubated with the dye for 2.5 min (final working concentration 2.5 µg/ml), constantly mixed by inverting the tube. At the end of the incubation time 3 ml of FCS was added to quench residual dye and cells were centrifuged at 1200 rpm for 5 min. Cells were then resuspended in cRPMI, and counted before further use.

2.2.3 T cell isolation by FACS

Freshly isolated spleen cells from naïve mice were washed and resuspended in a maximum volume of 1 ml of RPMI. Cells were surface stained with fluorescent antibodies directed against CD3 and γδ TCR (1/100 dilution). Double positive cells were purified by FACS with a MoFLo cell sorter (Beckman Coulter). Cellular purity was determined by flow cytometry and was consistently > 98%

2.2.4 γδ T cell isolation by MACS

γδ T cell were purified from pooled inguinal, auxiliary, brachial, cervical and mesenteric lymph node cells by sequential MACS sorting using TCRγδ T cell isolation kit (Miltenyi Biotec) as follows: first non-T cells were directly labelled with MicroBeads conjugated to monoclonal antibodies against mouse CD45R (B220; isotype: rat IgG2a), and CD11b (Mac-1 α-chain; isotype: rat IgG2b) while γδ T cells were labelled with biotin-conjugated monoclonal antibody against mouse TCRγδ. Non-T cells were depleted on an autoMACS machine using the "depl05" program. γδ T cells in remaining T cell enriched fraction were indirectly labelled with Anti-Biotin MicroBeads and purified by positive selection on an autoMACS machine using the "posseld2" program. Cellular purity of separated populations was determined by flow cytometric analysis and was routinely >90%. γδ T cells were seeded at 0.5 x 10^6 cells/ml and stimulated as required.

2.2.5 In vitro differentiation and stimulations

For all $CD4^+$ T cell stimulations cells were activated in the presence of plate bound anti-CD3 5μg/ml and anti CD28 5μg/ml. For Th17 differentiation cells were cultured in the presence of TGF-β (5 ng/ml), IL-6 (10 ng/ml), anti-IFN-γ (10 μg/ml) (XMG 1.2) and anti-IL-4 (10 μg/ml) (11B11). For Th1 differentiation cells were cultured with IL-12 (10 ng/ml) and anti- IL-4 (10μg/ml). iTreg cells were induced in the presence of TGF-β (5 ng/ml) and rIL-2 (10U/ml). After 72-96 hours supernatants were analysed by ELISA and cells were examined for intracellular cytokine/transcription factor expression by flow cytometry. Restimulation conditions for visualisation of intracellular cytokines are described in section 2.2.10

2.2.6 Measurement of cytokine concentration by ELISA

Cytokine concentrations were determined using Ready Set Go ELISA kits (eBioscience) according to the manufacturer guidelines. High binding microtitre plates (Greiner bio-one) were coated overnight at 4°C with 50 μl per well of capture antibody diluted in PBS. Plates were washed 5 times in ELISA wash buffer (PBS / 0.05% Tween 20) and non-specific binding sites were blocked by incubation with 150 μl of blocking buffer (eBiosciences) for 2 h at room temperature. Plates were again washed 5 times before the addition of 50 μl per well of sample supernatant or an eight point two-fold serial dilution from the top working standard recombinant protein for each cytokine diluted in ELISA assay diluent (eBiosciences). Plates were incubated overnight at 4°C. After washing, 50 μl of specific biotinylated detection antibody diluted in ELISA assay diluent was added per well and incubated at room temperature. Plates were again washed 5 times and incubated with 50 μl / well horseradish peroxidase (HRP)-conjugated streptavidin for 20 min at room temperature. Plates were washed 7 times and 50 μl of TMB substrate (eBiosciences) added to each well. The enzyme

reaction was stopped by the addition of 25 µl/well of 1 M H_2SO_4. The OD values were determined by measuring absorbance at 492 nm using a Synergy Mx microplate reader (BioTek) and cytokine concentrations for supernatant samples were calculated against the standard curve prepared using recombinant mouse cytokine provided in the Ready Set Go ELISA kit.

WT and *Tir8/Sigirr*$^{-/-}$ splenocytes or FACS sorted γδ T cells were cultured in the presence of IL-23 (10 ng/ml) and IL-23 (0.01 ng/ml - 10 ng/ml). These assays were performed in the absence of plate bound antibody stimulation for 72 hours to assess the innate responsiveness of the cells present to these stimuli before analysis of the cell culture supernatants by ELISA.

2.2.7 Induction, treatment and assessment of EAE

EAE was induced according to manufacturer's instructions using active EAE induction kit EK-0113 (Hooke Labs MA. U.S.A). Mice were injected sub-cutaneously on day 0 with equal 100µl volumes in the upper and lower back of preforumlated MOG_{35-55} for a total dose of 200µg/mouse. Pertussis toxin (200ng) was also injected on day 0 and 24 hours later Mice were monitored daily for signs of disease with disease severity scored as follows 0. Normal, 1. Limp tail, 2. Wobbly gait, 3. Severe hind limb weakness 4. Complete hind limb paralysis 5. Moribund or death. Recombinant sCD25 (25µg/mouse) was administered immediately prior to immunization and every 12 hours thereafter for the first 3 days through sub cutaneous injection close to the site of immunisation. Control mice were treated with PBS vehicle.

2.2.8 Mononuclear cell (MNC) isolation from CNS tissue

Mice were anesthetized by intra-peritoneal injection of 40 µl pentobarbital (Eutheathal) and perfused intra-cardially with 20 ml ice-cold PBS to remove peripheral blood from CNS tissue. Brain tissue was isolated, homogenised in ice-cold Hanks Balanced Salt Solution containing 3% FCS (HBSS/FCS), and passed through 100 µm cell strainer. Tissue homogenate was washed with HBSS/FCS and centrifuged at 170 g for 10 min. Cell pellet was then suspended in 2 ml of HBSS/FCS containing Collagenase D (1 mg/ml; Calbiochem) and DNase I (10 µg/ml; Calbiochem) and incubated for 45 min at 37°C with gentle shaking. Cells were then washed in HBSS/FCS and resuspended in 5 ml of 40% isotonic percoll solution. This was carefully overlayed on 5 ml of 70% isotonic percoll solution with a plastic Pasteur pipette. Percoll gradients were centrifuged at 2,000 g for 20 min at 18°C, without brake. After centrifugation MNC were found on the interface between 40% and 70% percoll solutions, myelin debris was floating on the surface of the 40% percoll. Cells were aspirated with a Pasteur pipette, washed twice with cRPMI and counted before further analysis. The yield of MNCs and $CD4^+$ T cells for a typical isolation from Brain and spinal chord is isted below. Due to low numbers of cells from spinal chord isolations individual mice were pooled before flowcytometric analysis.

Table 2.10 Yields and purities from a typical MNC isolation from CNS tissue

BRAIN	%$CD4^+$	Total Cells	Total $CD4^+$
PBS 1	2.52	$2.60x10^5$	$6.55 x10^3$
PBS 2	10.4	$1.46x10^5$	$1.52 x10^4$
PBS 3	23.7	$8.00x10^5$	$1.90 x10^5$
SCD25 1	36.7	$4.30 x10^5$	$1.58 x10^5$
SCD25 2	6.86	$3.00 x10^5$	$2.06 x10^4$
SCD25 3	19	$4.46 x10^5$	$8.47 x10^4$
SPINAL CHORD	**%$CD4^+$**	**Total Cells**	**Total $CD4^+$**
PBS 1	27.3	$4.60 x10^4$	$1.26 x10^4$
PBS 2	27.3	$1.20 x10^4$	$3.28 x10^3$
PBS 3	27.3	$8.60 x10^4$	$2.35 x10^4$

SCD25 1	34.2	4.30×10^5	1.47×10^5
SCD25 2	34.2	3.00×10^5	1.03×10^5
SCD25 3	34.2	2.73×10^5	9.34×10^4

2.2.9 Antigen specific restimulation

Single cell suspensions were prepared by homogenising spleen or inguinal lymph nodes through a 40 μm cell strainer. Cells were centrifuged at 1200 rpm for 5 min and red blood cells were lysed by incubating loose cell pellets with heated 0.87% ammonium chloride. Cells were washed with cRPMI, centrifuged at 1200 rpm for 5 min and counted using trypan blue solution. Cells were resuspended at 2×10^6 (spleens) or 1×10^6 (lymph nodes) and seeded in 200 μl in a 96-well U-bottomed plate. Premade MOG peptide solution (Hooke labs) was added to triplicate wells at final concentrations of 2-50 μg/ml. PMA at 10 ng/ml and anti-mouse CD3ε antibody at 1μg/ml were used as a positive control. Cells were incubated for 72 h at 37°C/5% CO_2 and cytokine concentration in the supernatants was quantified by ELISA.

2.2.10 Flow cytometry

Surface staining

Single cell suspensions, prepared from organs isolated from *in vivo* studies or obtained from in vitro cultures were added to a FACS tube at 1×10^6 cells in 100 μl per tube. The cells were washed in FACS buffer and centrifuged at 1200 rpm for 5 min. Cells were then incubated

with the appropriate dilution of FACS antibody directed against a surface antigen for 15 min at room temperature in the dark. In parallel, single stained compensation controls were prepared using FACS comp beads (BD). Fluorescence minus one (FMO) controls were also stained where required. Excess staining antibody was removed by washing each tube twice in FACS Buffer, 1 ml per tube, with centrifugation at 1200 rpm for 5 min between each wash. Cells were resuspended in 0.5 ml FACS buffer for the analysis. If the discrimination of live and dead cells was required 7-AAD (1/200 Miltenyi Biotech), which is a fluorescent DNA dye that only stains dead cells (as intact membranes prevent the ingress of the dye into live cells), was added to the tubes directly before acquisition by flow cytometery.

Restimulation prior to intracellular staining

Cell suspensions were added to a sterile FACS tube at 1-2 x 10^6 cells in 500 µl per tube. Cells were activated with PMA (10 ng/ml) and Ionomycin (1 µg/ml) in the presence of Brefeldin A (BFA) (5 µg/ml) in cRPMI supplemented with 2-ME (50 µM) and incubated at 37°C and 5% CO2 for 6 h. Alternatively, cells were incubated with BFA alone for 4 hours at 37°C. Cells restimulated *ex vivo* from aldara treated mice were inclubated in the presence of IL-23 (50 ng/ml) for 18 hours with BFA for the final 4 hours. After incubation, cells were washed twice with 3 ml of FACS buffer, pelleted by centrifugation at 300 g for 5 min and stained as required.

Intracellular staining

In order to detect intracellular proteins of interest cells were first stained with surface antibodies, as described above. Cell were washed with facs buffer and resuspended in Fix/Perm solution (eBioscience) and incubated for 30 min at 4oC in the dark. Cells were then

washed twice with Permeabilization Buffer (eBioscience) and stained with the appropriate fluorochrome-conjugated antibody (eBioscience) diluted in Permabilixation Buffer. After 30 min cells were washed twice with Permabilization buffer followed by centrifugation at 1200 rpm for 5 min, resuspended in 0.5 ml FACS buffer and acquired as described above.

Stained cell suspensions were acquired using LSR II or LSR FORTESSA (BD) flow cytometers according to the manufacturer's instructions. The flow cytometer was calibrated using single-stained suspensions of compensation beads (BD). For all experiments initial gating was performed on live lymphocytes from forward versus side scatter plots. $CD4^+$ T cells were gated on CD4 positive cells from the live lymphocyte gate. Gating for γδ T-cells and subsets were performed for CD45, γδ TCR and Vγ4 positivity as appropriate. Other unconventional cell subsets were gated on CD45 positive and CD3 negative staining. The extent of intracellular positive intracellular staining signal was determined using appropriate isotype control antibodies. Flow cytometry data analysis was performed using FlowJo (Treestar) software.

2.2.11 Subcutaneous footpad model and ex vivo analysis of BrdU incorporation

Mice were injected subcutaneously (s.c.) into the footpad with reagents dissolved in PBS in a total volume of 25 µl (50 µl/mouse). The lymphatics of the foot drain preferentially to the easily isolated popliteal LN (pLN), located behind the knee. pLN were removed 4 h after immunisation and cells were used as required.

BrdU flow kit (BD Pharmingen) was used according to manufacturer's instructions for *in vivo* BrdU labelling of cells. Mice were injected intraperitoneally with BrdU (1mg/mouse) 48hrs and 24hrs prior to initiation of the footpad model as described above. Upon isolation cells were stained for surface protein before fixation and permeabilisation with BD Cytofix/Cytoperm Buffer. Cells were subsequently treated with DNaseI to expose intracellular BrdU and stained with anti-BrdU antibody.

2.2.12 Aldara and IL-23 induced models of Psoriasis

Aldara™ model

Mice were shaved on their back before hair removal using a depilatory cream. 2 days later Imiquimod (5% Cream, Aldara™ MEDA Pharmaceuticals, Dublin, Ireland) or control cream was applied to the back (3.125mg/day) or ears (1mg/day) of mice for 1-7 days, depending on experimental requirements. Ear thickness was measured using a thickness gauge (Hitec) before the initiation of the experiment on day 0 and every day thereafter until the termination of the experiment. Cells from skin draining lymph nodes were restimulated with IL-23 (50 ng/ml) for 12 hours before further analysis by flow cytometry as described above. Ears from WT and *Tir8/Sigirr*-/- mice were also taken and homogenised in PBS containing a protease inhibitor cocktail (Sigma, UK). Protein concentrations were equalised and homogenate was analysed for expression of IL-1β and IL-23 by ELISA. For IL-17A neutralisation experiments mice were treated on day 0 and day 2 with 250μg/mouse of anti IL-17A antibody (17F3) or

mouse IgG1 isotype control (MOPC-21) (Bio-X-Cell, West Lebanon, NH, USA) and disease was monitored as previously described (194).

IL-23 intra-dermal injection model

WT and *Tir8/Sigirr*$^{-/-}$ mice were anesthetised and injected intradermally with either 200ng IL-23 or PBS in a 20µl volume every day using a 30g needle. Ear thickness was measured using a thickness guage (Hitec) before the initiation of the experiment on day 0 and every day thereafter for a period of 7 days. At the experimental endpoint ears were cut into small pieces and digested for 1 hour at 37 ^{0}C in complete RPMI supplemented with 1 mg/ml Collagenase D and 100 mg/ml DNASE I before passing through a 40µM filter to form a single cell suspension. Cells from psoriatic skin were restimulated with PMA (10 ng/ml) and ionomycin (1 µg/ml) for 2 hrs in the presence of Brefeldin A (5 µg/ml) before further analysis by flow cytometry.

2.2.13 Histopathological analysis of Tissue

Tissue was fixed overnight in 10% formalin before embedding into paraffin blocks. 8µm sections were subsequently cut and rehydrated in progressively decreasing concentrations of IMS (100%-70%) before stained with haematoxylin and eosin. Sections were then dehydrated in progressively increasing concentrations of IMS (70%-100%) and coverslipped. Sections were scored blindly for pathological manifestations of psoriatic inflammation on a scale of 0-4 (0, no observable difference over control; 1, mild; 2, moderate; 3, marked; 4, severe), for

the following parameters acantosis, desquamation, parakeratosis and infiltration. Scores for each parameter were combined for an overall histological severity score.

2.2.14RNA isolation

Extraction of RNA from whole tissue was performed using Isolate RNA mini Kit (Bioline) according to manufacturer instruction. Cells or tissue homegenates were lysed with 450 µl of lysis buffer R and passed through a 23 gauge needle. 1 volume of 70% ethanol was added to the lysates and samples were transferred to the spin column R2 and centrifuged for 2 minutes at 10,000 g after which the flow through was discarded. Column was washed once with 500 µl of AR wash buffer and then with 700 µl of BR wash buffer. After each wash column was centrifuged for 30 sec at 10,000 g and flow through was discharged. Finally column was placed in a new collection tube and RNA was eluted with 20 µl of RNase-free H2O by centrifugation for 2 min at 10,000 g. RNA concentration was determined spectrophotometrically using a Nanodrop (Thermo Scientific) according to the manufacturer's instructions.

2.2.15Reverse transcription and real time PCR reaction

RNA samples were reverse-transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems), according to the manufacturer's protocol and the cDNA reverse transcriptase product was diluted 1:4 with nuclease free water. Transcripts were quantified by real time quantitative PCR on an ABI

7500 Fast Real Time PCR System with Applied Biosystems predesigned Taqman Gene Expression Assays and reagents according to the manufacturer's instructions. For each sample, mRNA abundance was normalised to the amount of 18S ribosomal RNA (rRNA) and is expressed as fold difference compared to cells incubated in medium only or as fold difference compared to control mice.

2.2.16Statistical analysis

Statistical analysis was performed using the computer based mathematical package GraphPad Prism. Student's t test was used to compare the mean values between two groups. Statistical differences in mean values between more than two experimental groups were determined by One-way Analysis of Variance (ANOVA) followed by Tukeys post test or by Two-way ANOVA with repeated measures where appropriate. P values less than 0.05 were considered significant.

Chapter 3: sCD25 enhances the generation of Th17 responses and autoimmunity

3.1 Introduction

Upon its discovery IL-2 was initially classified as a pro-inflammatory cytokine due to its ability to enhance the proliferation of activated T cells. However with the fatal T cell driven autoimmunity observed in mouse strains deficient in IL-2 or its receptor CD25 it has become clear that the primary non-redunant role for IL-2 is in the maintenance of peripheral T cell tolerance (195, 196). IL-2 exerts these pleiotrophic effects through its receptor, a heterotrimeric complex expressed on the surface of activated T cells consisting of the α chain (CD25), β chain (CD122) and the common γ chain (CD132). A number of studies have explored the mechanisms through which IL-2 elicits its tolergenic effects. IL-2 signalling is essential for the maintenance of competitive fitness of peripheral regulatory T cells, cells which are established as important for modulation of autoreactive T cells responses (197). It also functions to directly inhibit the generation of Th17 responses both *in vitro* and *in vivo* (154), furthermore there is a strong genetic association between mutations at the *il-2* and *cd25* gene loci and a number of T cell driven autoimmune disorders (113). Interestingly a number of these susceptibility alleles correlate with elevated levels of the soluble form of the IL-2R alpha chain, sCD25 in the serum of patients (113, 198). Despite such observations suggesting a role for sCD25 in disease pathogenesis its precise role in directing pathogenic T cell subsets, if any, is currently unclear. sCD25 is shed from the surface of activated T cells with the rate of shedding directly correlating with the proliferation of these cells. The levels of sCD25 in the serum of patients are remarkably stable in steady state conditions and as such sCD25 is classically viewed as a biomarker of activation in disorders with a T cell component (167) but the possibility that these elevated levels of sCD25 could be contributing to the progression of disease has yet to be determined. A number of other soluble cytokine receptors have been identified as both having antagonistic properties, IL-6R (168) or agonistic

properties IL-1RI relavant to their respective signalling recpetors in immune responses (169). Furthermore soluble receptors such as IL-4R have been described as acting as chaperones or carrier proteins for their ligands (169).

The role sCD25 plays in the context of IL-2R signalling is also currently unclear, although there are a number of studies which describe an inhibitory role for sCD25 in IL-2 mediated responses *in vitro* (113, 199) but whether these observations are of relevance *in vivo* has not been investigated.

The primary focus of this section of the study was to investigate a potential role for sCD25 in the progression of autoimmune disease and provide novel insight into its impact on both the pathogenic and regulatory subsets *in vivo.* Furthermore we wanted to assess the impact of sCD25 on the *in vitro* differentiation of $CD4^+$ T cell subsets and explore the pathways implicated in their regulation.

3.2 Results

3.3.1 sCD25 treatment enhances antigen specific IL-17A but does not impact FOXP3 generation in the periphery

Serum sCD25 is present at elevated levels in patients carrying alleles known to confer susceptibility to autoimmune diseases. However despite these observations a specific role for elevated sCD25 in the pathogenesis of such conditions has yet to be formally identified. In order to address the possibility that sCD25 could enhance Th17 generation *in vivo* C57BL/6J mice were immunized sub-cutaneously with MOG_{35-55} emulsified in complete freunds adjuvant (CFA). MOG_{35-55} is a myelin antigen reported to be targeted by pathogenic T cells in EAE (114). Mice were treated sub-cutaneously with sCD25 (12.5ug) immediately prior to immunisation, and every 12 hours thereafter for the first 3 days. After 7 days cells from draining lymph nodes were restimulated *ex vivo* in the presence of MOG_{55-55} for 72hrs. Levels of IL-17A and IFN-γ in the cell culture supernatants were subsequently assessed by ELISA. Levels of antigen specific IL-17A were significantly enhanced from mice treated with sCD25 whilst there was were no significant differences in the levels of IFN- γ (Fig. 3.1A). Furthermore analysis of IL-17 expression by flow cytometric analysis demonstrated enhanced expression of IL-17A from mice treated with sCD25 (Fig. 3.1B). To assess the potential impact of sCD25 treatment on Treg cells expression of FOXP3 in the lymph nodes was also assessed by flow cytometric analysis. No differences in percentages (Fig. 3.1C) or relative levels (Fig. 3.1D) of Tregs were observed between mice treated with PBS compared to those treated with sCD25. Data shown is representative of two separate experiments comprising 3 mice per group. Together these data demonstrate that sCD25 is capable of enhancing antigen specific IL-17A expression without any apparent impact on Tregs.

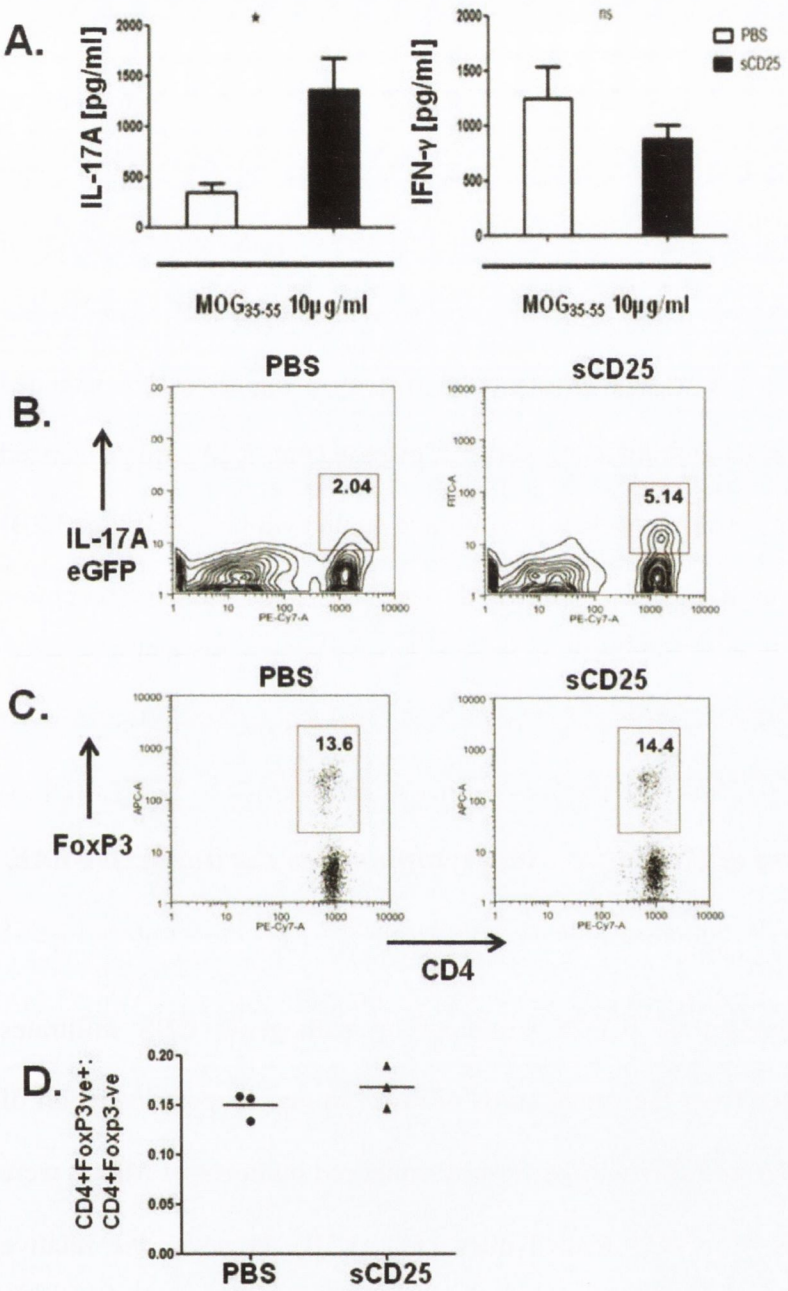


Fig 3.1 sCD25 enhances peripheral antigen specific Th17 responses.
(A) Levels of IL-17A and IFNγ secreted by draining lymph node cells of mice immunized and treated as above (3 per group), isolated after 9 days, and restimulated *ex vivo* with MOG_{33-55} (10 µg/ml) for 72 hours. (B) Percentage of $CD4^+$ IL-17A-eGFP+ve cells after *ex vivo* restimulation in the presence of $MO5_{35-55}$ (10µg/ml) for 72 hours. (C) Percentage and (D) relative number ratios of $CD4^+FoxP3^+$:$CD4^+FoxP3$- T cells in the draining lymph nodes of both sCD25 and control treated immunized mice. 3 mice per group were analysed. Statistical significance determined by unpaired student's t-test, * $p \leq 0.05$, ** $p \leq 0.01$.

3.3.2. Enhanced EAE severity in mice treated with sCD25

As sCD25 enhances MOG antigen specific T cell responses *in vivo* it was important to examine whether these effects could modulate disease pathogenesis in EAE. Mice were immunised with MOG_{35-55} peptide emulsified in CFA and treated with the same regimen of PBS or sCD25 as described above. Disease progression was monitored and scored using established scoring protcols. Mice treated with sCD25 display a significantly enhanced disease phenotype during the onset and induction phase of disease (Fig. 3.2A), this enhanced disease was also associated with a higher weight loss in mice treated with sCD25 (Fig. 3.2B) These data demonstrate that enhanced sCD25 in EAE leads to exacerbated autoimmune disease.

3.3.3 Intracellular staining of CNS infiltrating lymphocytes during active EAE.

In order to determine the impact of sCD25 treatment on pathogenic CNS infiltrates mononuclear cells were isolated from the spinal cords of mice on day 15 post induction of EAE before staining for IL-17A and IFN-γ. Significantly enhanced numbers of MNCs were detected in the spinal chords of sCD25 treated mice (Fig. 3.3B), however the relative percentages of infiltrating Th1 versus Th17 cells was unaltered between groups (Fig. 3.3A), the observed disease phenotype correlated with significantly increased numbers of pathogenic Th1 and Th17 cells infiltrating into the spinal cord of sCD25 treated mice compared to mice treated with PBS (Fig. 3.3C).

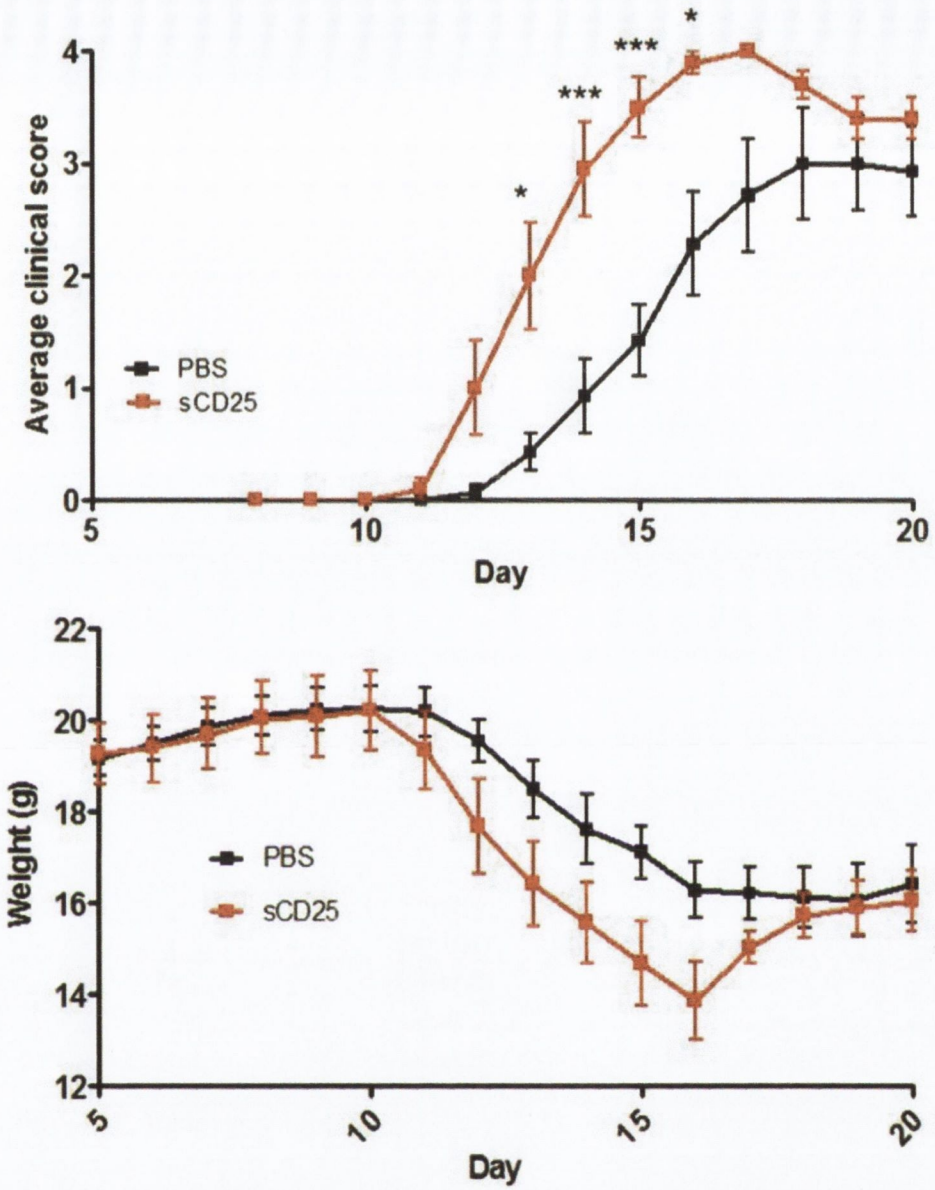


Fig 3.2 Exogenous sCD25 enhances autoimmunity.
(A) MOG_{33-55} immunized C57BL/6 mice developed EAE and (B) lost weight from day 12 after immunization with a peak of disease severity observed from day 15. Subcutaneous administration of recombinant sCD25 (25 μg/mouse) immediately prior to immunization and every 12 hours thereafter for 72 hrs resulted in a significant exacerbation in severity of symptoms during disease onset and induction. 6–7 mice used per group. Statistical significance determined two way anova, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

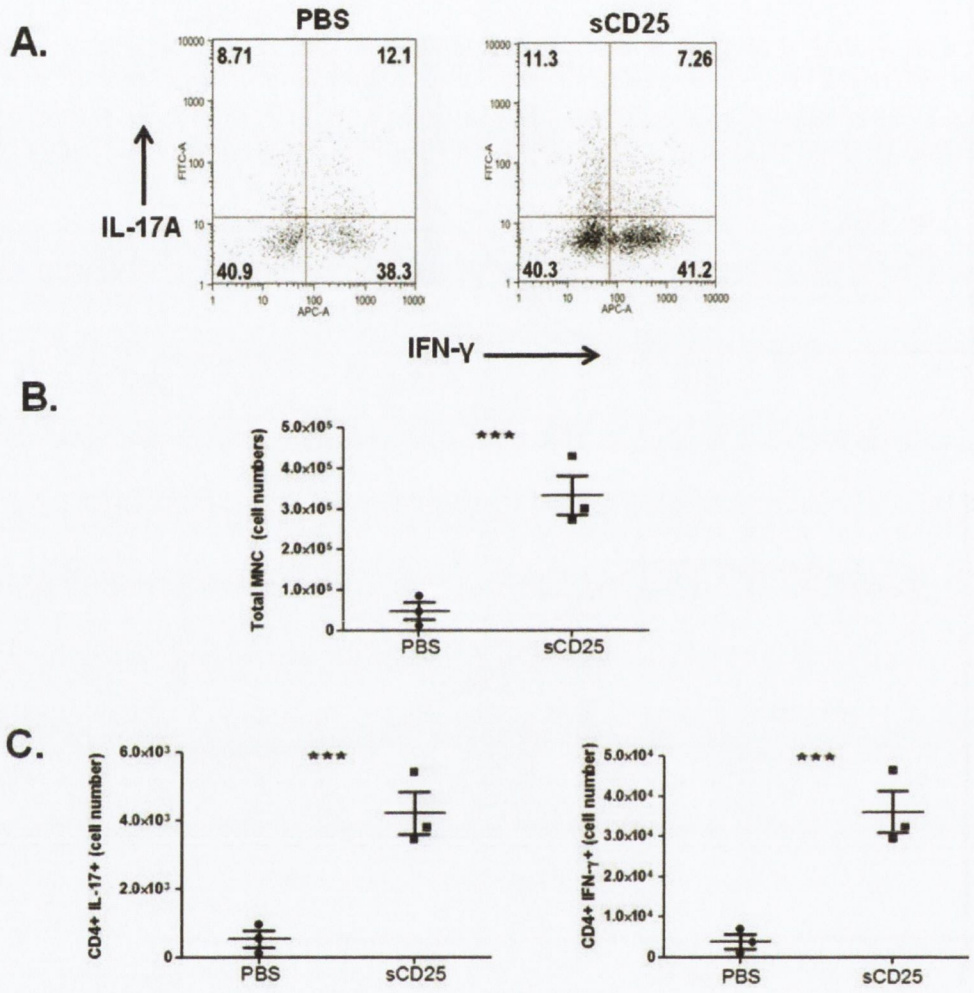


Fig. 3.3 Exacerbated disease phenotype with exogenous sCD25 is associated with enhanced infiltration of Th1 and Th17 cells into the CNS.

(A) Mononuclear cells harvested from spinal cords of control (PBS) and sCD25 treated IL-17A-eGFP reporter mice (3 per group) on day 15 after immunization with MOG $_{35-55}$ and analysed for expression of IL-17 and IFN-γ by $CD4^+$ cells. (B) Total number of MNC cells isolated (C) Cell numbers of $CD4^+IL\text{-}17^+$ and $CD4^+$ $IFN\gamma^+$ cells in spinal cords of IL-17A-eGFP reporter mice at day 15. Cells were gated initially on live lymphocyte populations before gating on $CD4^+$ and subsequent analysis of $CD4^+$ cells for dual expression of IL-17A and IFN-γ. Cell counts were performed initially on each individual mouse prep before pooling each mouse in the group for flow cytometric analysis. significance determined by unpaired student's t-test, *$p \leq 0.05$ **$p \leq 0.01$, ***$p \leq 0.001$

3.3.4 sCD25 enhances the generation of Th17 responses in vitro.

IL-2 has been implicated as a negative regulator of Th17 differentiation both in vitro and *in vivo* (154), as we have demonstrated the ability of sCD25 to exacacerbate autoimmune disease we sought to determine the mechanistic basis behind these events by investigation of the impact sCD25 has on the generation of Th17, Th1 and Treg responses *in vitro*. $CD4^+$ T cells were differentiated towards a Th17 phenotype in the presence of TGF-β, IL-6, anti-IFN-γ and anti-IL-4 alone or with 1 μg/ml, 5 μg/ml, 10 μg/ml or 20μg/ml sCD25. Levels of IL-17A in the cell culture supernatants were assessed by ELISA with a dose dependant increase in IL-17A expression from the cells treated with sCD25 was observed compared to Th17 conditions (Fig. 3.4A). A threefold increase was observed at the highest concentration of sCD25 compared to Th17 conditions alone. Flow cytometric analysis for expression of IL-17A from these cells demonstrated a similar dose dependant relationship with sCD25 capable of greatly enhancing expression of IL-17A compared to Th17 cultures alone (Fig 3.4B). The data shown is representative of more than five independent experiments. These data demonstrate that exogenous sCD25 is capable of enhancing Th17 responses *in vitro* and suggest a mechanism whereby sCD25 enhances the severity of autoimmune disease observed through *in vivo* administration.

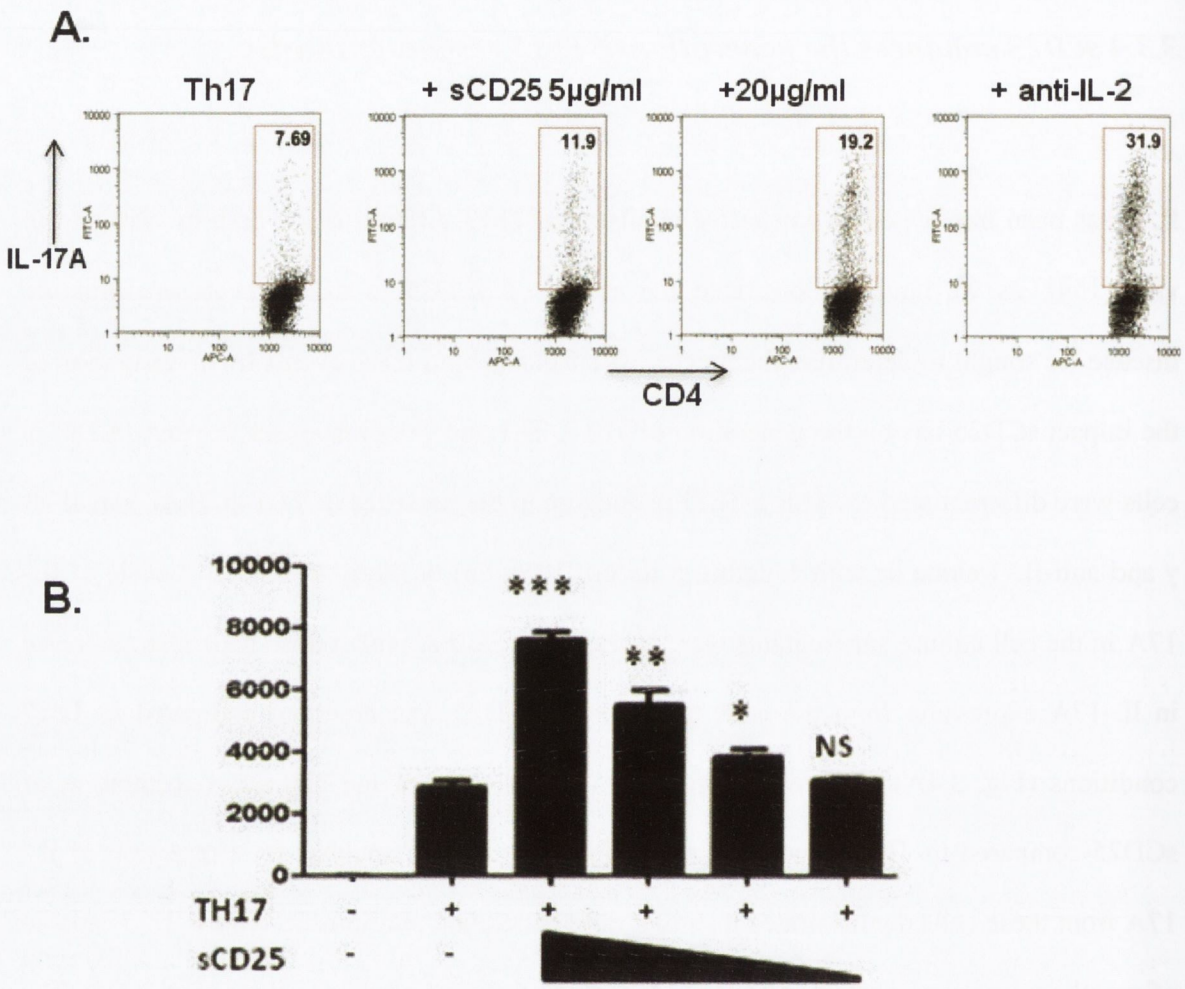


Fig. 3.4 sCD25 enhances the generation of Th17 responses in vitro.
Purified naïve $CD4^+$ T cells were activated with plate bound anti-CD3 (5µg/ml) and anti-CD28 (5µg/ml) and cultured under Th17 inducing condition, TGF-β (5 ng/ml), IL-6 (10 ng/ml), anti IL-4 (10 µg/ml) and anti IFN-γ (10 µg/ml) in the presence of a range of concentrations of sCD25 (20, 10, 5 or 1 µg/ml) or anti-IL-2 (10 µg/ml). Cells were activated Levels of IL-17A or IFNγ expression were determined after 96 hrs by (A) Flow cytometric analysis and (B) ELISA. ™). These data are representative of at least four independent experiments with similar results. Cells were gated initially on live lymphocytes followed by side scatter against CD4 positive before CD4 against the intracellular marker of interest Statistical Significance determined by unpaired student's t-test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3.5 sCD25 does not impact the generation of Th1 responses in vitro.

Following on from our observations of the capability of sCD25 to impact T cell polarisation, we were interested to assess a potential role for sCD25 in the regulation of Th1 responses *in vitro.* IL-2 has been shown to play a role in differentiation to this lineage so conceivably interference with available IL-2 from exogeneous sCD25 could modulate Th1 lineage commitment and IFN-γ expression (200) .

$CD4^+$ T cells were differentiated towards a Th1 phenotype in the presence of IL-12 and anti-IL-4 antibody alone or with increasing concentrations of sCD25. Intracellular cytokine staining yielded no observable difference in IFN-γ expression in the presence of sCD25 (Fig. 3.5A). Levels of IFN-γ in the cell culture supernatants were also assessed by ELISA after 72hrs (Fig. 3.5B), however no significant differences were observed between cultures with sCD25 added compared to those cultured under Th1 conditions alone. Together, these data demonstrate that sCD25 does not impact the development of Th1 responses *in vitro.*

3.3.6 sCD25 does not impact the generation of Tregs in vitro.

Treg cells are accepted as being exquisitely sensitive to homeostatic levels of IL-2, such that it is indispensible for their development and maintenance (201). Therefore we also examined the potential for sCD25 to inhibit the development of FoxP3 positive Treg cells *in vitro.*

$CD4^+$ T-cells were differentiated to a Treg phenotype with TGF-β and IL-2 in the presence or absence of sCD25. After 72 hours, cells were assessed for FoxP3 expression by flow cytometric analysis. No differences in FoxP3 levels were observed between cells treated with sCD25 and those that treated with media alone (Fig. 3.6A), indicating that sCD25 does not inhibit the *in vitro* generation of Tregs.

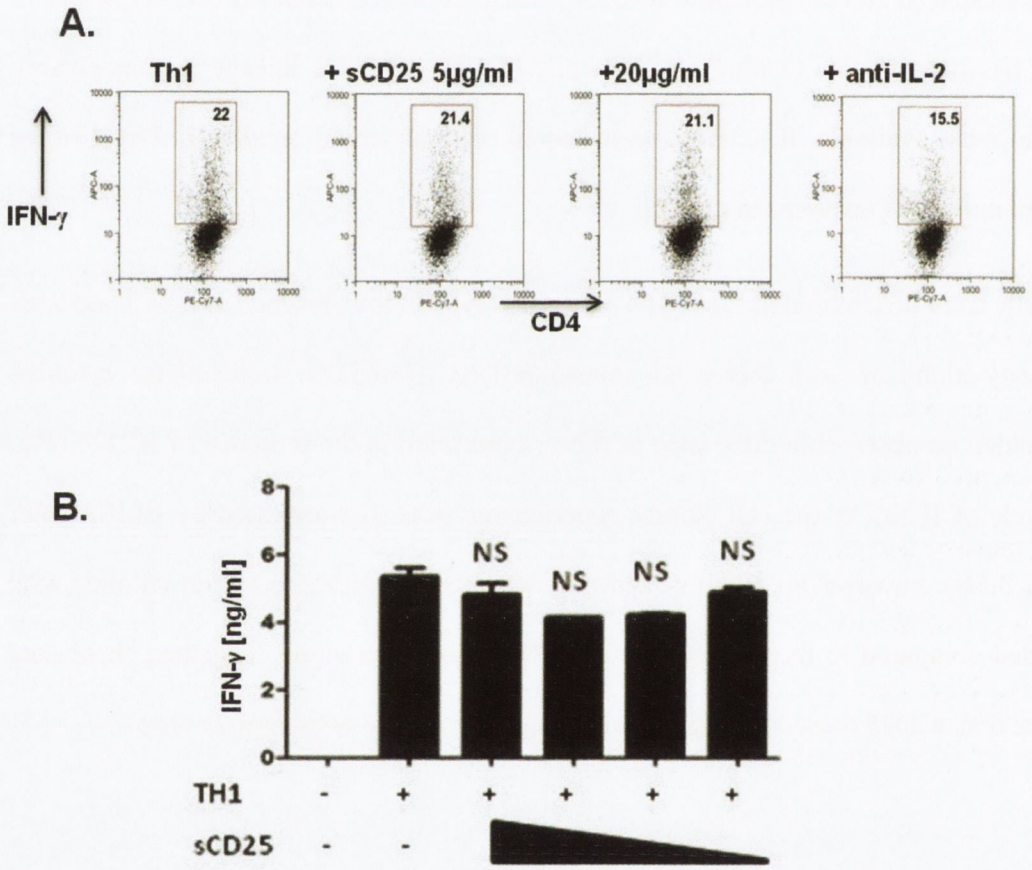


Fig. 3.5 sCD25 does not impact the generation of Th1 responses *in vitro.*
Purified naïve $CD4^+$T cells were activated with plate bound anti-CD3 (5µg/ml) and anti-CD28 (5µg/ml) and cultured under Th1 inducing condition, IL-12 (10 ng/ml), anti IL-4 (10 µg/ml) in the presence of a range of concentrations of sCD25 (20, 10, 5 or 1 µg/ml) or anti-IL-2 (10 µg/ml). Levels of IFNγ expression were determined after 96 hrs by (A) Flow cytometric analysis and (B) ELISA. ™). Statistical significance was determined by students T test. Cells were gated initially on live lymphocytes followed by side scatter against CD4 positive before CD4 against the intracellular marker of interest. These data are representative of at least two independent experiments with similar results

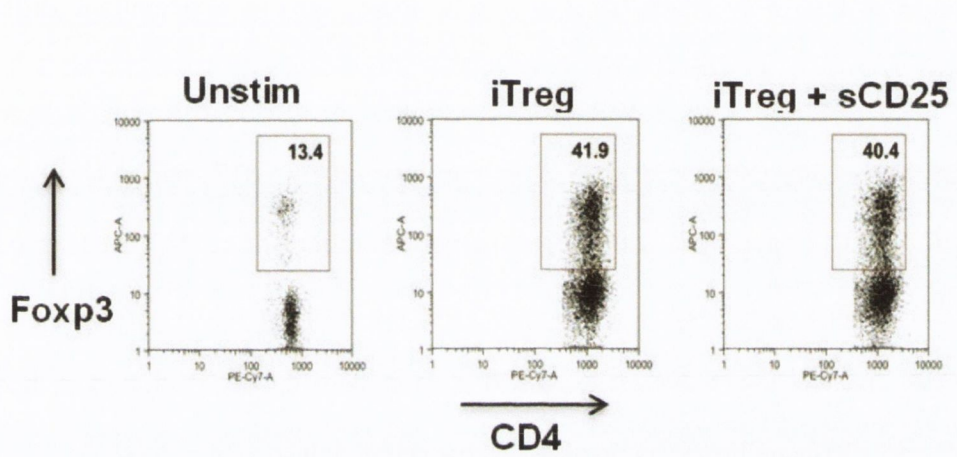


Fig. 3.6 sCD25 does not impact the generation of Tregs *in vitro*
(A) Purified naïve $CD4^+$ T cells were activated plate bound anti-CD3 (5μg/ml) and anti-CD28 (5μg/ml) under Treg inducing conditions, TGF-β (5 ng/ml) and rIL-2 (10U/ml) for 96 hours, in the presence or absence of sCD25 (20 μg/ml) and FoxP3 expression determined by flow cytometric analysis. These data are representative of at least two independent experiments with similar results. Cells were gated initially on live lymphocytes followed by side scatter against $CD4^+$ positive before $CD4^+$ against the intracellular marker of interest

3.3.7 sCD25 does not impact the proliferation or survival of Th17 cells but facilitates IL-6 receptor activity

A reported role of IL-2 signalling is the promotion of the survival and proliferation of activated T cells (202). We wanted to explore the possibility that exogenous sCD25 could impact proliferation and/or the viability of Th17 cells through possible interference with this

signalling activity. To assess the impact on proliferation $CD4^+$ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and polarized to a Th17 phenotype with increasing concentrations of sCD25 added to cultures as before. After 72h the proliferative state of the cells was examined by flow cytometry for CFSE dilution as well as intracellular cytokine staining for IL-17A expression. Although an increase in IL-17A expression in the presence of sCD25 was observed (Fig 3.7A), there was no concomitant difference in proliferation of these cells (Fig. 3.7B).

To assess a role for exogenous sCD25 in the survival of Th17 cells $CD4^+$T cells were polarized to a Th17 phenotype with increasing concentrations of sCD25 added to cultures as before. After 48hrs, cells were stained with 7-Amino-Actinomycin D (7-AAD) a common cell viability dye, no observable difference in survival between cells cultured in the presence of sCD25 and Th17 conditions alone was observed (Fig. 3.7C).

Activation of STAT3 downstream of the IL-6R is established as indispensible for Th17 generation (30). STAT3 signalling was examined after 48 hours and consistent with the enhanced generation of the Th17 response, a pronounced increase in STAT3 phosphorylation at tyrosine 705 was observed in cells treated with sCD25 compared to Th17 cultures alone (Fig. 3.7D). Together these data confirm the ability of sCD25 to enhance the generation of Th17 responses without adversely impacting the viability or proliferation of these cells.

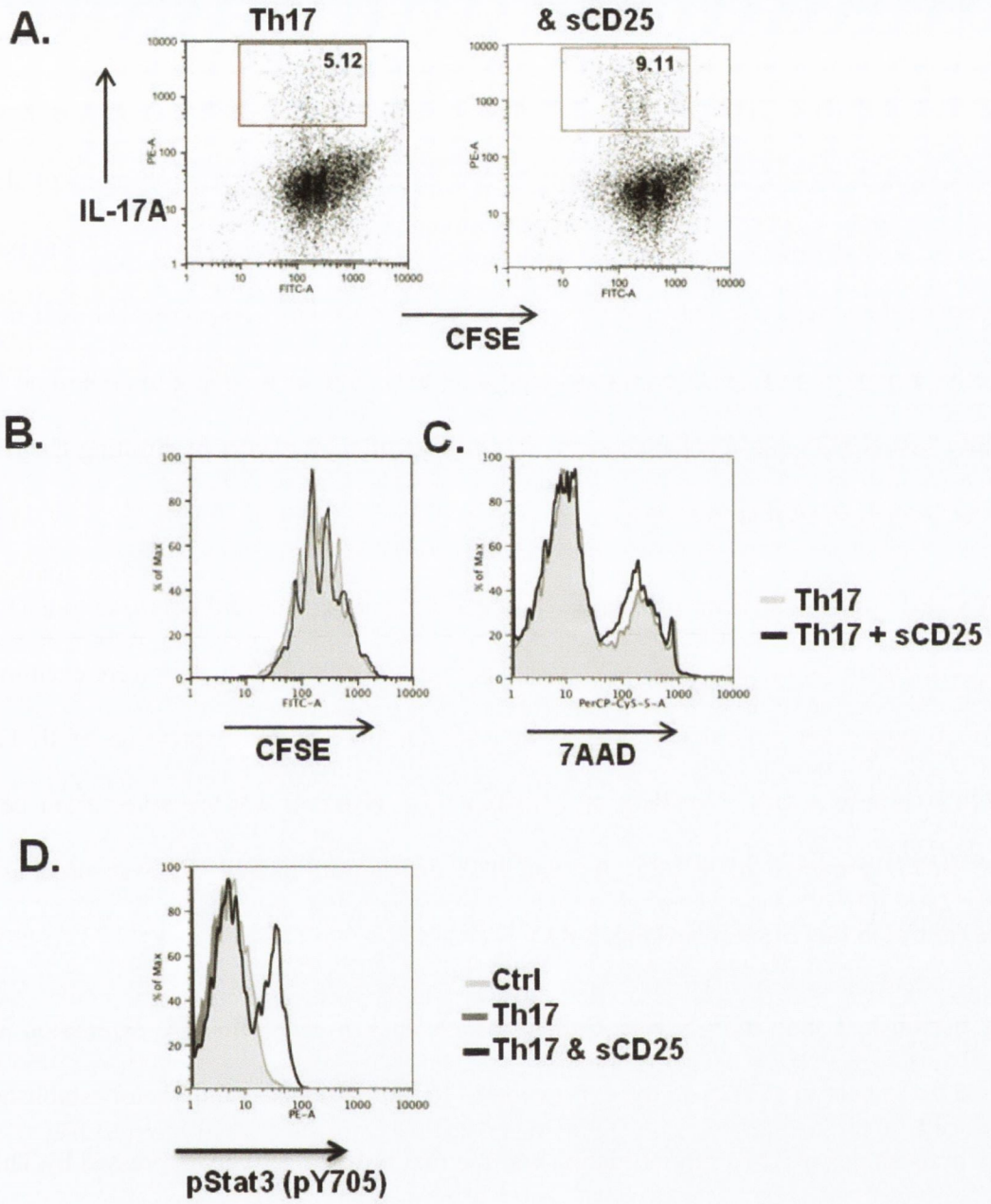


Fig. 3.7 sCD25 enhances Th17 respones *in vitro* without impacting proliferation or survival
Naïve $CD4^+$ T cells were stained with CFSE (2.5 μM) prior to activation under Th17 conditions in presence or absence of sCD25 (20 μg/ml). After 96 hours, levels of intracellular (A) IL-17A expression and (B) CFSE dilution or (C) 7AAD incorporation were determined by flow cytometric analysis. Purified naïve $CD4^+$ T cells were activated under Th0, Th17 and Th17& sCD25 (20 μg/ml) conditions for 72 hours and levels of (D) P-Stat3 (pY705) were assessed by flow cytometric analysis. These data are representative of at least two independent experiments with similar results. Cells were gated initially on live lymphocytes followed by side scatter against CD4 positive before CD4 against the cell membrane or intracellular marker of interest

3.3.8 sCD25 enhances the development of Th17 cells at early time-points.

Upon *in vitro* activation $CD4^+$ T-cells can initiate expression of IL-2 within a matter of hours with peak expression commonly observed between 24 and 36 hours. The presence of IL-2 during T cell differentiation is inhibitory to the generation of Th17 cells (154) and it has been demonstrated that Tregs can enhance the early generation of Th17 responses through their constitutive expression of CD25 on the cell surface (203). We next sought to determine the possibility that sCD25 could enhance early Th17 differentiation events by limiting the IL-2 signalling cascade in similar manner.

Initially $CD4^+$T cells were isolated from IL-17A eGFP reporter mice and polarised to a Th17 phenotype alone or in the presence of 20 μg/ml sCD25 and after 48 hrs cells were examined for IL-17A expression by flow cytometric analysis. Despite limited expression of IL-17A from Th17 cultures alone, a striking upregulation in expression was observed from cells cultured in the presence of sCD25. An anti-IL-2 neutralisation antibody was used as a positive control in this experiment (Fig. 3.8A).

Having established that sCD25 was capable of enhancing early IL-17A expression we examined the impact of sCD25 on the expression of RORγt, a transcription factor established as a key orchestrator of Th17 differentiation and the first to be selectively expressed by Th17 cells (31). $CD4^+$ T cells were polarised to a Th17 phenotype in the presence of sCD25 as before and after 48h were examined for expression of RORγt by intracellular nuclear staining. Consistent with the IL-17A expression data from Fig. 3.8A, sCD25 enhanced the expression of RORγt in a concentration dependant manner (Fig. 3.8B)

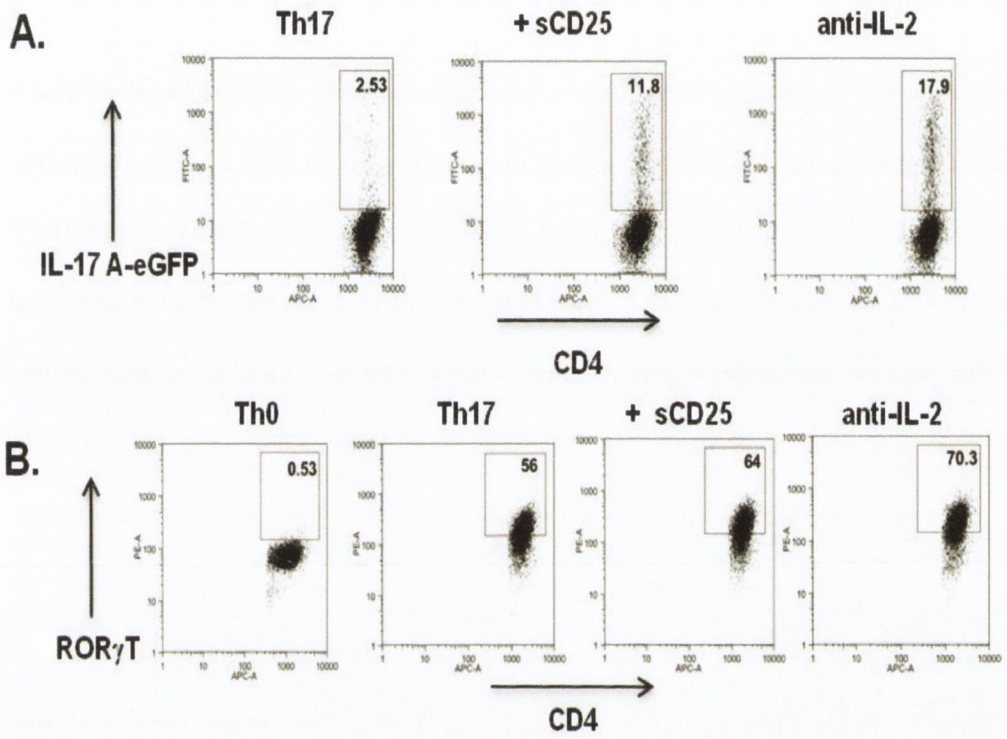


Fig. 3.8 sCD25 acts early during Th17 development.
Purified naïve $CD4^+$ T cells from IL-17AeGFP reporter mice activated under Th17 inducing conditions in the presence of sCD25 (20 μg/ml) or anti-IL-2 (10 μg/ml) for 48 hrs and examined for levels of (A) IL-17A and (B) RORγT expression by flow cytometric analysis. All data are representative of at least 3 independent experiments. Cells were gated initially on live lymphocytes followed by side scatter against CD4 positive before CD4 against the intracellular marker of interest

3.3.9 sCD25 inhibits IL-2 receptor signalling but does not impact expression of IL-2 or act at the cell surface.

In order to elucidate the mechanism whereby sCD25 enhances early differentiation of Th17 cells we examined the phosphorylation state of the transcription factor STAT5, a downstream signalling mediator of the IL-2R, which is required for IL-2s negative regulatory role in Th17 differentiation (154). Cells undergoing Th17 differentiation were cultured in the presence or absence of sCD25 and the phosphorylation of STAT5 at tyrosine 694 determined after 24hrs. A marked reduction in STAT5 phosphorylation was observed in the sCD25 treated cells compared to Th17 conditions alone (Fig. 3.9A). In order to determine the mechanism through which sCD25 mediates this inhibition we assessed the frequency of expression of intracellular IL-2 upon cellular activation in the presence or absence of sCD25, however no observable differences in IL-2 expression were noted (Fig. 3.9B). To examine the possibility that sCD25 could directly bind to the cell surface thereby interfering with efficient formation of the IL-2 receptor signalling complex or inhibiting IL-2 binding, we utilized a HIS-tag on the soluble form to discriminate between surface bound and the soluble CD25. However surface binding sCD25 was undetectable during the first 24 hours of activation (Fig. 3.9C).

One of the noted roles of IL-2 is its ability to drive the surface expression of CD25 upon T cell activation by acting in an autocrine loop. Cells treated with sCD25 displayed diminished expression of surface CD25 after 24h compared to those cultured under Th17 conditions alone (Fig. 3.9D). This observation is further indicative of abrogated IL-2 signalling activity in the presence of sCD25. These data demonstrate the ability of sCD25 to inhibit IL-2 receptor signalling *in vitro*. However this inhibitory ability is not mediated through direct

inhibition of IL-2 expression or interaction with the cell surface and heterotrimeric IL-2 receptor complex.

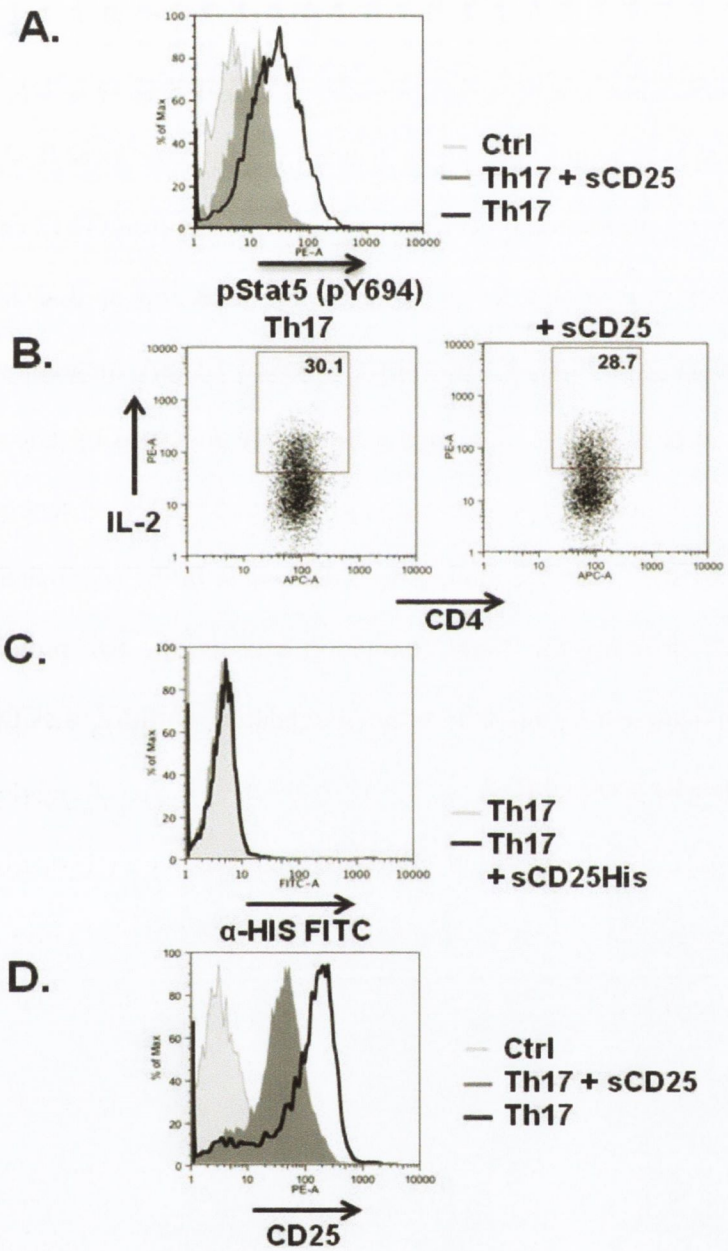


Fig. 3.9 sCD25 inhibits IL-2R signalling but does not impact IL-2 expression or act at the cell surface.

(A–D) Purified naïve $CD4^+$T cells activated for 24 hrs under Th17 inducing conditions in the presence or absence of sCD25 and examined for levels of (A) p-Stat5 pY694, (B) intracellular IL-2 expression (C) surface binding of sCD25-His and (D) surface expression of endogenous CD25 by flow cytometric analysis. These data are representative of at least two independent experiments with similar results. Cells were gated initially on live lymphocytes followed by side scatter against CD4 positive before $CD4^+$ against the cell membrane or intracellular marker of interest

3.3.10 sCD25 inhibits IL-2 signalling by sequestering secreted IL-2

Surface bound CD25 on Tregs is known to enhance early Th17 generation through its ability to limit the availability of inhibitory IL-2 to Th17 cells undergoing differentiation (203). To address the possibility that sCD25 was acting in a similar manner to sequester local IL-2 in the microenvironment, ELISAs were performed on cell culture supernatants from Th17 cells treated with sCD25. Importantly sCD25 was found to inhibit the detection of IL-2 in a concentration dependant manner (Fig. 3.10A). Furthermore, a mixed ELISA method was devised whereby a plate was coated with anti IL-2 capture antibody followed by an anti sCD25 antibody as the secondary antibody to allow detection of IL-2 and sCD25 present in a complex. Significant levels of this IL-2/sCD25 complex were detectable in the supernatents of cells treated with sCD25 demonstrating the direct interaction between the two proteins (Fig 3.10B). Together these data demonstrate that the immunomodulatory abilities of sCD25 are mediated by its ability to sequester secreted IL-2

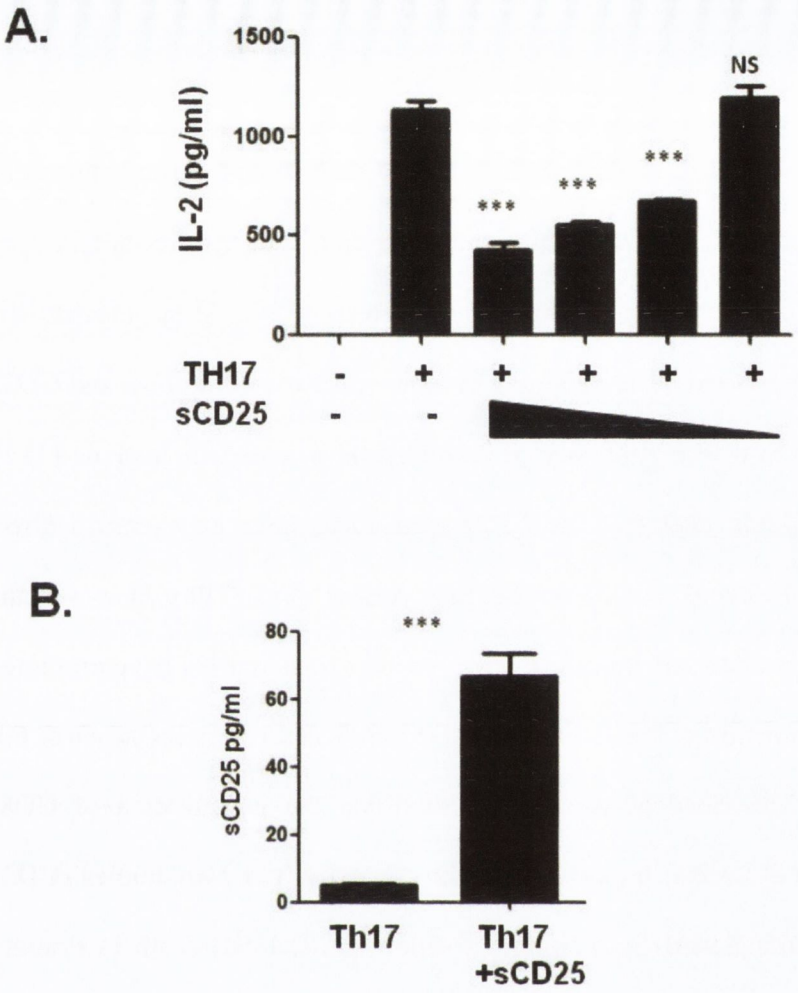


Fig. 3.10 sCD25 inhibits IL-2R signalling by sequestering local IL-2
(A) Levels of secreted IL-2 detected by ELISA 24 hours after Th17 activation in the presence of decreasing doses of sCD25 (20, 10, 5, 1 μg/ml). (B) Levels of IL-2/sCD25 complex in the supernatants of Th17 cells activated for 24 hours in the presence or absence of sCD25 (20 μg/ml). Data shown as mean +/− standard deviation from triplicate experiments. All data are representative of at least 3 independent experiments. Statistical significance determined by unpaired student's t-test, ***$p \leq 0.001$.

3.3 Discussion

The primary non-redundant role of IL-2 is the maintenance of peripheral T cell tolerance. IL-2 activity confers these tolerogenic effects through the generation and maintenance of Treg cells (197) and ability to inhibit the generation of Th17 cells (154). These mechanistic observations are further underscored by correlations between mutation at the *IL-2* and *CD25* gene locus and the susceptibility to a number of autoimmune diseases in humans (113). Recent analysis of susceptibility alleles at the *CD25* gene locus have uncovered a direct association with elevated levels of sCD25 in patients' serum (113, 198); however the functional consequences of such observations are unknown. Previous reports have presented conflicting information with regard to the impact of sCD25 on T cell responses *in vitro.* For instance, sCD25 has been described as capable of inhibiting the proliferation of $CD8^+$ cytotoxic T cell lines (199). However, importantly in the context of our own studies sCD25 reduced IL-2 receptor signalling whilst inhibiting activation induced cell death in primary human $CD4^+$ T cells (113).

Here we report a previously unappreciated role for sCD25 in the modulation of T cell driven autoimmune disease *in vivo*. Mice treated for a short time post immunisation with the myelin component peptide MOG_{35-55} express signficantly enhanced antigen specific IL-17A during the peripheral priming of pathogenic T cells. These mice go on to develop an exacerbated disease phenotype in EAE which is associated with increased infiltration of pathogenic Th17 and Th1 cells into the CNS of mice treated with sCD25. This observation is broadly in line with reports whereby neutralisation of circulating IL-2 by anti-IL-2 monoclonal antibody for a limited period elicits spontaneous autoimmune autoimmunity in mice. Similar treatment of diabetes-prone nonobese diabetic mice triggers early onset of diabetes and produces a wide

spectrum of T cell-mediated autoimmune diseases, including gastritis, thyroiditis, sialadenitis, and notably, severe neuropathy (204). The study by Setoguchi *et* al., study notes that chronic IL-2 neutralisation in this setting will reduce the numbers of CD25 expressing FoxP3 positive $CD4^+$ T cells, therefore it is surprising due to the established role for IL-2 in the maintenance of Treg homeostasis (205) that we don not see a similar phenomenon in mice treated with sCD25. It is possible that sCD25 treatment impacted the suppressive ability of Treg however, more likely is that the length of the dosage regimen and the relatively low dosage of (3 x 25µg) compared to the study by Setoguchi et al. (1mg of anti-IL-2 Mab) wasn't sufficient to impact the number or incidence of Treg cells. Tregs which express higher levels of CD25 to Th17 cells could conceivably be less sensitive to the level of IL-2 deprivation presented by sCD25 treatment (206). Similarly IL-2 is established as important for the generation of Th1 responses (207) however we report no impact on the differentiation of Th1 cells at the concentrations used in this study.

It is clear that sCD25 is capable of enhancing the generation of Th17 responses *in vitro* and *in vivo.* However, despite elevated antigen specific IL-17A in the periphery cells infiltrating the CNS express IL-17A at a similar level on a per cell basis in sCD25 and vehicle treated mice. The use of an IL-17-eGFP reporter mouse which is capable of identifying cells that carry a legacy of IL-17A expression does not suggest that inter lineage plasticity is at play as an increase in cells co expressing both IL-17A and IFN-γ would be clearly evident by flow cytometric analysis. It is likely that sCD25 treatment promotes the peripheral expansion of antigen specific Th17 cells which facilitates increased early infiltration to the site of inflammation, an observation which has been previously reported (208).

In vitro sCD25 enhanced the generation of Th17 responses through its ability to sequester secreted IL-2. Th17 cells differentiated in the presence of sCD25 display enhanced STAT3 phosphorylation, a signalling mediator downstream of the IL-6 receptor whose activity is

indispensible for the generation of Th17 cells (30). Furthermore these cells also display abrogated IL-2 receptor signalling in the presence of sCD25. This observation when considered in conjunction with our STAT3 data is clearly in line with a recent report which describes the magnitude of Th17 differentiation as a subtle interplay between the levels of STAT3 and STAT5 activity

Classically CD25 is thought of as having a low affinity for IL-2, however this dogma has developed because of *in vitro* comparisons with the binding efficiency with the heterotrimeric IL-2 receptor complex as a whole. Despite this perceived lower affinity, sCD25 is capable of binding IL-2 efficiently and eliciting immunomodulatory effects *in vitro* (113, 209). This ability of sCD25 to efficiently bind IL-2 and act as a decoy receptor *in vitro* provides the mechanistic basic for our *in vivo* observations. The use of humanised antibodies against CD25, are performing with some promise in clinical trials for multiple sclerosis (210). However while the definitive mechanism of action is currently unclear it is noteworthy that this antibody can bind sCD25 as well as block it ability to sequester IL-2. Coupled with the observations that elevated sCD25 levels in MS patients correlates with specific susceptibility alleles at the *CD25* gene locus, evidence is growing for a non redundant role for sCD25 in the progression of autoimmune disease. As such this study potentially provides a novel mechanism which could explain these observations.

Chapter 4: TIR8/SIGIRR regulates innate IL-17A expression and psoriasiform inflammation through direct action on γδ T cells

4.1 Introduction:

In the previous chapter we have demonstrated a novel role for sCD25 in the enhancement of Th17 responses and autoimmunity. Following on from this work we have sought to examine alternative enhancers of the type 17 response and more specifically investigate the role for novel signalling mediators in the regulation of these pathways and autoimmunity. The specific focus of this chapter relates to the role of TIR8/SIGIRR in the regulation of innate IL-17A and dermal inflammation in murine models of psoriasis.

Psoriasis is a chronic immune driven disease of the skin manifesting itself macroscopically as scaly inflamed skin lesions occurring due to dysregulated keratinocyte proliferation (123). This thickening of the epidermal layer is also associated with a significant immune cell infiltrate composed of T cells, dendritic cells macrophages and neutrophils (124). Recent advances have identified IL-23 driven $CD4^+$ and γδ T cells expressing IL-17A as important drivers of disease pathogenesis (129, 130), with a number of novel therapeutic interventions for the treatment of psoriasis focusing on this pathway (133, 134).

IL-23 is a myeloid derived cytokine which acts as a key growth factor inducing the expression of a number of type 17 cytokines including IL-17A, IL-17F and IL-22 from both Th17 cells and innate γδ cells, which a number of recent studies have implicated as central drivers of psoriatic inflammation (36, 72, 119, 129). IL-23 shares its p40 subunit with the Th1 instructive cytokine IL-12 and an anti-p40 monoclonal antibody (Ustekinumab) has met with some successes for the treatment of psoriasis in the clinic (211). Whilst the role IL-23 plays in the development of type 17 responses and associated autoimmunity is well established, a number of other cytokines are also known to contribute to, and indeed complement, the actions of IL-23 in similar settings. One such example is IL-1, a cytokine

which exists in two distinct forms IL-1β and IL-1α. These signalling mediators both share the same receptor, IL-1 receptor 1 type 1 (IL-1R1) and elicit remarkably similar effects despite significant differences in their expression and activity (173). It has long been surmised that IL-1R1 signalling is dysregulated in autoimmune disorders such as psoriasis however its specific role has proved controversial (212, 213). Recently reports have demonstrated that IL-1R1 signalling is indispensable for acanthosis, neutrophil recruitment and Munro's abscess formation in the Aldara™ induced model of psoriasis (214). These findings coupled with its established role in complimenting the proinflammatory actions of IL-23 highlight the need to further examine the role IL-1R1 signalling plays in psoriasis pathogenesis and in particular investigate the action of signalling mediators which are known to regulate its effects.

One such mediator TIR8/SIGIRR, which serves as a negative regulator of inflammation driven by the Toll/IL-1 receptor superfamily members (178, 215). TIR8/SIGIRR is currently designated as an orphan receptor for reasons attributed to its unique structure within the Toll/IL-1 receptor superfamily. It possesses a single extracellular Ig domain, distinct from the characteristic three domains of other family members such as the IL-1R1 or the IL-18 receptor and current consensus suggests the small size of this single extracellular portion precludes binding of a cognate extracellular ligand. Recently TIR8/SIGIRR has been identified as a modulator of autoimmune disease through its ability to inhibit the IL-1R1 driven proliferation of Th17 cells (181). Little is known of the impact TIR8/SIGIRR may elicit in human disease, however it is noteworthy that its expression is significantly down regulated in the peripheral blood of psoriatic arthritis patients (192). This observation coupled with the results of murine studies implicating an important role in the regulation of IL-23/IL-17 signalling strongly indicates a potential role in the regulation of psoriatic inflammation.

This portion of the study utilised a transgenic mouse deficient in *Tir8/Sigirr* $^{-/-}$ in order to characterise the impact of TIR8/SIGIRR deficiency on expression of innate IL-17A γδ T cells

driven by IL-1β and IL-23 stimulation. Furthermore, as these cells are established as important drivers of disease pathogenesis in psoriasis we sought to examine the role TIR8/SIGIRR plays in the regulation of dermal inflammation in this setting and provide a potential mechanistic link to published clinical observations (192).

4.2 Results

4.2.1 sCD25 and IL-1β enhance expression of IL-17A from Th17 cells.

To confirm previous reports of enhanced IL-1 mediated Th17 generation in the absence of SIGIRR/TIR8 (181), $CD4^+$ T cells were differentiated to a Th17 phenotype in the presence of either IL-1β or sCD25 as a positive control. IL-17A expression was examined by intracellular staining after 72h and a similar enhancement of Th17 polarisation was observed between the two treatments (Fig. 4.1A). Wild type (WT) or TIR8 -/- $CD4^+$ T cells were then differentiated to a Th17 phenotype in the presence of absence of IL-1β. After 72hours, levels of IL-17A in the cell culture supernatants were assessed by ELISA. IL-1β treatment enhanced expression of IL-17A, an effect which was further enhanced in cells lacking TIR8 (Fig. 4.1B). These data indicate that both sCD25 and IL-1 are capable of enhancing IL-17A expression from Th17 cells and reflect the opposing roles of both IL-1β and IL-2 in regulating Th17 responses (154, 174). These data confirm previous observations concerning the effects of IL-1β in enhancing Th17 responses and the key restraining role TIR8 plays in such settings, allowing us to examine these events in the context of regulation by TIR8 more closely (216).

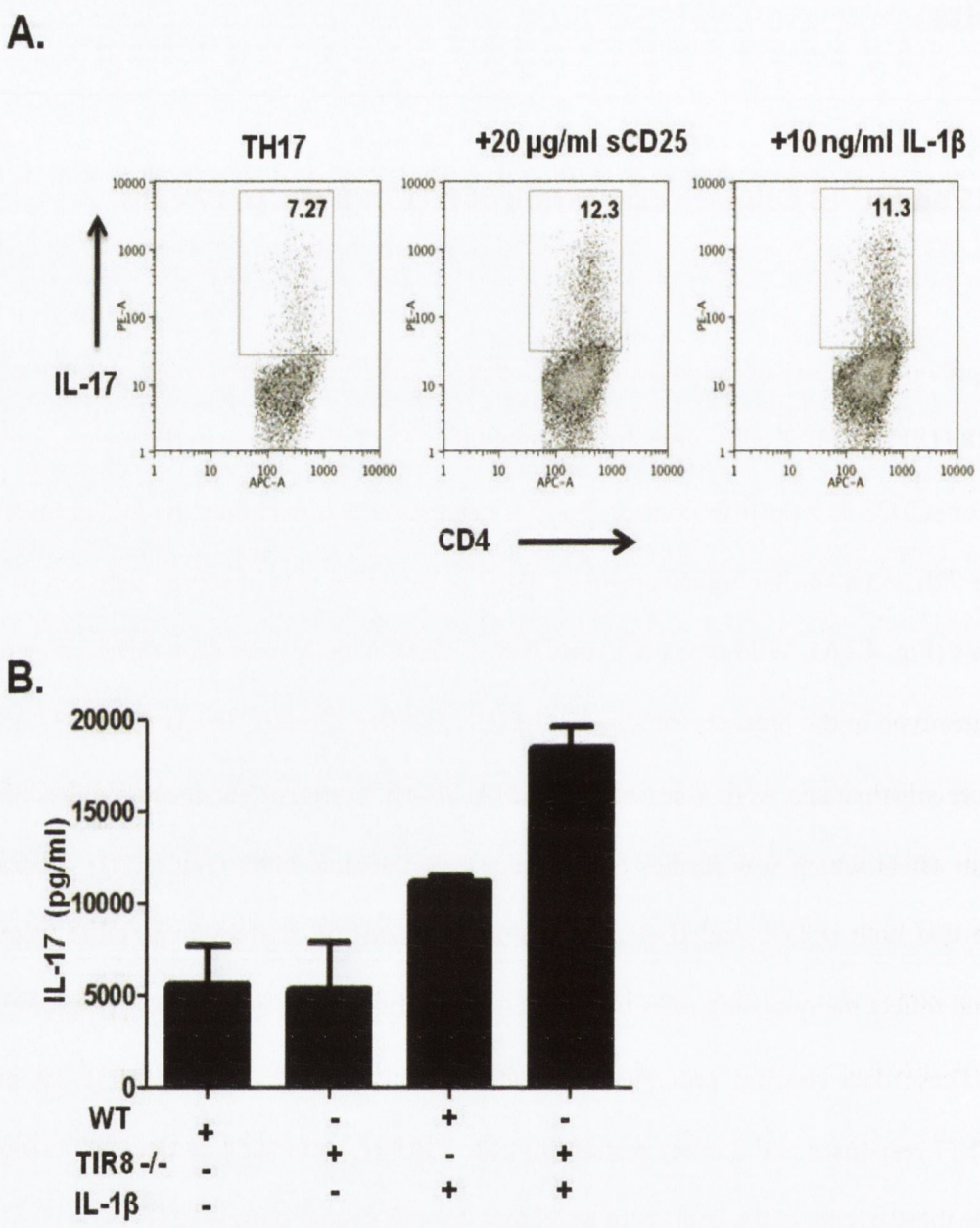


Fig. 4.1 sCD25 and IL-1β enhance expression of IL-17A from Th17 cells.
(A) $CD4^+$ T cells were purified by magnetic bead separation kit and differentiated to a Th17 phenotype in the epresence of sCD25 or IL-1 and IL-17 expression analysed by intracellular staining. (B) WT and *Tir8/Sigirr*$^{-/-}$ $CD4^+$ T cells were differentiated to a Th17 phenotype in the presence of absence of IL-1 before performing an ELISA for IL-17A Cells were gated initially on live lymphocytes followed by side scatter against CD4 positive before $CD4^+$ against the intracellular marker of interest.

4.2.2 TIR8$^{-/-}$ splenocytes express enhanced levels of Type 17 cytokines in response to IL-1β and IL-23

TIR8 has been identified as a negative regulator of IL-1β mediated Th17 cell development and proliferation (216). Whilst its role in the development of Th17 cells and impact on associated IL-17A expression and autoimmune pathology has been established, its role in the modulation of innate IL-17A from T cell subsets has yet to be clarified. Initial experiments focused on examining the role of TIR8 in the modulation of IL-1R1 signalling events in promoting innate type 17 responses.

Wild type and TIR8$^{-/-}$ splenocytes were cultured in the presence of IL-23 and increasing concentrations of IL-1β and after 72h levels of IL-17A in the cell culture supernatants were assessed by ELISA. IL-23 alone was not sufficient to induce detectable levels of IL-17A however addition of IL-1β drove IL-17A expression in a concentration dependant manner with significantly enhanced levels observed from TIR8 $^{-/-}$ cells compared to WT cells (Fig. 4.2A). As no exogenous antigen receptor stimulation was provided under these stimulatory conditions, these data demonstrate that innate IL-17A expression mediated by IL-1R1 is regulated by TIR8 activity. Moreover a number of other cytokines classically associated with type 17 responses were also enhanced including IL-22 (Fig. 4.2B), GM-CSF (Fig. 4.2F) and the IL-17A/F heterodimer (Fig. 4.2C). There were no significant differences in the expression of IFN-γ (Fig. 4.2D). Interestingly however, no differences were detected in IL-17F levels, suggesting possible differential regulation between expression of IL-17A and IL-17F in this setting.

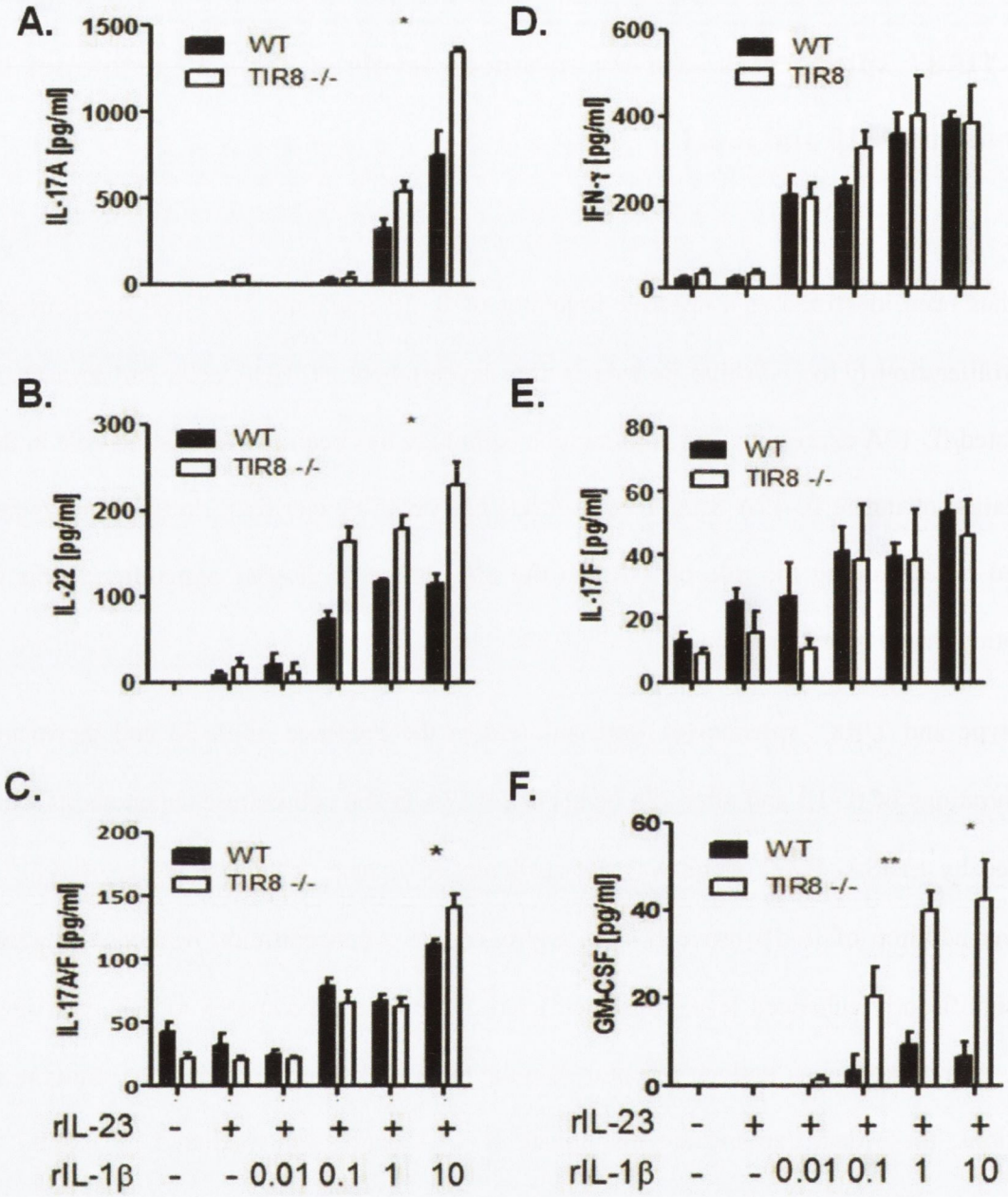


Fig. 4.2 *TIR8-/-* splenocytes express enhanced levels of Type 17 cytokines in response to IL-1β and IL-23

WT and *Tir8/Sigirr*$^{-/-}$ splenocytes were innately stimulated with IL-23 (10 ng/ml) and increasing concentrations of IL-1β for 96hrs. Cell culture supernatants were analysed by ELISA for levels of (A) IL-17A, (B) IL-22, (C) IL-17A/F (D) IFN-γ, (E) IL-17F, (G) GM-CSF. These data are representative of at least two independent experiments with similar results Statistical significance determined by unpaired student's t-test *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

4.2.3. IL-1β and IL-23 driven expression of IL-17A from splenocytes is mTOR dependant. Functional blockade *in vivo* of γδ T cells reduces IL-17A expression from splenocytes.

It has previously been demonstrated that γδ T cells represent a significant source of innate type 17 cytokine responses (66). To determine whether TIR8 regulated innate γδ responses in our system WT mice were injected intraperitoneally with an appropriate isotype control or an anti γδ-TCR antibody which is capable of functional blockade or depletion of these cells *in vivo*. Splenocytes from these mice were subsequently cultured with IL-1β and IL-23 as described above before analysis of cell culture supernatants by ELISA. A marked decrease in IL-17A expression was observed from mice treated with 250μg of anti γδ TCR antibody compared to the isotype control (Fig. 4.3A). This observation implicates γδ T cells as the likely source of the majority of IL-17A in these assays.

TIR8/SIGIRR is established as a negative regulator IL-1R signalling on Th17 cells, this dysregulation leads to enhanced proliferation and expression of IL-17A in an mTOR dependant mechanism (181). To examine the potential for similar dysregulation in our *in vitro* splenocyte assays we treated cells from WT and TIR8-/- mice with specific inhibitors of two known IL-1 dependant signalling pathways, JNK and mTOR, before culturing in the presence of IL-1β and IL-23 in a similar manner to that described above. While inhibition of IL-17A expression was observed in the presence of the JNK inhibitor SP600125, the impact of TIR8/SIGIRR deficiency was abolished in the presence of the mTOR inhibitor temsirolimus confirming the role of TIR8/SIGIRR in the modulation of mTOR mediated IL-1R signalling in γδ T cells (Fig. 4.3B).

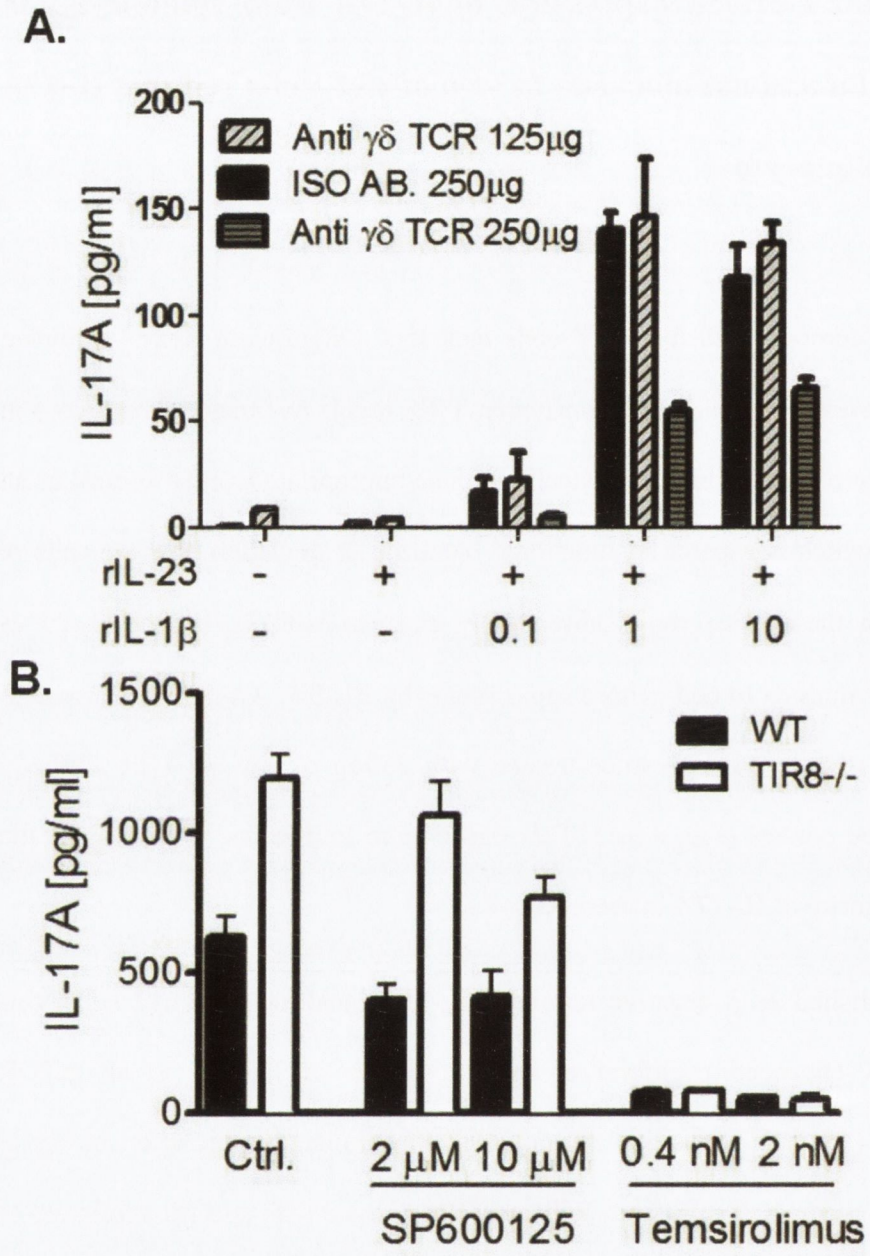


Fig. 4.3 Functional blockade *in vivo* of γδ T cells reduces IL-17A expression from spleoncytes.

IL-1β and IL-23 driven expression of IL-17A from splenocytes is mTOR dependant.

Functional blockade of γδ TCR by *in vivo* treatment was performed with indicated doses of anti γδ TCR antibody 48 hours prior to innate *in vitro* stimulation with IL-1β and IL-23. After 72hrs cell culture supernatants were analysed for levels of IL-17A by ELISA (B). WT and *Tir8/Sigirr* $^{-/-}$ splenocytes were pre-treated for 2 hours with the indicated doses of SP600125 or Temsirolimus before washing and innate stimulation with IL-23 (10 ng/ml) and IL-1β (10ng/ml) for 72hrs Cell culture supernatants were subsequently analysed for levels of IL-17A (A). These data are representative of at least two independent experiments with similar results Statistical significance determined by unpaired student's t-test *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

4.2.4 FACS purified γδ T cells express enhanced IL-17A and IL-22 in response to IL-1β and IL-23 stimulation but not TGF-β and IL-6

γδ T cells have been previously shown to express IL-17A directly in response to IL-1β and IL-23 stimulation (66). Similar to the splenocyte experiments previously described, purified γδ T cells from *tir8/sigirr*$^{-/-}$ mice express enhanced IL-17A when stimulated with IL-1β and IL-23 (Fig.4 A&B). Furthermore TIR8/SIGIRR also regulates the expression of IL-22 (Fig.4.4 C) and GM-CSF (Fig.4.4 D) in response to IL-1β and IL-23 stimulation

Conversely, other cytokines known to induce type 17 responses in $CD4^+$ T cells were not capable of inducing IL-17A expression from purified γδ T cells (Fig. 4.5A), even in the presence of TLR stimulated antigen presenting cells or by direct stimulation with the TLR7 agonist imiquimod (Fig. 4.5B). These data confirm the dependency of innate IL-17A from γδ T cells on IL-1β and IL-23 and further highlights the ability of TIR8/SIGIRR to regulate this signalling axis.

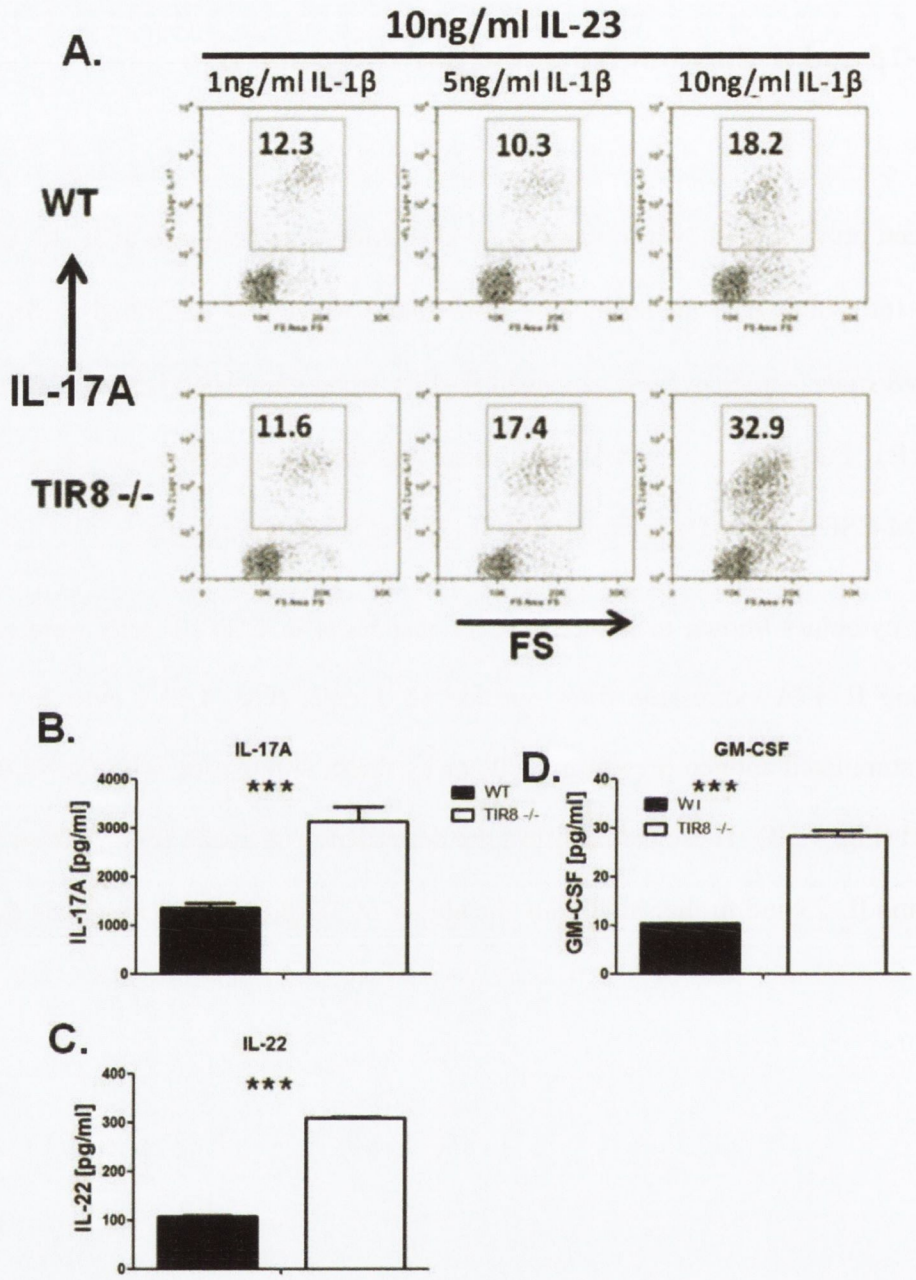


Fig. 4.4 TIR8/SIGIRR $^{-/-}$ γδ T cells express enhanced type 17 cytokines in response to IL-1β and IL-23

(A) FACS purified WT and *Tir8/Sigirr*$^{-/-}$ γδ T cells were innately stimulated with IL-23 (10 ng/ml) and increasing concentrations of IL-1β for 96hrs before analysis of IL-17A expression by intracellular staining. Cell culture supernatants were also analysed for expression of (B) IL-17A and (C) IL-22 and (D) GM-CSF after 96 hours Cells were gated initially on live lymphocytes before forward scatter against IL-17A. Statistical significance determined by unpaired student's t-test *p≤0.05, **p≤0.01, ***p≤0.001

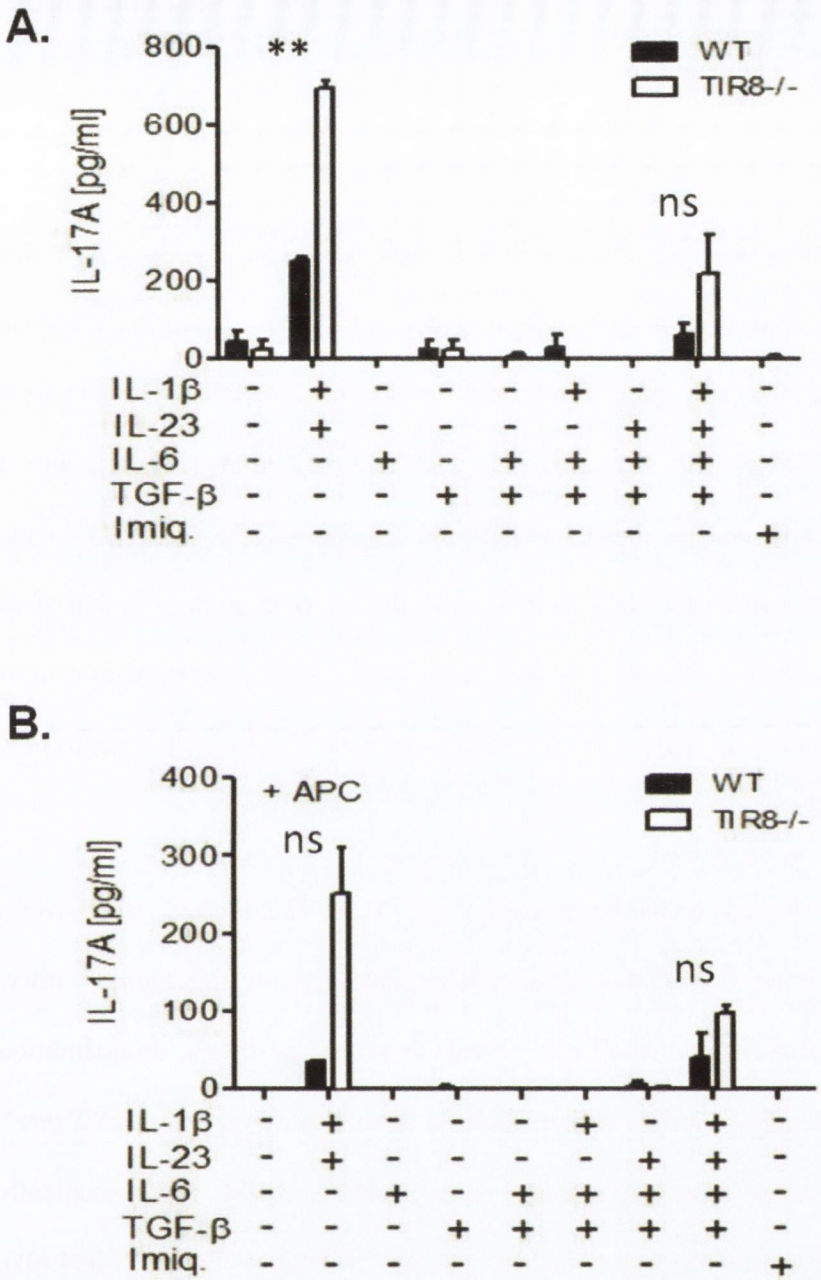


Fig. 4.5 γδ T cells do not express IL-17A in response to TGF-β and IL-6
Auto-MACS purified γδ T cells were cultured (A) in the absence or (B) presence of TLR activated APCs along with the indicated combinations of cytokines for 72 hours before analysis of the cell culture supernatants for IL-17A. These data are representative of a single experiment Statistical significance determined by unpaired student's t-test *p≤0.05, **p≤0.01, ***p≤0.001

4.2.5 TIR8-/- mice display enhanced disease in the Aldara™ induced model of psoriasiform inflammation.

Innate γδ T cells which express IL-17A in response to IL-1R1 stimulation are thought to play a key pathological role in a variety of autoimmune disorders with recent emerging evidence suggesting a critical role in the progression of psoriasis (66, 72). Interestingly it has been observed that TIR8/SIGIRR expression is significantly downregulated in the peripheral blood of psoriatic patients. However despite these observations the role of TIR8/SIGIRR in the regulation γδ T cell driven pathology, and more specifically its role in the regulation of dermal inflammation in psoriasis, have yet to be addressed (192). To answer these questions we utilised a murine model of psoriasiform inflammation where γδ T cells are known to play a key role in disease progression.

Aldara™ cream applied to the ears of wild type control (WT) and *Tir8/Sigirr* $^{-/-}$ mice over a period of 5-7 days results in an exacerbated inflammatory phenotype in *Tir8/Sigirr* $^{-/-}$ mice. Analysis of H&E stained sections of treated ears reveals enhanced acantosis, desquamation (scaling and outer layer shedding) and dermal infiltration of nucleated cells in *Tir8/Sigirr*$^{-/-}$ mice (Fig.4.6A). This enhanced disease phenotype is also clearly visible macroscopically with enhanced desquamation and erythema on the ears of *Tir8/Sigirr*$^{-/-}$ mice (Fig.4.6B). Furthermore, measurement of the overall thickness of treated ears revealed significantly enhanced epidermal thickening in *Tir8/Sigirr*-/- mice (Fig. 4.6C). The enhanced disease phenotype is also visible histologically. (Fig.4.7A) and macroscopically (Fig.4.7B) on *Tir8/Sigirr*$^{-/-}$ mice when Aldara ™ is applied to the shaved back area.

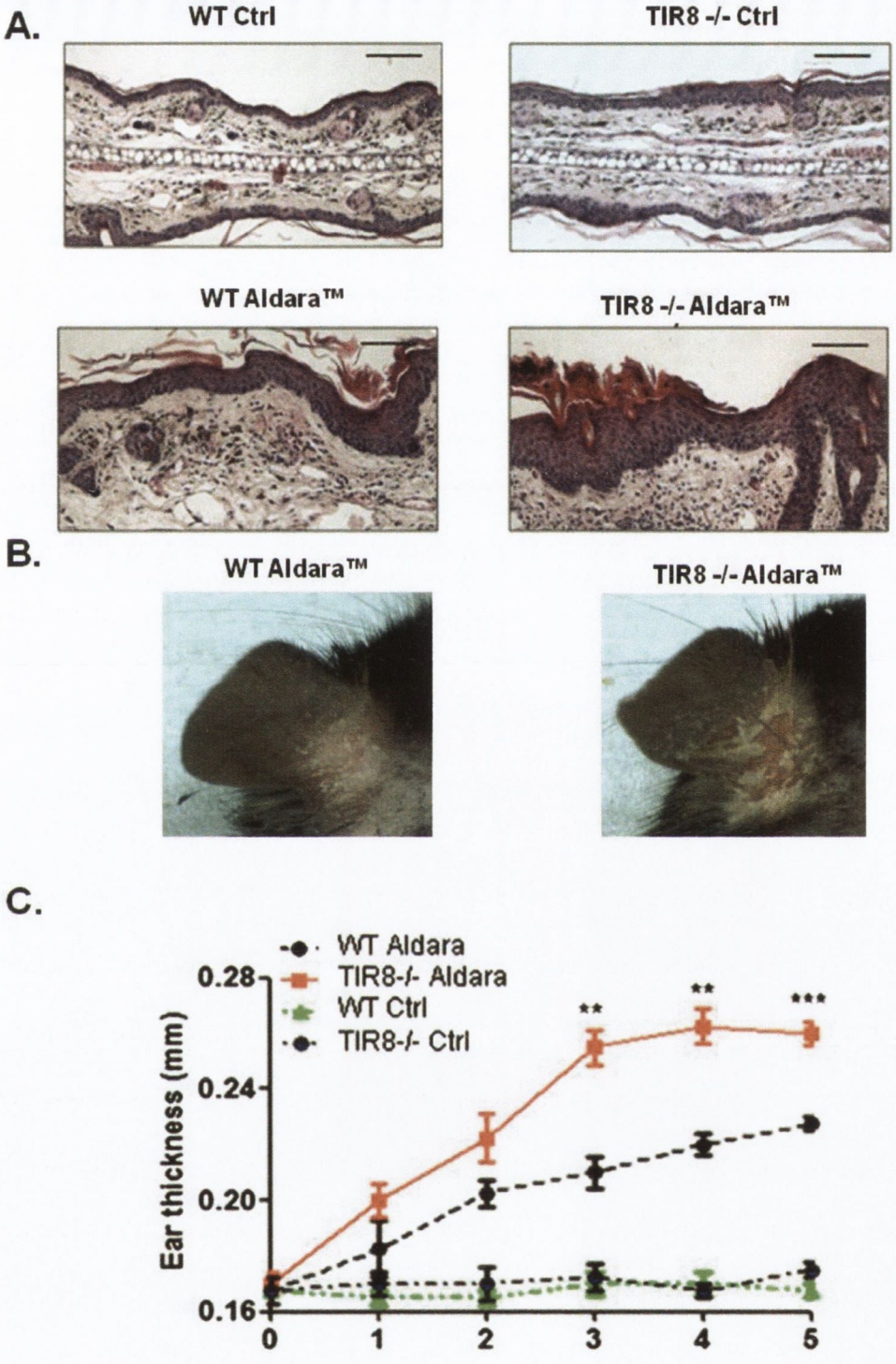


Fig. 4.6 TIR8/SIGIRR$^{-/-}$ mice display enhanced disease in the Aldara™ induced model of psoriasiform inflammation.

(A) H&E stained sections from ears of WT and *Tir8/Sigirr*-/- mice treated with Aldara™ (1mg/day) or control cream for 5 days (Scale bar, 50μm). (B) Representative macroscopic photographs of ears from WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara™. (C) Increased epidermal thickening in *Tir8/Sigirr*-/- mice treated with Aldara™. . These data are representative of at least five independent experiments with similar results Statistical significance determined by unpaired student's t-test, *p≤0.05 **p≤0.01, ***p≤0.001.

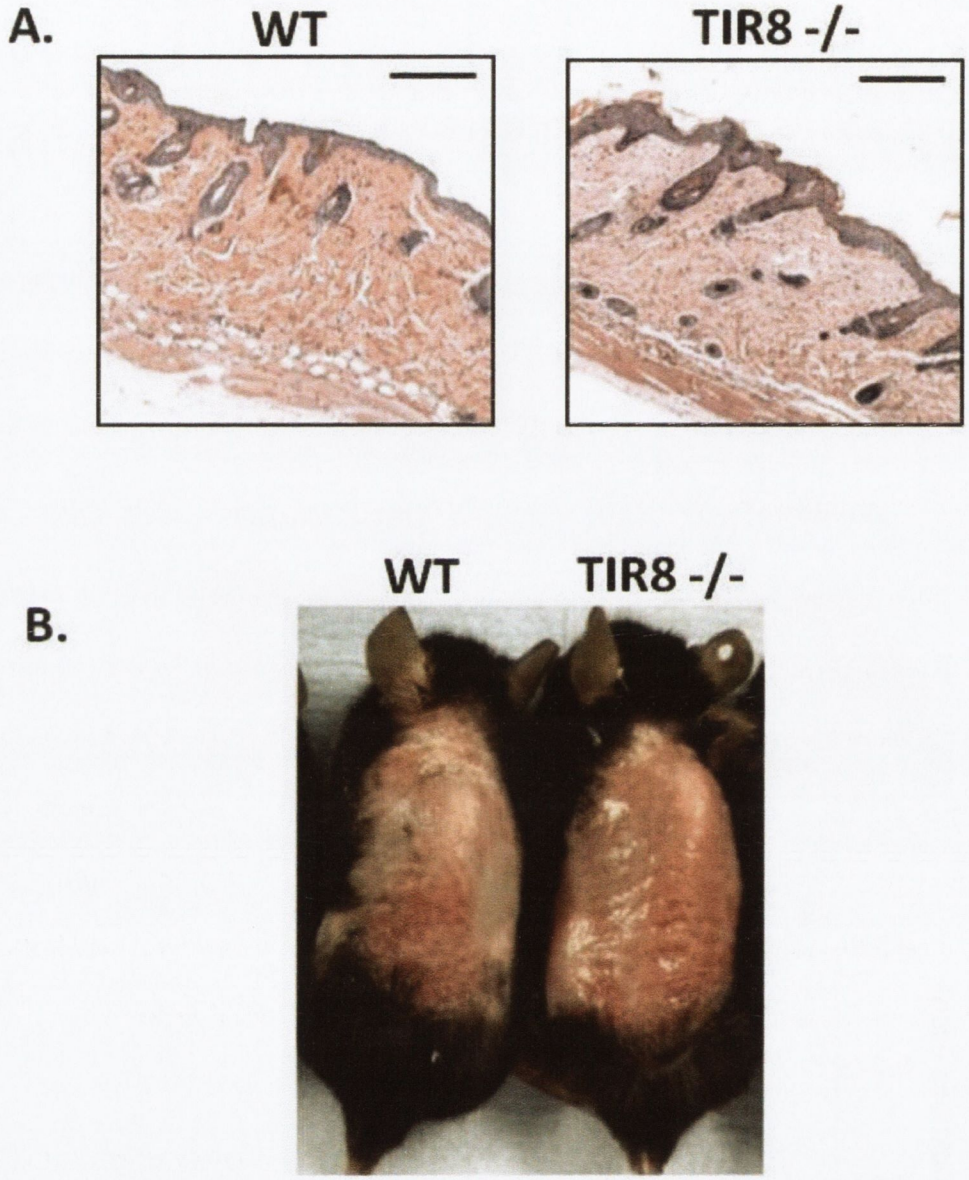


Fig 4.7 TIR8/SIGIRR$^{-/-}$ mice display enhanced disease in the Aldara™ induced model of psoriasiform inflammation when applied to the back

(A) H&E stained sections from back skin of WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara™ or control cream for 5 days (Scale bar, 50μm). (B) Representative macroscopic photographs of WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara™). These data are representative of at least two independent experiments with similar results

4.2.6 TIR8/SIGIRR expression is relatively high on γδ T cells compared with other immune cells.

TIR8/SIGIRR expression has been identified on $CD4^+$ T cells (181, 182) however expression levels on innate γδ T cells, cells which have been demonstrated as playing a key role in the progression of inflammation in this setting, has yet to be determined. We examined CD3+, $CD4^+$, $CD3^+$ γδ- and γδ T cells along with murine skin expression of *Tir8/Sigirr* by real time PCR analysis. Strikingly basal expression of the gene was found to be highest in γδ T cells compared to other cells and tissues examined (Fig. 4.8A).

Application of Aldara™ is thought, in part, to drive psoriasiform inflammation through induction of IL-23 and IL-1β from dendritic cells and keratinocytes (142, 214). Furthermore IL-1 and IL-23 are known to act synergistically to drive expression of IL-17A from γδ T cells and γδ T cells expressing large amounts of IL-17A are established as key drivers of inflammation in settings of ALDARA™ induced psoriasiform inflammation. In order to investigate the possibility that an enhancement of either, or both, of these cytokines in the *Tir8/Sigirr*$^{-/-}$ mouse was accounting for the disease phenotype observed, skin biopsies from treated mice were homogenised and analysed for protein expression levels of both IL-1β (Fig.4.8B) and IL-23 (Fig. 4.8C). Aldara™ treatment clearly increased levels of both IL-1β and IL-23 over time, however no significant differences were observed in the levels of expression of IL-1β or IL-23 between WT and *Tir8/Sigirr*$^{-/-}$ mice. Interestingly we also observed significantly enhanced expression of GM-CSF at this timepoint in the Aldara™ treated skin of *Tir8/Sigirr* $^{-/-}$ mice. (Fig.4.8D). Together these data indicate that TIR8/SIGIRR plays a role in the regulation of Aldara™ induced psoriasiform inflammation in mice, however due to lack of difference observed between IL-1β and IL-23 protein levels this

activity is likely not mediated through dysregulated myeloid or stromal cell activity. Importantly, elevated GM-CSF was observed in *Tir8/Sigirr* $^{-/-}$ mice which is suggestive of hematopoetic cell involvement.

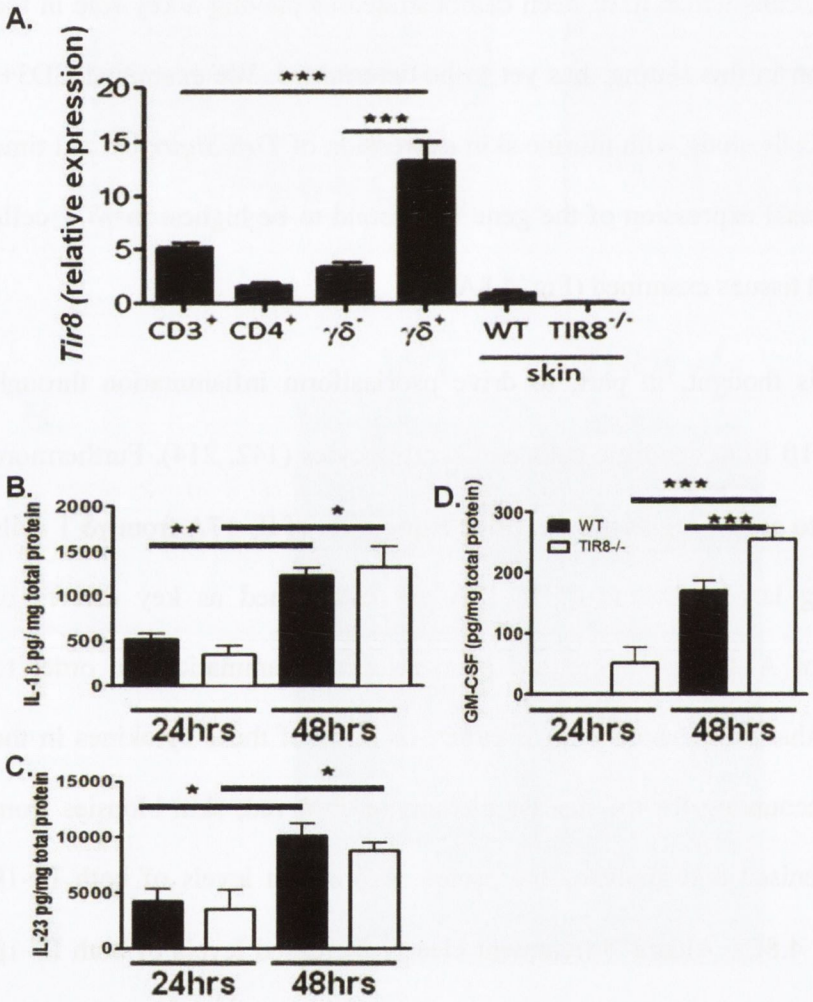


Fig. 4.8 TIR8/SIGIRR expression is relatively high on γδ T cells compared with other immune cells. Unaltered protein expression of IL-1β and IL-23 between WT and TIR8/SIGIRR$^{-/-}$ in skin lysates

(A) Real time PCR analysis for basal expression of *Tir8/Sigir* in the indicated cells and tissues. CD3^{+}, CD4^{+} and γδ T cells were purified by magnetic bead separation. Skin sections were isolated from the shaved backs of naïve WT and *Tir8/Sigirr*$^{-/-}$ before isolation of RNA for analysis. WT and *Tir8/Sigirr*$^{-/-}$ - mice were treated for either 24hrs or 48hrs by topical application of Aldara™ cream. ELISAs for (B) IL-1β , (C) IL-23 and (D) GM-CSF were performed on tissue lysates from ears from WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara™ for the indicated times. significance determined by unpaired student's t-test, *p≤0.05 **p≤0.01, ***p≤0.001

4.2.7 Elevated IL-17A transcripts in skin biopsies of TIR8-/- mice treated with Aldara.

Skin biopsies from WT and TIR8$^{-/-}$ mice treated with Aldara for 5 days were subjected to real time PCR analysis for IL-17A (Fig. 4.9A), IL-1β (Fig. 4.9B), IL-22 (Fig 4.9C) and IL-23 (Fig 4.9D). No differences were observed in the detectable levels of IL-1β or IL-23. Although IL-22 was also unchanged IL-17A was significantly elevated from the skin of *Tir8/Sigirr-/-* mice. These data further indicate dysregulated hematopeoietic cell involvement rather than a defect in myeloid or stromal cells in the enhanced disease observed in this setting.

4.2.8 Elevated IL-17A from γδ T cells in the skin draining lymph nodes of ALDARA™ treated mice.

γδ T cells expressing IL-17A and IL-22 have been implicated as important drivers of disease pathogenesis in the Aldara™ induced model as well as human psoriasis. (36, 72, 119, 129). To assess any impact of *Tir8/Sigirr* deficiency on the expression of these cytokines from T cell populatoins in this disease model skin draining lymph nodes of ALDARA™ treated mice were isolated and stained for IL-17A and IL-22. Significantly enhanced levels of IL-17A, but not IL-22, expression was observed from *Tir8/Sigirr*$^{-/-}$ γδ T cells compared to WT cells. (Fig. 4.10A&B). γδ T cells, rather than Th17 cells, appear to be the primary source of IL-17A in this setting as limited expression of IL-17A and IL-22 from CD4^{+} T cells was observed (Fig. 4.10C&D). These data demonstrate that in the absence of TIR8/SIGIRR there is increased

Aldara™ induced psoriasiform dermal inflammation that occurs in association with increased IL-17A expression by γδ T cells.

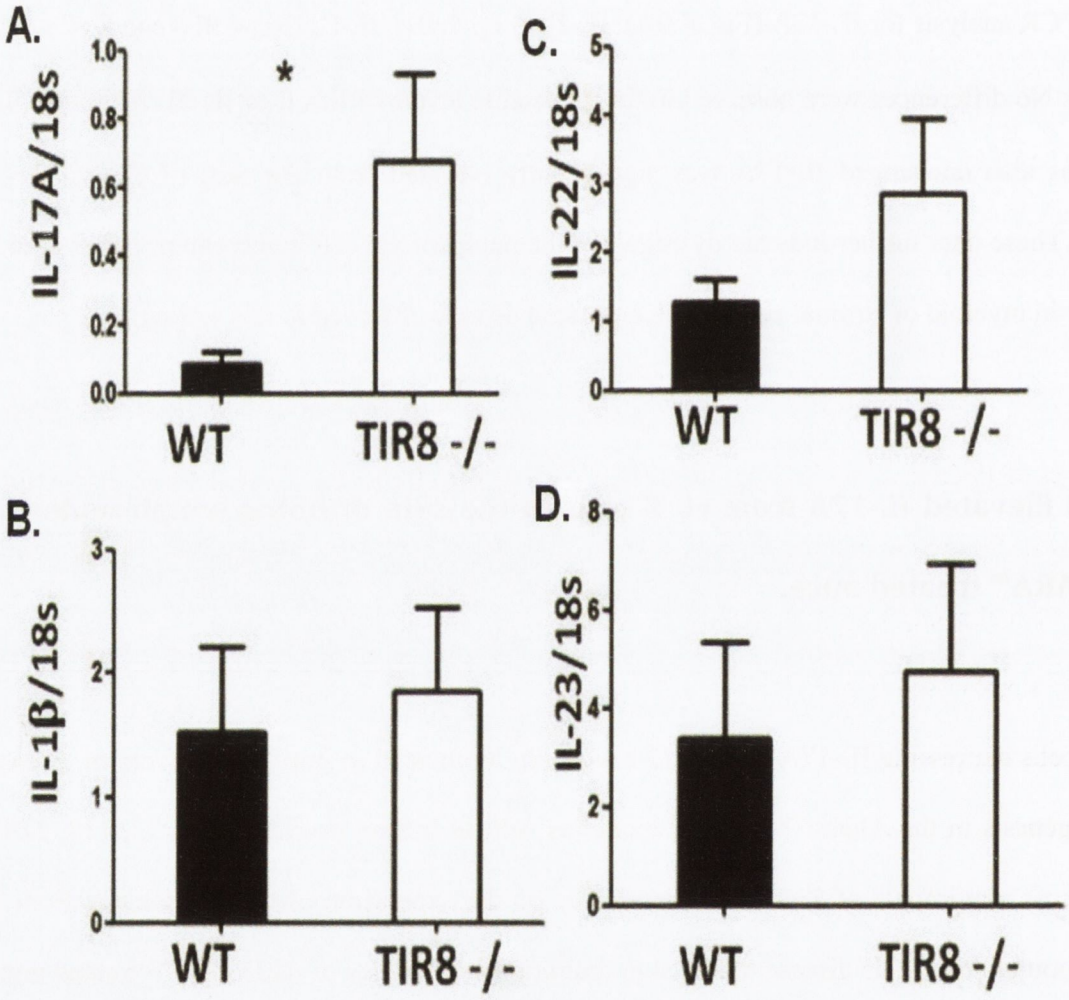
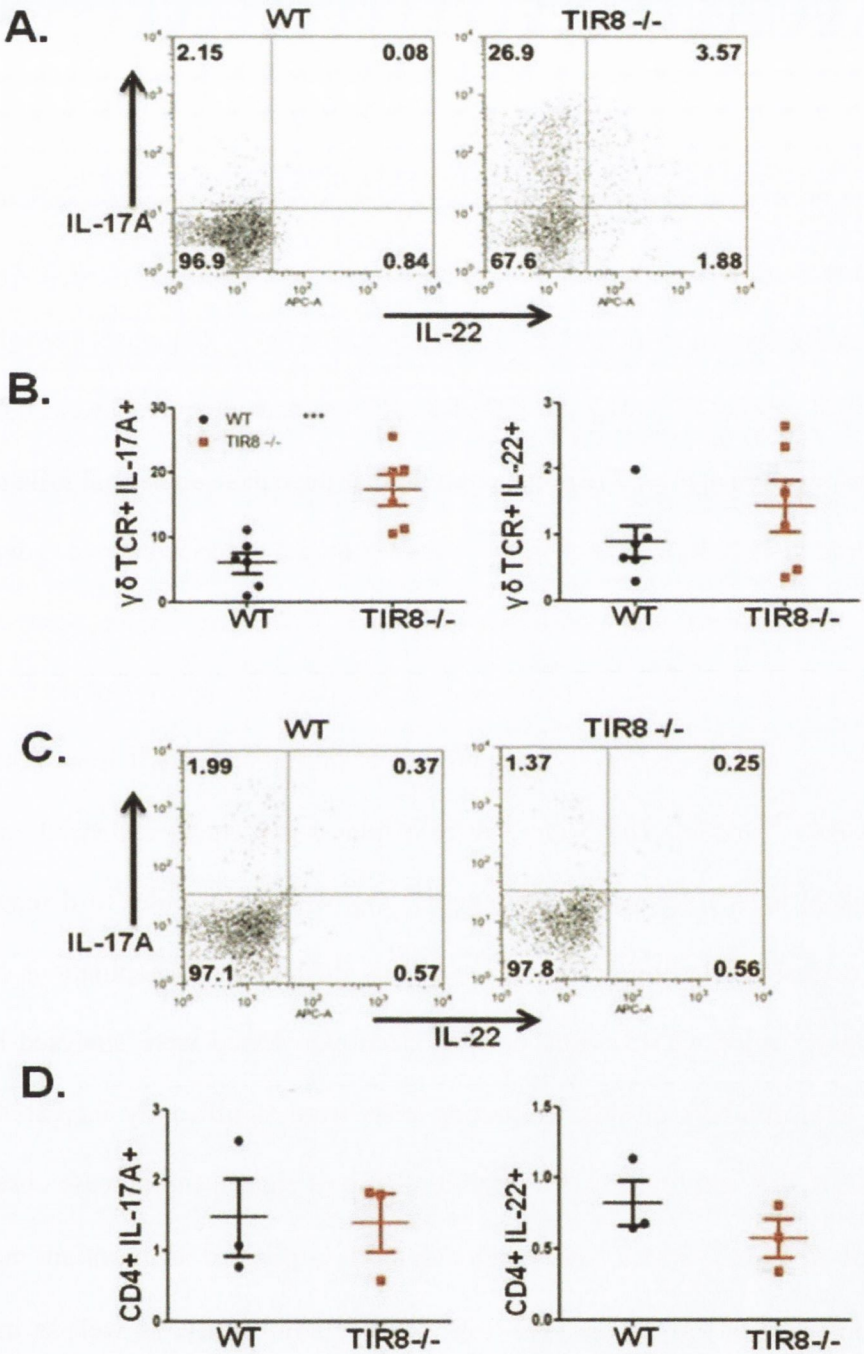


Fig 4.9 Elevated IL-17A transcripts in the skin of TIR8/SIGIRR$^{-/-}$ mice
Biopsies of skin treated with Aldara™ for 5 days were subjected to real time PCR analysis for (A) IL-17A (B) IL-1β (C) IL-22 (D) IL-23 Statistical significance determined by unpaired student's t-test, These data are representative of at least two independent experiments with similar results
*$p \leq 0.05$.



4.10 Elevated IL-17A from γδ T cells in the skin draining lymph nodes of ALDARA™ treated mice.

Cells from skin draining lymph nodes of WT and *Tir8/Sigirr*$^{-/-}$ mice were examined by flow cytometry for intracellular IL-17A and IL-22 expression from (A&B) γδ T cells and (C&D) CD4^{+} T cells. These data are representative of at least three independent experiments with similar results. Cells were gated initially on live lymphocytes followed by CD3 against γδ TCR or side scatter against CD4. γδ TCR or CD4 positive cells were then gated agains the intracellular markers of interest. Statistical significance determined by unpaired student's t-test***p≤0.001.

4.2.9 Intradermal IL-23 injection results in significantly enhanced dermal inflammation in Tir8/Sigirr$^{-/-}$ mice.

Intradermal injection of IL-23 is another widely used model of psoriasiform inflammation which is robustly comparable to the Aldara™ model in terms of the key cellular mechanisms involved such as the induction of an IL-1R1-dependant type-17 response through direct stimulation of T cell subsets (72, 148). As TIR8/SIGIRR has been characterised as a negative regulator of TLR7 signalling (217, 218), it is possible that the enhanced dermal inflammatory phenotype observed in Tir8/Sigirr$^{-/-}$ mice in the Aldara™ model described above is dependent on this activity. To address this possibility WT and Tir8/Sigirr$^{-/-}$ mice were injected intradermally with recombinant IL-23 or vehicle control daily for up to 7 days. Consistent with previous reports IL-23 injection induced epidermal thickening in WT mice (148, 219). Similar to the Aldara^{TM} model, *Tir8/Sigirr*$^{-/-}$ mice display significantly enhanced epidermal thickening compared to WT controls in this model (Fig. 4.11A). In order to determine the composition of dermal infiltrating cells and assess any differences in populations between WT and *Tir8/Sigirr*$^{-/-}$ mice, CD45^{+} cells purified from skin tissue were analysed by flow cytometry. The total numbers of skin infiltrating cells were significantly increased in the absence of TIR8/SIGIRR activity (Fig. 4.11B) with the most significant increase observed in the Vγ4 subset of γδ T cells, which have previously been implicated as important mediators of dermal inflammation in this model (72) (Fig. 4.11C). Importantly, as well as increased numbers, expression levels of IL-17A on a per cell basis were also significantly enhanced among *Tir8/Sigirr*$^{-/-}$ Vγ4 cells, with these cells accounting for the vast majority of IL-17A expressing cells in this setting (Fig. 12A&D). Although increased numbers of CD4^{+} T cells were also observed in inflamed skin and their expression of IL-17A was elevated, their overall contribution to IL-17A levels was limited when compared to that observed from

innate subsets (Fig. 4.12B&D). Finally, expression of IL-17A by Vγ4-ve γδ T cell subsets and a small population of $CD45^+$ non-T cells was also significantly enhanced in *Tir8/Sigirr*$^{-/-}$ mice (Fig. 4.12C&D). These data demonstrate that intradermal administration of IL-23 also leads to enhanced psoriasiform inflammation in *Tir8/Sigirr*$^{-/-}$ mice and this occurs in association with increased skin infiltration of Vγ4 γδ T cells expressing elevated levels of IL-17A .

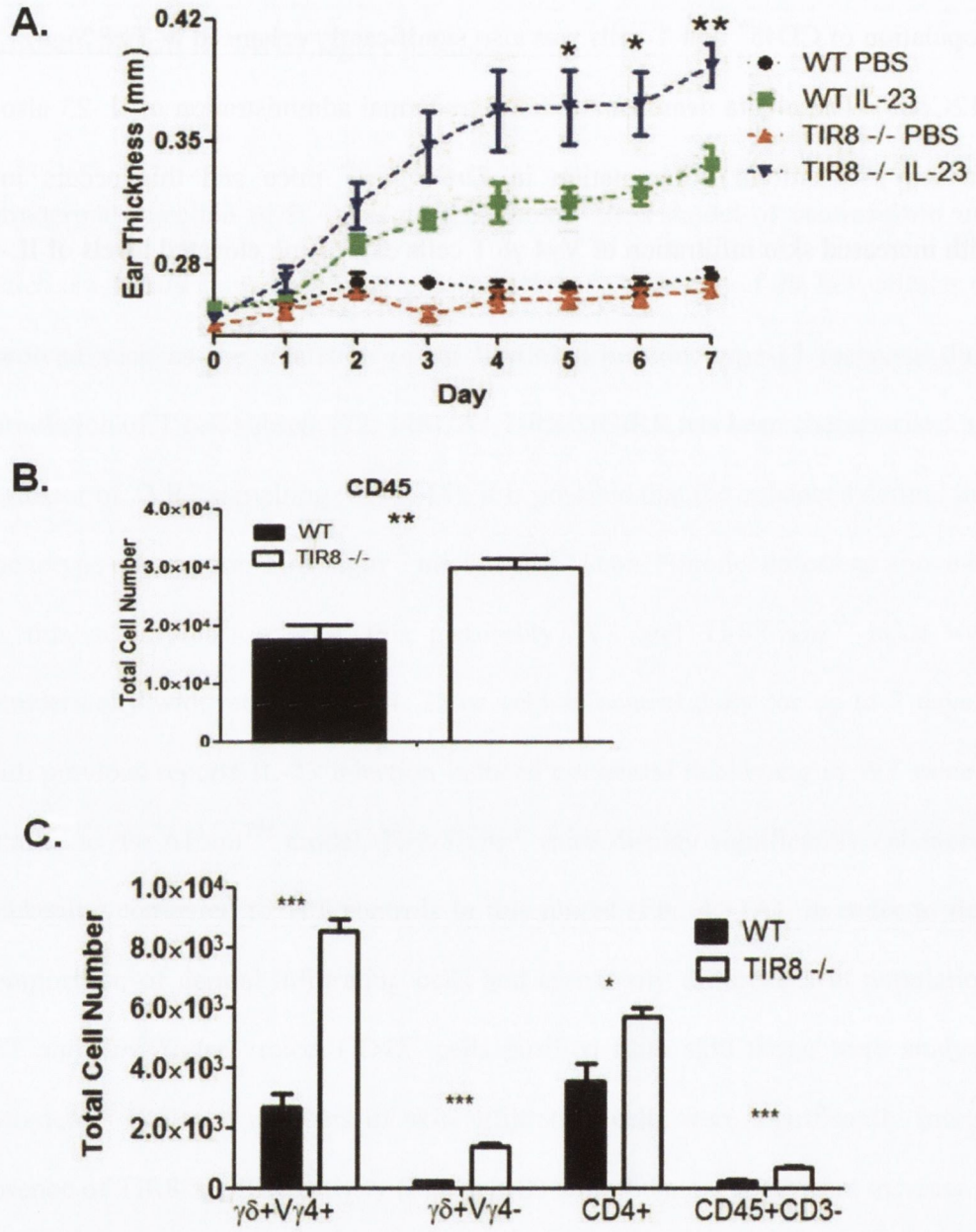


Fig. 4.11 Intradermal IL-23 injection results in significantly enhanced dermal inflammation in Tir8/Sigirr$^{-/-}$ mice.

(A) Intradermal injection of IL-23 (200ng) or vehicle control into the ears of WT and *Tir8/Sigirr*$^{-/-}$ mice resulted in enhanced epidermal thickening in *Tir8/Sigirr*$^{-/-}$ mice. Increased numbers of (B) total and (C) specific immune cell subsets infiltrating the skin of *Tir8/Sigirr*$^{-/-}$ mice after rIL-23 treatment for 7 days. Cells were gated initially on live lymphocytes followed by CD3 against γδ TCR or side scatter against CD4. γδ TCR or CD4 positive cells were then gated agains the intracellular markers of interest These data are representative of at least two independent experiments with similar results. Statistical significance determined by unpaired student's t-test, *p≤0.05 **p≤0.01, ***p≤0.001.

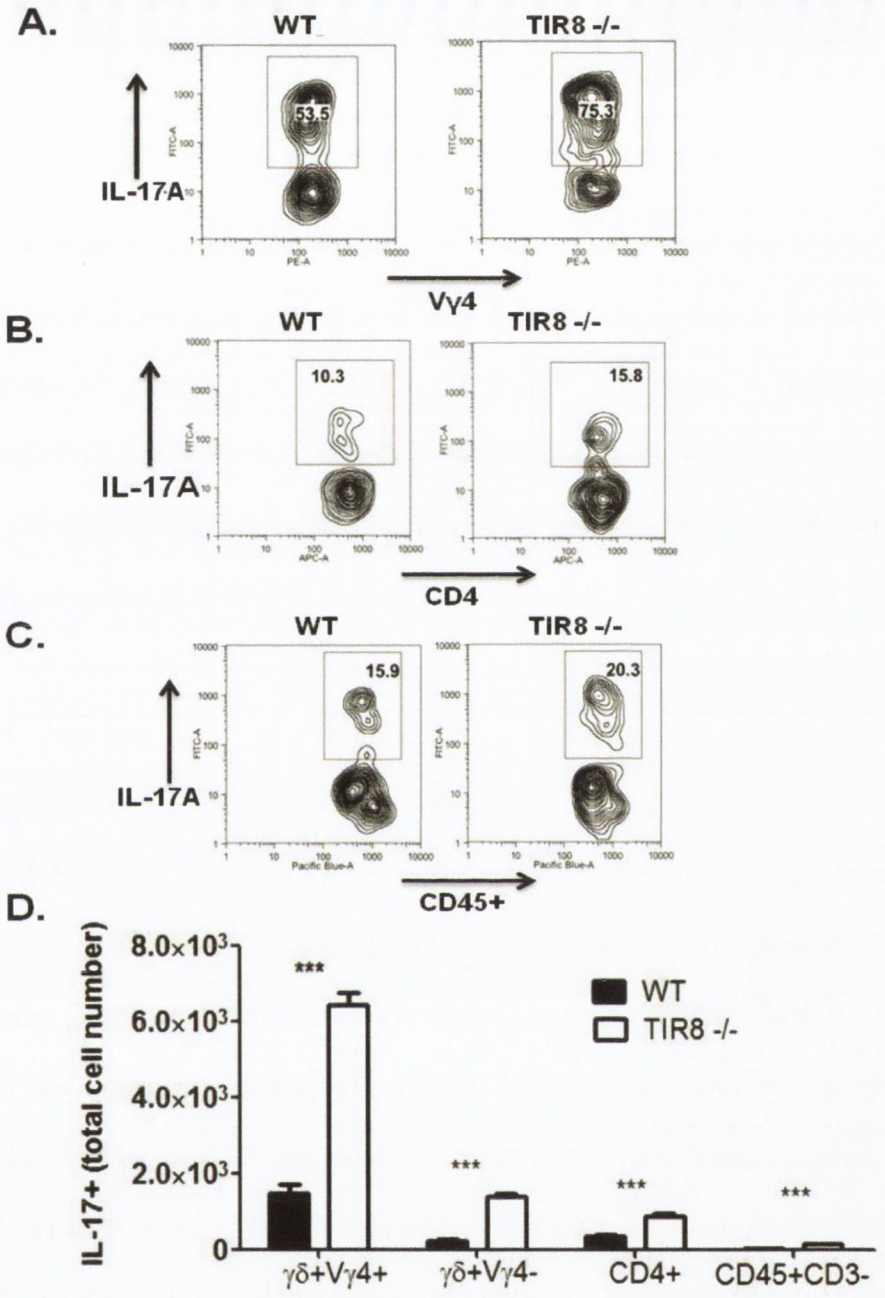


Fig. 4.12 Enhanced IL-17A expression from infiltrating immune cell after IL-23 intradermal injection.

Skin infiltrating immune cell subsets were analysed by flow cytometry for intracellular IL-17A expression by (D) Vγ4 cells (E) $CD4^+$ T cells and (F) CD3- $CD45^+$ cells (G) IL-17A positive cell numbers of skin infiltrating cell subsets from IL-23 treated WT and *Tir8/Sigirr*$^{-/-}$ mice. ™). . Cells were gated initially on live lymphocytes followed by CD3 against γδ TCR or side scatter against $CD4^+$. γδ TCR or CD4 positive cells were then gated agains the intracellular markers of interest These data are representative of at least two independent experiments with similar results Statistical significance determined by unpaired student's t-test, $^{**}p\leq0.01$, $^{***}p\leq0.001$.

4.2.10 IL-1β and IL-23 induce enhanced innate IL-17A and IL-22 expression from Tir8/Sigirr $^{-/-}$ γδ T cell subsets *in vivo*.

In support of the role IL-23 plays in the progression of IL-17A-driven dermal inflammation, we were interested to assess the potential role TIR8/SIGIRR plays in regulating IL-1 and IL-23 signalling in the context of innate IL-17A expression by γδ T cell subsets. To address these questions we utilised an *in vivo* innate stimulation system whereby WT and *Tir8/Sigirr*$^{-/-}$ mice were injected in the hind footpad with a combination of IL-1β and IL-23 (100ng/mouse of each) as described previously (7). After 4 hours, popliteal lymph node cells were harvested from treated mice and untreated controls and cultured in the presence of Brefeldin A, without further *ex vivo* stimulation, before analysis by flow cytometry. No significant expression of IL-17A was detected in the untreated controls compared to isotype (Fig. 4.13A), however significantly elevated expression of IL-17A was observed from *Tir8/Sigirr*$^{-/-}$ γδ T cells from mice treated with IL-1β and IL-23 (Fig. 4.13B&13E). IL-22 was also elevated (Fig. 4.13C&13E) but there were no differences in IL-17F expression, which was detected at relatively low levels (Fig. 4.12&12E). The Vγ4 subset once again was the predominant subset expressing elevated IL-17A, but it is noteworthy that another population of Vγ4 negative γδ T cells also expressed increased levels of IL-17A (Fig. 4.14A). As TIR8/SIGIRR has previously been reported to suppress Th17 proliferation driven by IL-1β we assessed the possibility that the enhanced IL-17A observed in this assay system was as a consequence of dysregulated expansion of *Tir8/Sigirr*$^{-/-}$ cells (181). However, no detectable differences in γδ T cell BrdU incorporation over normal homeostatic levels were observed (Fig. 4.14B and Fig. 4.14C). This observation indicates a direct effect of TIR8/SIGIRR activity in regulating innate IL-17A expression without altering cell expansion *in vivo*.

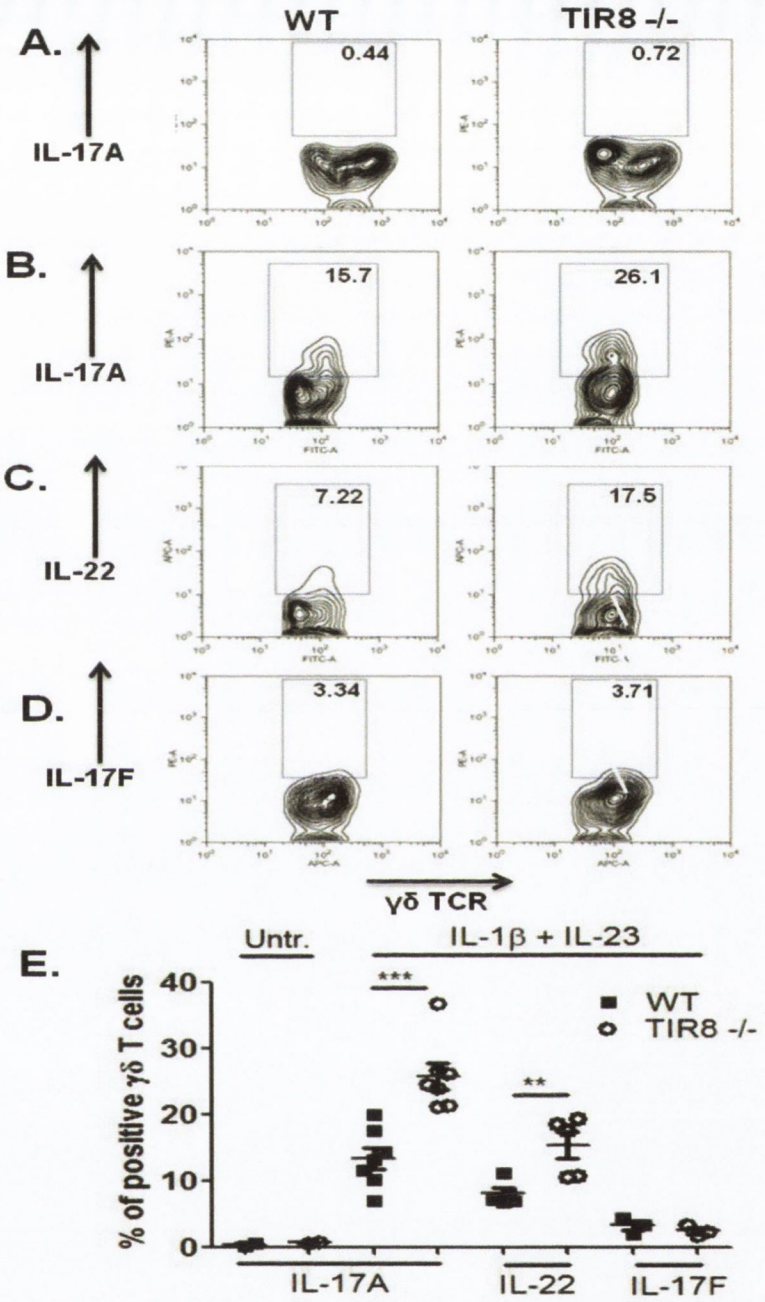


Fig. 4.13 IL-1β and IL-23 induce enhanced innate IL-17A and IL-22 expression from Tir8/Sigirr $^{-/-}$ γδ T cell subsets *in vivo*.

WT and *Tir8/Sigirr*$^{-/-}$ mice injected in the hind footpad with a combination of IL-1β (100ng) and IL-23 (100ng). After 4 hours popliteal lymphnodes were harvested from treated mice and untreated controls. γδ T cells were subsequently analysed by flow cytometry for intracellular expression of the following cytokines. (A) IL-17A from untreated mice, (B) IL-17A from treated mice (C) IL-22 from treated mice and (D) IL-17F from treated mice. (E) Expression of cytokines by γδ T cells from untreated and treated mice. Each dot indicates one mouse.. These data are representative of at least two independent experiments with similar results. . Cells were gated initially on live lymphocytes followed by CD3 against γδ TCR. γδ TCR positive cells were then gated agains the intracellular markers of interest Statistical significance determined by unpaired student's t-test $^{**}p\leq0.01$, $^{***}p\leq0.001$.

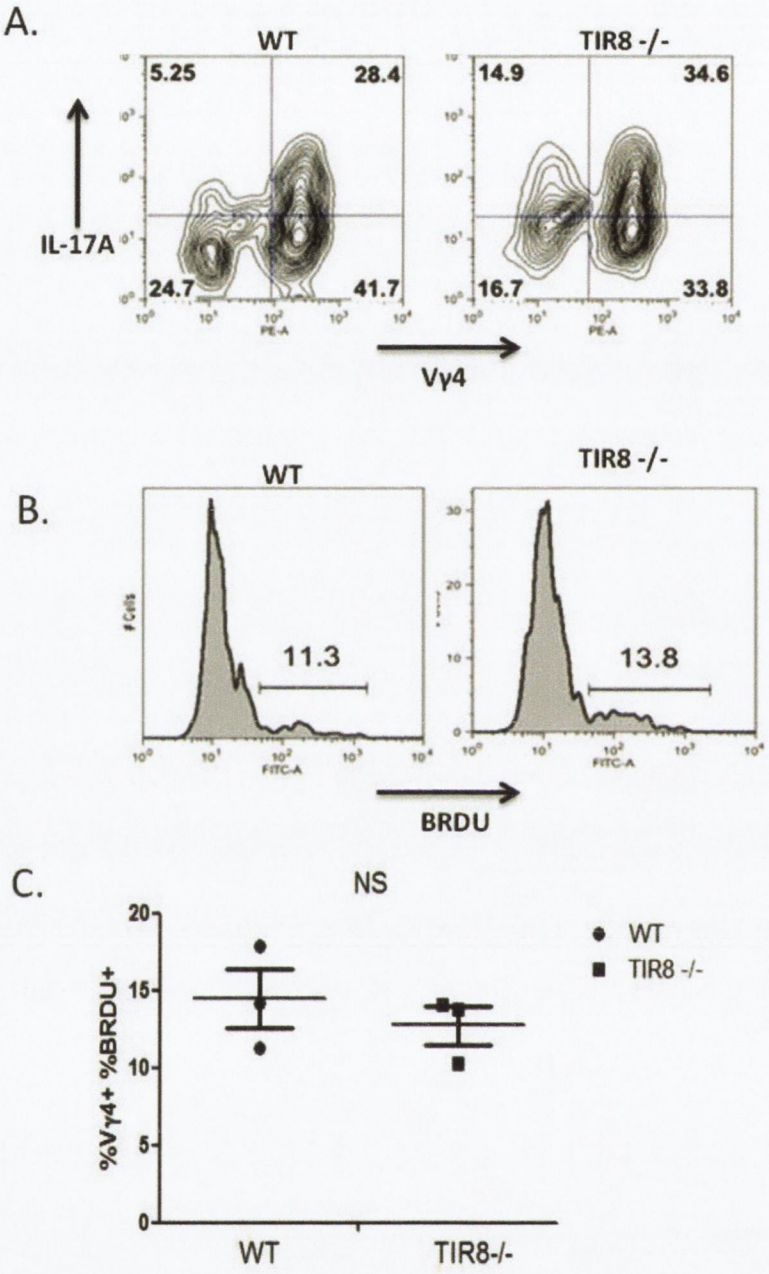
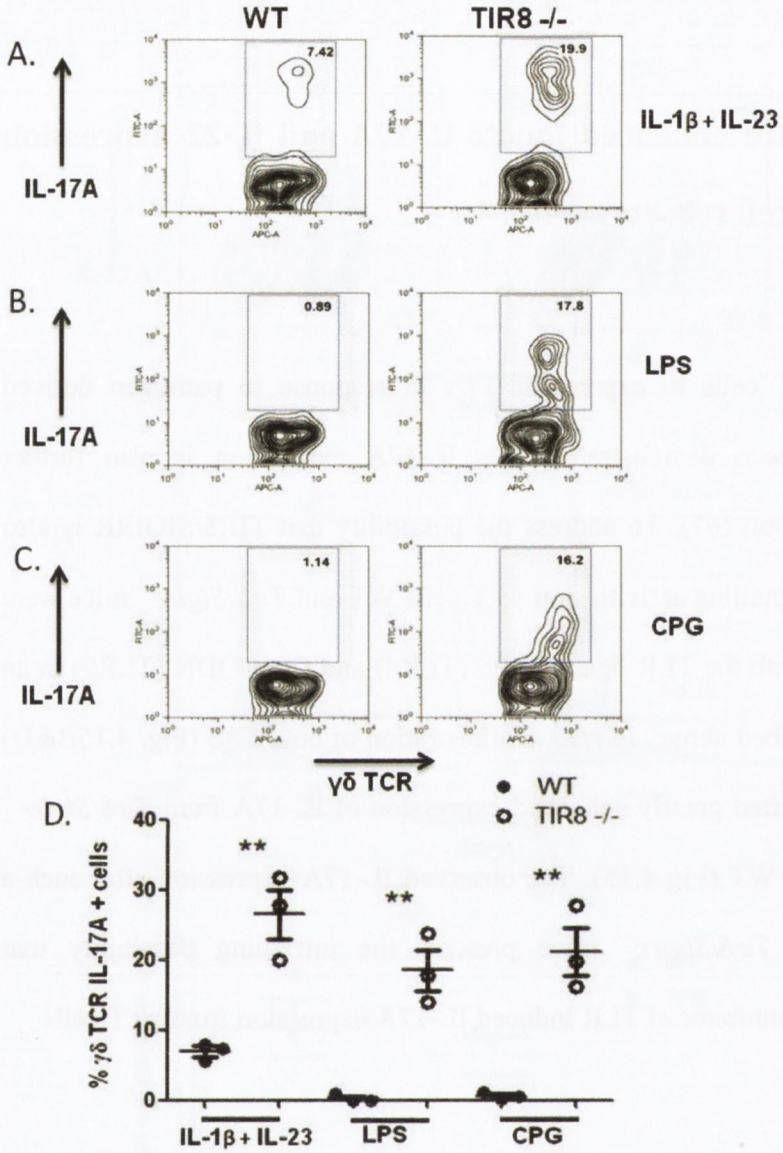


Fig. 4.14 TIR8/SIGIRR directly regulates expression of IL-17A from Vγ4 γδ T cells *in vivo*
(A) Expression of innate IL-17A by the Vγ4 subset of γδ T cells from WT and *Tir8/Sigirr*$^{-/-}$ *mice* treated with IL-1β and IL-23 in the footpad. (B) Analysis of BrdU incorporation by the Vγ4 subset after *in vivo* stimulation with IL-1β and IL-23. (C) Each dot represents a single mouse from (B).. Statistical significance assessed by unpaired student's t-test. Cells were gated initially on live lymphocytes followed by CD3 against γδ TCR . γδ TCR positive cells were then gated agains the intracellular markers of interest significance determined by unpaired student's t-test, *p≤0.05 **p≤0.01, ***p≤0.001

4.2.11 TLR ligands induce enhanced innate IL-17A and IL-22 expression from Tir8/Sigirr $^{-/-}$ γδ T cell subsets *in vivo*.

Recently the ability of γδ T cells to express IL-17A in response to pathogen derived environmental signals has been demonstrated, this IL-17A expression is also further enhanced with IL-23 stimulation (67). To address the possibility that TIR8/SIGIRR is also capable of regulating these signalling activities on γδ T cells WT and *Tir8/Sigirr*$^{-/-}$ mice were treated by footpad injection with the TLR ligands LPS (TLR4) and CPG-ODN (TLR9) in an identical manner to that described above. *In vivo* administration of both LPS (Fig. 4.15B&D) and CPG (Fig. 4.15C&D) elicited greatly enhanced expression of IL-17A from *Tir8/Sigirr*$^{-/-}$ γδ T cells when compared to WT (Fig 4.15). The observed IL-17A expression after such a short period of time from *Tir8/Sigirr*$^{-/-}$ mice presents the intriguing possibility that TIR8/SIGIRR acts as a direct inhibitor of TLR induced IL-17A expression from γδ T cells.



4.2.15 TLR ligands induce enhanced innate IL-17A and IL-22 expression from Tir8/Sigirr $^{-/-}$ γδ T cell subsets *in vivo*.

WT IL-17AeGFP and *Tir8/Sigirr*$^{-/-}$ eGFP mice were injected in the hind footpad with either a combination of (A) IL-1β (100ng) and IL-23 (100ng), (B) LPS (500ng) or (C) CpG-ODN (5μg). After 4 hours popliteal lymph nodes were harvested and γδ T cells were analysed by flow cytometry for expression of IL-17A.. Cells were gated initially on live lymphocytes followed by CD3 against γδ TCR . γδ TCR positive cells were then gated agains the intracellular markers of interest Statistical significance determined by unpaired student's t-test, **p≤0.01.

4.2.12 Enhanced severity of psoriasiform inflammation in Tir8/Sigirr$^{-/-}$ mice is dependent on IL-17A.

As we have identified a novel mechanism through which TIR8/SIGIRR regulates innate IL-17A expression we next sought to determine whether the observed exacerbation of psoriasiform dermatitis in *Tir8/Sigirr*$^{-/-}$ mice was mediated by increased IL-17A expression. To achieve this, we utilised an IL-17A neutralisation antibody in the Aldara™ disease model whereby mice were treated with anti IL-17A or isotype control (250µg/mouse) on day 0 and day 2. Similar to our earlier observations *Tir8/Sigirr*$^{-/-}$ mice treated with isotype control antibody displayed enhanced disease characterised by enhanced acantosis, desquamation and infiltration of nucleated cells into the dermal layers when compared to isotype control antibody treated WT mice. Strikingly, treatment of *Tir8/Sigirr*$^{-/-}$ mice with an anti-IL-17A neutralising antibody reduced disease severity to levels comparable with those observed WT mice (Fig. 4.16A&B). This reduction in disease severity was evident from histological scoring performed on H&E stained tissue sections from diseased skin and also through analysis of epidermal skin thickening in treated mice (Fig. 4.16C&D). Taken together, these data confirm that the enhanced dermal inflammation observed in *Tir8/Sigirr* deficient mice is mediated by dysregulated expression of IL-17A.

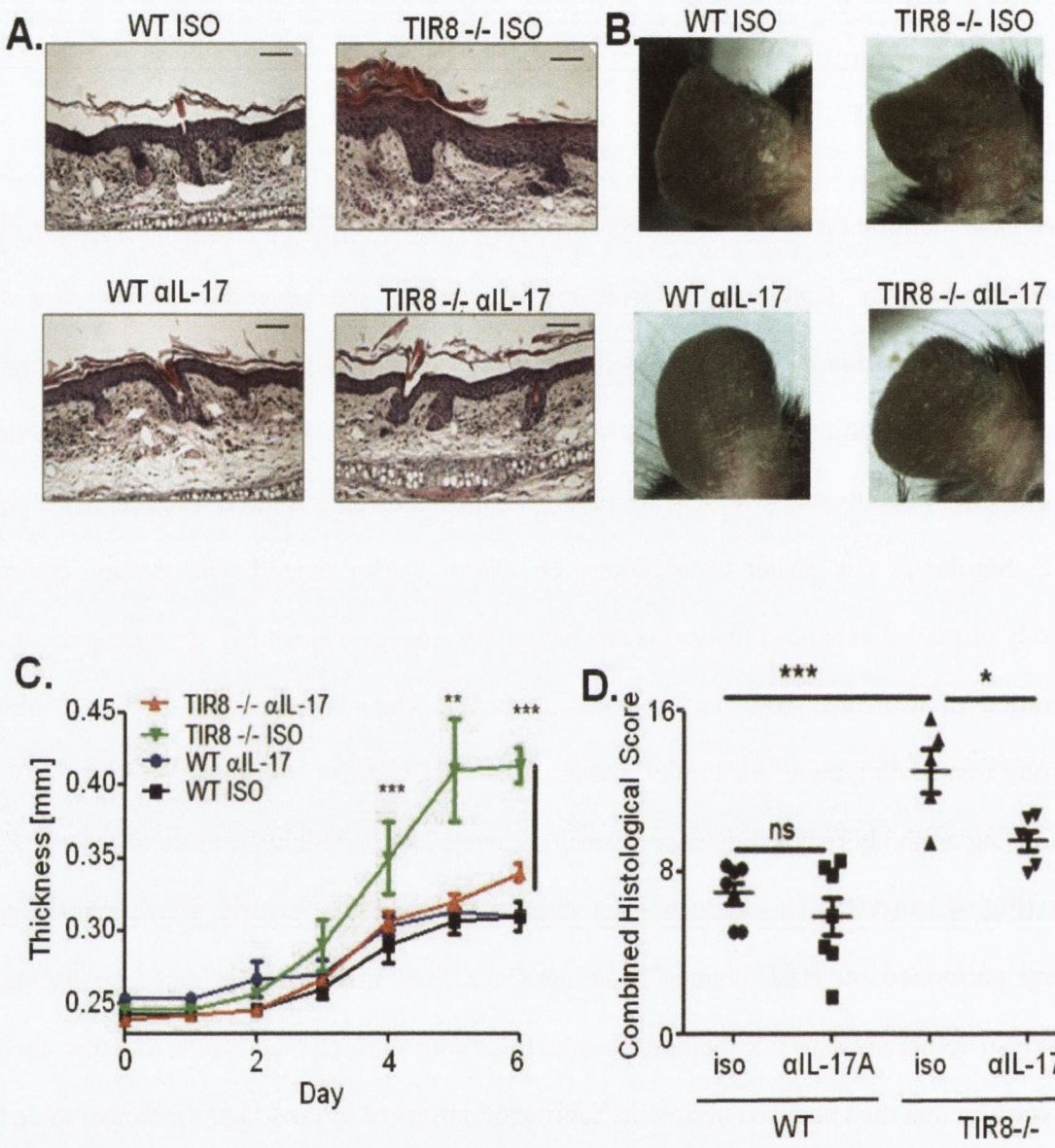


Fig 4.16 Enhanced severity of psoriasiform inflammation in Tir8/Sigirr$^{-/-}$ mice is dependent on IL-17A.

Mice were injected intraperitoneally with an anti IL-17A neutralisation antibody or isotype control (250μg/mouse) on day 0 and day 2 of the experiment. (A) H&E stained sections from ears of WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara™ (1mg/day) or control cream for 5 days. (Scale bar, 50μm). (B) Representative macroscopic photographs of ears from WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara™. (C) Increased epidermal thickening in *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara™.. These data are representative of at least three independent experiments with similar results Statistical significance determined by unpaired student's t-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

4.3 Discussion

Psoriasis is a common autoimmune disease of the skin which continues to pose a significant socioeconomic burden. Despite major advances in our understanding of the specific mechanisms which contribute towards disease pathogenesis significant questions remain to be answered (123). In recent times it has become evident that innate cell subsets provide a major source of IL-17A expression in settings of autoimmunity and host defence, particularly during the early stages of the response (220). A number of studies in murine models of psoriasiform inflammation have defined a role for innate cells governed by the IL-23/IL-17 signalling axis as central mediators of disease (54, 72, 147, 148). However, specific pathways which regulate the responses of these innate subsets remain to be identified. This study uncovers a novel role for the receptor TIR8/SIGIRR as a regulator of innate IL-17A driven dermal inflammation in psoriasis, which further underscores its importance as signalling mediator with therapeutic potential for interventions focusing on IL-1R1 and its interplay with the IL-23/IL-17 signalling axis.

The role of IL-1R1 signalling has received renewed focus in the context of psoriasiform inflammation as a consequence of observations implicating the IL-23/IL-17 axis as a central mediator of disease (214) . Interestingly mice deficient in IL-1R1 antagonist, an endogenous suppressor of IL-1R1 signalling, develop spontaneous autoimmune inflammation in the skin (221). This phenotype is strikingly similar to common manifestations of human psoriasis, with marked acantosis, hyperkeratosis and a nucleated cell infiltrate. Furthermore, these mice also display severe aortic inflammation; a serious cardiovascular complication observed in some chronic psoriasis suffers (222). These observations highlight the importance of examining IL-1R1 mediated inflammation in psoriasis and further highlight the potential

importance of TIR8/SIGIRR as a critical signalling mediator in this context. TIR8/SIGIRR has been described as a negative regulator of inflammation in settings of fungal infection and murine autoimmunity (155, 181). However, these studies have not addressed the role of TIR8/SIGIRR in the regulation of innate IL-17A and how this may impact the progression of autoimmune pathology. We have demonstrated that *Tir8/Sigirr*$^{-/-}$ mice develop enhanced inflammation, displaying a number of the histological characteristics of human psoriasis upon topical application of Aldara^{TM} containing the TLR7 agonist imiquimod. *Tir8/Sigirr*$^{-/-}$ mice display enhanced acantosis, desquamation, infiltration of nucleated cells and also ear thickening compared to WT mice. This model has been described as IL-23 and IL-1 dependant, with innate γδ T cells, expressing IL-17A, established as critical mediators of disease pathogenesis. Protein expression levels of IL-1β and IL-23 in the skin whilst upregulated in response to Aldara™ treatment were unaltered between WT and *Tir8/Sigirr*$^{-/-}$ mice. This lack of difference in protein expression levels is suggestive of a less prominent role for dysregulated stromal cell or myeloid cell involvement in the progression of dermal inflammation in the absence of TIR8/SIGIRR. Furthermore the overall higher expression of TIR8/SIGIRR on γδ T cells compared to CD4^{+} T cells and skin underscores the importance TIR8/SIGIRR plays in the regulation of inflammation driven by these cells in such settings of dermal inflammation.

Examination of the skin draining lymph nodes revealed significantly enhanced expression of IL-17A by γδ T cells from *Tir8/Sigirr*$^{-/-}$ mice. IL-22, another type 17 cytokine important for driving inflammation in the Aldara™ model (223), was unaltered between WT and *Tir8/Sigirr*$^{-/-}$ mice. Both IL-17A and IL-22 expression were virtually undetectable from CD4^{+} T cells, which is in line with previous reports implicating γδ T cells as the primary source of IL-17A in this setting (54). The low expression of IL-22 in both WT and *Tir8/Sigirr*$^{-/-}$ mice is somewhat surprising in particular as it is clear from our *in vivo* and *in vitro* stimulation

experiments that TIR8/SIGIRR activity can also inhibit IL-1R1 driven IL-22 expression. However, the levels of IL-22 detected *in vitro* are in the order of 5-10 fold less than the levels of IL-17A detected which may explain our failure to detect any such difference in the Aldara™ model. It is also noteworthy that non-T cells have previously been implicated as important sources of IL-22 in this model (54, 223). Imiquimod stimulation of TLR7 on myeloid cells is thought to drive IL-23 expression in the Aldara™ induced model of psoriasiform inflammation (72, 142). TIR8/SIGIRR has also been described as a negative regulator of TLR7 signalling (217, 218) and as such, the observed enhancement of inflammation in *Tir8/Sigirr*$^{-/-}$ mice, could be as a consequence of this activity. Therefore, we also used an established model of intradermal injection of IL-23 to assess the impact of TIR8/SIGIRR activity on dermal inflammation. In this model, *Tir8/Sigirr*$^{-/-}$ mice also displayed an enhanced disease phenotype compared to their WT counterparts confirming that TIR8/SIGIRR activity more broadly regulates dermal inflammation independently of its role as an inhibitor of TLR7 signalling. The IL-23 model is also primarily dependent on the direct stimulation of proinflammatory T cell subsets strongly indicating that TIR8/SIGIRR activity plays a prominent regulatory role on these cells in the context of psoriasiform inflammation. In line with previous reports, we observed the Vγ4 subset of γδ T cells as the primary source of innate IL-17A expression. Notably, these cells are also present in the inflamed skin in significantly elevated numbers. TIR8/SIGIRR has previously been described as a negative regulator of IL-1 driven proliferation of Th17 cells which could account for this observed expansion of infiltrating cells (181) . These elevated cell numbers could also be as a consequence of a feed forward mechanism in what are remarkably inflamed tissues which is facilitative of further uncontrolled infiltration of proinflammatory cells. Interestingly, we have also demonstrated that TIR8/SIGIRR can directly negatively regulate innate IL-1 driven IL-17A expression *in vivo* without altering expansion. As well as γδ T cells we have also

identified $CD4^+$ T cells and a population of $CD45^+$ $CD3^-$ non-T cells as sources of elevated IL-17A in the inflamed skin of *Tir8/Sigirr*$^{-/-}$ mice. These cells have also been identified in a number of similar studies as sources of IL-17A in this setting (54). However their relative contribution towards disease progression, especially given their low numbers, warrants further investigation. These data confirm dysregulated activity in dermal infiltrating haematopoietic cells in the absence of TIR8/SIGIRR activity and identify a novel role for TIR8/SIGIRR as a suppressive signalling mediator of the innate arm of the IL-23/IL-17 pathway. We have also demonstrated enhanced expression of IL-17A, IL-22 and IL-17A/F but not IL-17F from *Tir8/Sigirr*$^{-/-}$ splenocytes *in vitro* and *in vivo* in an IL-1 dependant manner. This enhanced expression appears to be dependent on dysregulated mTOR activity, an observation in line with a previous report describing the role TIR8/SIGIRR plays in the regulation of mTOR mediated IL-1 receptor activity (181). Despite the unaltered expression of IL-23 and IL-1β in the inflamed skin of WT and *Tir8/Sigirr*$^{-/-}$ mice, the enhanced sensitivity of the *Tir8/Sigirr*$^{-/-}$ cells to IL-1R1 stimulation coupled with the established role aberrant IL-1R1 signalling plays in the progression of dermal inflammation provides a potential mechanistic explanation for the enhanced disease observed in these mice.

IL-17A expression is established as a key cytokine driving inflammation in humans and murine models of psoriasis (129). *Tir8/Sigirr*$^{-/-}$ mice display an enhanced psoriatic disease phenotype associated with enhanced IL-17A expression from γδ T cells in both the skin draining lymph nodes and tissue infiltrating immune cells. Treatment of *Tir8/Sigirr*$^{-/-}$ mice with an IL-17A neutralisation antibody reverses the enhanced disease to levels observed in WT mice. These observations in *Tir8/Sigirr*$^{-/-}$ mice are broadly in line with observations in the clinic where neutralisation of elevated IL-17A in human patients results in amelioration of disease symptoms (134). Interestingly in this study, WT mice treated with anti-IL17A displayed a mild but not significant reduction in disease severity after histological

examination (Fig. 4.15D). This lack of efficacy in WT mice is potentially explained by compensatory mechanisms associated with other proinflammatory IL-17 family members such as IL-17C, which is significantly upregulated in psoriatic plaques and utilises IL-17A receptor for its signalling activities (224). Also pathways independent of IL-17A and IL-17 receptor A have recently been identified as playing an important role in the progression of Aldara™ induced disease in WT mice (225). As anti-IL-17A is demonstrating considerable promise in clinical trials among psoriasis patients, and decreased TIR8/SIGIRR expression/activity has previously been reported in PBMCs of psoriatic patients, these data identify an important mechanism which may have direct relevance in human disease. These observations also highlight the requirement for greater understanding of the molecular and cellular mechanisms at play in such settings.

Taken together, this study has for the first time identified TIR8/SIGIRR as a central regulator of innate type 17 responses with particular significance in the context of psoriasiform inflammation where its role as a critical mediator of IL-1R1 signalling identifies it as a potential target for future therapeutic interventions.

Chapter 5: General Discussion Chapter

5.1 General Discussion

The discovery of a third subset of $CD4^+$ T cells which preferentially express IL-17A has sparked intensive research into the mechanisms surrounding their generation and maintenance. These cells have been implicated as central mediators of inflammation in numerous setting of autoimmunity as well as playing essential roles in host defence against infection. Furthermore, their characterisation has resulted in significant revisions of the consensus surrounding the cellular and molecular mechanisms at play in the pathogenesis of a number of T cell driven autoimmune diseases. Despite notable advances in the understanding of type 17 immune responses, significant gaps in our knowledge remain relating to the molecular cellular mechanisms at play, particularly *in vivo.* These issues are compounded by the recent discovery that a large proportion of IL-17A expression at early time points and indeed throughout the immune response is attributable to innate cellular sources. Such observations place IL-17A directly at the interface between innate and adaptive immunity, with increasing clinical evidence supporting a role for both innate and adaptive sources of IL-17A in human disease states (72, 226).

This study has sought to address the potential for sCD25 and TIR8/SIGIRR to act as signalling mediators in settings of autoimmune disease where IL-17A activity is known to play a significant pathogenic role. Previous clinical observations have suggested a potential role for these proteins in the regulation of inflammation in settings of MS and psoriasis. However their potential *in vivo* relevance has yet to be satisfactorily addressed (113, 192).

Specifically, this work has identified a novel role for sCD25 as a modulator of autoimmune disease *in vivo* (227). Mice treated with sCD25, for a short time post immunisiation, develop

significantly enhanced disease which is associated with increased antigen specific Th17 cells in the periphery and enhanced infiltration of Th17 cells to the CNS. Furthermore, sCD25 enhanced the generation of Th17 cells *in vitro* through inhibition of IL-2R signalling. sCD25 mediated these events through direct interaction with IL-2, acting as a 'sink' and thereby limiting its bioavailability to Th17 cells. These data demonstrated the immunomodulatory abilities of sCD25 for the first time *in vivo*. Furthermore, the association between elevated levels of sCD25 found in the sera of autoimmune patients and the presence of specific susceptibility alleles at the CD25 gene locus offer perhaps the clearest indication that sCD25 plays a role in autoimmune pathogenesis (113).

Whilst sCD25 was shown to enhance the generation of adaptive Th17 responses, the second part of this study demonstrates the importance of innate IL-17A in autoimmune inflammation and identified a novel signalling mediator which negatively regulates these events. The orphan receptor TIR8/SIGIRR was shown to regulate dermal inflammation driven by IL-17A expressing γδ T cells through direct inhibition of IL-17A. *Tir8/Sigirr*$^{-/-}$ mice displayed enhanced susceptibility in both chemical and cytokine induced models of psoriasis. This increased disease severity was associated with enhanced infiltration of Vγ4^{+} γδ T cells that express significantly elevated levels of IL-17A. Importantly the enhanced disease phenotype was reversible upon treatment with an anti-IL-17A antibody (228). This arm of the study further highlights the central role IL-17A plays not only in the pathogenesis of psoriasis but also its importance in bridging the gap between innate and adaptive immunity as a consequence of its expression by innate γδ T cells. Furthermore, it identifies TIR8/SIGIRR as a novel intrinsic regulator of innate type 17 responses and identifies a previously unappreciated role for this receptor as a regulator of dermal inflammation.

In recent times a number of autoimmune disorders, characterised by T cell driven inflammation and previously thought to be mediated by Th1 cells, have now been revisited

with a growing appreciation for the significant IL-17A component involved (229, 230). These observations have directly led to the development of a number of novel frontline therapeutics which have successfully targeted components of the IL23/IL-17 pathway. These early successes in the clinic, in particular with antibodies against IL-17A and IL-17RA, have led profound improvements in the quality of life for patients suffering from chronic psoriasis and rheumatoid arthritis. Strikingly, a single dose of the anti-IL-17A antibody secukinumab resulted in significantly reduced psoriatic plaque area as well as reduced number of proinflammatory cells in lesional skin (231). This therapy has also demonstrated potential effectiveness in ankylosing spondylitis (231). Similar effectiveness is also observed in psoriasis patients treated with alternative antibodies targeting both IL-17A and IL-17RA (133, 134). However, despite the common inflammatory pathways involved in a number of autoimmune diseases, direct targeting of IL-17A in Crohn's disease has proven ineffective and even exacerbated disease in some cases (232, 233). Similarly, deficiency in IL-23 has even been suggested to accelerate wound healing settings of coilitis (234). These clinical anomalies highlight the complexity of the role IL-17A plays in such settings and emphasises the critical importance of further elucidating the cellular and molecular mediators at play in the regulation of this pathway.

The mechanisms through which type 17 immune responses are generated depend on the ability of innate immune cells to respond to environmental stimuli leading to the expression of instructive signals which include IL-23 and IL-1. With the discovery of Th17 cells a number of classic immune regulatory mechanisms were implicated as playing a role modulating the activities of these cells such as IL-10 (235) and STAT1 mediated counter regulation by Th1 cells (236). This array of regulatory proteins has expanded rapidly over time.

For example, IL-27 is a member of the IL-12 cytokine family which has been shown to have anti-inflammatory properties in EAE (152, 237). This protective effect occurs as a consequence of IL-27 activity which inhibits Th17 responses and also its ability to suppress GM-CSF (238) a highly pathogenic cytokine which is expressed by Th17 cells in settings of neuroinflammation (239, 240). IL-27 is thought to inhibit Th17 cells through mechanisms distinct from interlineage regulation as lack of IL-27 also results in enhanced Th1 mediated tissue pathology. IL-27 has also been demonstrated to induce the expression of IL-10 from T cells, with IL-27 deficient T cells displaying defects in IL-10 expression, suggesting the possibility that IL-27 plays a role in the induction of peripheral Tregs (241-243). Th17 cells share similarities in their developmental programs with Tregs, with signals such as STAT3 activation enhancing the generation of Th17 responses but inhibiting the generation of Tregs. The role of IL-2 as an early inhibitory signal in the generation of Th17 cells, whilst promoting the generation of Tregs is well documented. Tregs act to suppress the inflammation and associated tissue pathology associated with dysregulated Th17 cell activity, however somewhat paradoxically Tregs have been shown to enhance the generation of Th17 cells independently of TGF-β signalling and Th1 regulation (203). This phenomenon, which only occurs at early time points during the generation of Th responses is mediated through the ability of Tregs to consume IL-2 as a consequence of their high surface CD25 expression. The early induction of Th17 cells described in these studies is similar in nature to our observations which demonstrate the ability of sCD25 to enhance the generation of Th17 cells at early time points. It is tempting to speculate that the reported enhancement by Tregs is mediated, at least in part by sCD25 shed from the surface of activated Tregs, further indicating of a role for sCD25 in the modulation of T cell responses and autoimmunity.

The interplay between classically described components of the immune system clearly plays a significant role in the regulation of Type 17 responses in settings of inflammation.

Recently, the scope and complexity of regulatory networks at play in Type 17 responses was further expanded by reports implicating a high salt diet as a powerful enhancer of Th17 cells and exacerbates disease in EAE (244, 245). High-salt conditions during *in vitro* Th17 differentiation activated the p38/MAPK pathway and serum/glucocorticoid-regulated kinase 1 (SGK1), an important mediator of sodium transport. Gene silencing or chemical inhibition of p38/MAPK pathway components or SGK1 abrogates the high-salt-induced Th17 cell development (244, 245) Furthermore, WT mice fed a high salt diet display a more severe EAE than mice fed a normal diet and this increased severity was dramatically reduced in SGK1-deficient mice (245). These data provide previously unappreciated insight into the role environmental factors play in the generation of Th17 responses and further highlight the importance of understanding the cellular regulation of innate sources of IL-17A by cells which can respond rapidly to environmental ques to express IL-17A and other associated cytokines (66, 67). Although such innate cells can apparently respond without recognition of specific peptide antigens presented by APC, it is clear that they can express abundant amounts of IL-17A which has important implications at the mucosal and barrier sites where these cells predominantly reside. IL-17A maintains tight barrier function, recruits neutrophils and drives expression of defensins which all contributes to the maintenance of mucosal integrity (101). These innate IL-17A expressing cells likely contribute to the maintenance of gut homeostasis through their ability to rapidly express IL-17A in response to microbial insult whilst providing other pro-resolving cytokines such as IL-22 which acts in repair of the epithelium. The opposite appears to be the case in settings of dermal inflammation where IL-17A expression by innate cells promotes inflammation through similar mechanisms contributing to dysregulated keratinocyte proliferation, erythema and lesion formation. Our studies have demonstrated TIR8/SIGIRR as playing a key role in the regulation IL-17A by γδ T cells in such settings. Interestingly, we also observe a population of $CD45^+$ CD3 negative

cells which express significantly elevated IL-17A in the absence of TIR8/SIGIRR. These alternative innate sources of IL-17A have sparked fervent interest in recent times as the repertoire of IL-17A expressing cells expands rapidly and in particular as it becomes clear that IL-17A dependant autoimmune inflammation is inducible in mice that are deficient in adaptive immune cells (246).

These novel populations of IL-17A expressing non T cells cells have proven difficult to characterise as they lack the expression of surface markers in many cases make them difficult to identify. A number of specific cell types expressing IL-17A under the apparent governance of RORγT have been identified with suggested roles in the maintenance of tissue homeostasis and potentially pathogenic roles in IBD (64). Strikingly the biology of these cells has revealed many similarities with adaptive T cells in their cytokine secretion profiles and transcription factors, further blurring the lines between innate and adaptive type 17 responses. Furthermore, our observations in γδ T cells and murine psoriasis models provide solid rationale for the investigation of TIR8/SIGIRR as a potential regulator of these nonconventional cells going forward. These cells are potentially very interesting new players in settings of type 17 immune responses however due to their overall low incidences compared to alternative immune lineages their specific contributions to such inflammation in humans are not completely clear.

It is clear that IL-17A expressing cells can also co express an array cytokines apart from IL-17A (247, 248), which can act in a tissue specific manner to enhance or inhibit the inflammatory response. A prime example is IL-22 which is pathogenic in the skin, has repair functions in the gut and is non essential in EAE (73, 249, 250). It is clear from our *in vivo* footpad studies that TIR8/SIGIRR has a role to play in the regulation of this cytokine further underscoring its potential importance as a key modulator of type 17 responses in such settings. Furthermore, in settings of EAE where IL-17A plays a prominent proinflammatory

role there are certain highly pathogenic cytokines such as GM-CSF whose expression can also be attributed to Th17 cells. It is also clear from our data that TIR8/SIGIRR plays a role in the regulation of innate GM-CSF expression further highlighting its importance in such settings.

IL-17A biology has made significant advances since the discovery of its predominant cellular source more than 10 years ago. It has become clear that IL-17 and the repertoire of cytokines associated with these cells play fundamental roles in setting of autoimmunity and host defence against infection. We have identified and characterised two novel regulators of IL-17A expression in settings of autoimmune inflammation. We further demonstrate the role for IL-2 receptor signalling in the maintenance of T cell tolerance and provide a potential mechanistic insight into the progression of autoimmune disease in certain susceptible MS patient cohorts. In a similar fashion we show that the regulation of Toll/ILR signalling through the actions of TIR8/SIGIRR plays a central role in the modulation of innate immune responses driven by IL-17A.

Taken together these data provide further evidence of the global role IL-17A plays in inflammatory settings across the innate and adaptive immune systems and more importantly identifies two therapeutic targets through which overt inflammation attributable to dysregulated activity of this cytokine could potentially be resolved.

5.2 Future work

Despite extensive evidence across the literature over the last number of years implicating TIR8/SIRIRR as in important player in the regulation of inflammation driven by TLR/ILR family members specific insights into the mechanisms through which it exerts these effects are currently limited (177, 184). It will be important going forward to investigate these mechanisms, specifically in the context of our data relating to role TIR8/SIGIRR plays in the regulation of innate IL-1R1 dependant IL-17A production. Previous reports have implied that certain subunits of TIR8/SIGIRR are specifically required for the regulation of such activities (183) however these findings have not been validated in primary murine T cell populations. We plan to construct a range of truncation mutants which specifically lack either the extracellular Ig domain, the TIR domain or the C terminal domain and ectopically express these mutants in *tir8/sigirr* $^{-/-}$ T cells to determine which specific subunits are involved in the inhibition of IL-1 driven innate IL-17A expression from these cells. This system once up and running has the potential for use in a wide variety or other primary cell types which are subject to regulation by TIR8/SIGIRR activities.

We also plan to assess potential ligand binding candidates for TIR8/SIGIRR through surface plasmon resonance studies where full length recombinant TIR8/SIGIRR as well as its individual recombinant subunits will be used to assess potential interacting partners with TIR8/SIGIRR and individual subunits. These studies have the potential to uncover potential ligands for TIR8/SIGIRR but could also reveal a role for TIR8/SIGIRR in the formation of signalling complexes of other IL-1 receptor and TLR family members. For example, TIR8/SIGIRR has been shown to interact with IL-1 receptor accessory protein (183) as does the receptor for the novel IL-1 family member IL-36 (251) however the possibility that

TIR8/SIGIRR acts as a regulator of IL-36 receptor responses has yet to be addressed. Furthermore, the newest IL-1 family member IL-38 has been demonstrated to bind to IL-36 receptor (252), as such it will also be interesting to assess a potential role for TIR8/SIGIRR as a putative regulator of both IL-36 receptor and IL-38 mediated signalling events.

Chapter 6: References

1. Janeway, C., P. Travers, M. Walport, and K. Murphy. 2008. *Janeway's immunobiology*. Garland Science, New York ; London.

2. Murray, P. J., and T. A. Wynn. 2011. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 11: 723-737.

3. Steinman, R., and Z. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *The Journal of Experimental Medicine* 137: 1142-1162.

4. Cutler, C. W., R. Jotwani, and B. Pulendran. 2001. Dendritic Cells: Immune Saviors or Achilles' Heel? *Infection and Immunity* 69: 4703-4708.

5. Kaufmann, S. H. 1996. gamma/delta and other unconventional T lymphocytes: what do they see and what do they do? *Proc Natl Acad Sci U S A* 93: 2272-2279.

6. Cua, D. J., and C. M. Tato. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 10: 479-489.

7. Russell, S. E., and P. T. Walsh. 2012. Sterile inflammation - do innate lymphoid cell subsets play a role? *Front Immunol* 3: 246.

8. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136: 2348-2357.

9. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775-787.

10. Sakaguchi, S., and N. Sakaguchi. 2005. Regulatory T cells in immunologic self-tolerance and autoimmune disease. *Int Rev Immunol* 24: 211-226.

11. Gregori, S., K. S. Goudy, and M. G. Roncarolo. 2012. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. *Front Immunol* 3: 30.

12. Crotty, S. 2011. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 29: 621-663.

13. Kaplan, M. H. 2013. Th9 cells: differentiation and disease. *Immunol Rev* 252: 104-115.

14. Tan, C., and I. Gery. 2012. The unique features of Th9 cells and their products. *Crit Rev Immunol* 32: 1-10.

15. Bottomly, K. 1988. A functional dichotomy in CD4+ T lymphocytes. *Immunol Today* 9: 268-274.

16. Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* 260: 547-549.

17. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655-669.

18. Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang, and S. L. Reiner. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292: 1907-1910.

19. Usui, T., J. C. Preiss, Y. Kanno, Z. J. Yao, J. H. Bream, J. J. O'Shea, and W. Strober. 2006. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *J Exp Med* 203: 755-766.

20. Cox, M. A., L. E. Harrington, and A. J. Zajac. 2011. Cytokines and the inception of CD8 T cell responses. *Trends Immunol* 32: 180-186.

21. Curtsinger, J. M., and M. F. Mescher. 2010. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol* 22: 333-340.

22. Zhu, J., and W. E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112: 1557-1569.

23. Deeths, M. J., R. M. Kedl, and M. F. Mescher. 1999. CD8+ T cells become nonresponsive (anergic) following activation in the presence of costimulation. *J Immunol* 163: 102-110.

24. Williams, M. A., A. J. Tyznik, and M. J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441: 890-893.

25. Shedlock, D. J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300: 337-339.

26. Allard, E. L., M. P. Hardy, J. Leignadier, M. Marquis, J. Rooney, D. Lehoux, and N. Labrecque. 2007. Overexpression of IL-21 promotes massive CD8+ memory T cell accumulation. *Eur J Immunol* 37: 3069-3077.

27. Ansel, K. M., I. Djuretic, B. Tanasa, and A. Rao. 2006. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu Rev Immunol* 24: 607-656.

28. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587-596.

29. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties. *Annual Review of Immunology* 7: 145-173.

30. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6: 1123-1132.

31. Ivanov, II, B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORgammat directs the

differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121-1133.

32. Ye, P., F. H. Rodriguez, S. Kanaly, K. L. Stocking, J. Schurr, P. Schwarzenberger, P. Oliver, W. Huang, P. Zhang, J. Zhang, J. E. Shellito, G. J. Bagby, S. Nelson, K. Charrier, J. J. Peschon, and J. K. Kolls. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 194: 519-527.

33. Huang, W., L. Na, P. L. Fidel, and P. Schwarzenberger. 2004. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J Infect Dis* 190: 624-631.

34. Gran, B., N. Chu, G. X. Zhang, S. Yu, Y. Li, X. H. Chen, M. Kamoun, and A. Rostami. 2004. Early administration of IL-12 suppresses EAE through induction of interferon-gamma. *J Neuroimmunol* 156: 123-131.

35. Willenborg, D. O., S. A. Fordham, M. A. Staykova, I. A. Ramshaw, and W. B. Cowden. 1999. IFN-gamma is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *J Immunol* 163: 5278-5286.

36. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201: 233-240.

37. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6: 1133-1141.

38. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235-238.

39. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441: 231-234.

40. Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jager, T. B. Strom, M. Oukka, and V. K. Kuchroo. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448: 484-487.

41. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299: 1033-1036.

42. Zhou, L., Ivanov, II, R. Spolski, R. Min, K. Shenderov, T. Egawa, D. E. Levy, W. J. Leonard, and D. R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 8: 967-974.

43. Das, J., G. Ren, L. Zhang, A. I. Roberts, X. Zhao, A. L. Bothwell, L. Van Kaer, Y. Shi, and G. Das. 2009. Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. *J Exp Med* 206: 2407-2416.

44. McGeachy, M. J., K. S. Bak-Jensen, Y. Chen, C. M. Tato, W. Blumenschein, T. McClanahan, and D. J. Cua. 2007. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 8: 1390-1397.

45. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179-189.

46. Khader, S. A., S. L. Gaffen, and J. K. Kolls. 2009. Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal Immunol* 2: 403-411.

47. Liang, S. C., X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L. A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 203: 2271-2279.

48. Yao, Z., S. L. Painter, W. C. Fanslow, D. Ulrich, B. M. Macduff, M. K. Spriggs, and R. J. Armitage. 1995. Human IL-17: a novel cytokine derived from T cells. *J Immunol* 155: 5483-5486.

49. Gaffen, S. L. 2009. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* 9: 556-567.

50. Yao, Z., W. C. Fanslow, M. F. Seldin, A. M. Rousseau, S. L. Painter, M. R. Comeau, J. I. Cohen, and M. K. Spriggs. 1995. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 3: 811-821.

51. Chang, S. H., H. Park, and C. Dong. 2006. Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. *J Biol Chem* 281: 35603-35607.

52. Schwandner, R., K. Yamaguchi, and Z. Cao. 2000. Requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in interleukin 17 signal transduction. *J Exp Med* 191: 1233-1240.

53. Chang, S. H., and C. Dong. 2010. Signaling of interleukin-17 family cytokines in immunity and inflammation. *Cell Signal* 23: 1069-1075.

54. Pantelyushin, S., S. Haak, B. Ingold, P. Kulig, F. L. Heppner, A. A. Navarini, and B. Becher. 2012. Rorgammat+ innate lymphocytes and gammadelta T cells initiate psoriasiform plaque formation in mice. *J Clin Invest* 122: 2252-2256.

55. Liang, S. C., A. J. Long, F. Bennett, M. J. Whitters, R. Karim, M. Collins, S. J. Goldman, K. Dunussi-Joannopoulos, C. M. Williams, J. F. Wright, and L. A. Fouser. 2007. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. *J Immunol* 179: 7791-7799.

56. Sonnenberg, G. F., L. A. Fouser, and D. Artis. 2011. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol* 12: 383-390.

57. Renauld, J. C. 2003. Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators. *Nat Rev Immunol* 3: 667-676.

58. Nagalakshmi, M. L., E. Murphy, T. McClanahan, and R. de Waal Malefyt. 2004. Expression patterns of IL-10 ligand and receptor gene families provide leads for biological characterization. *Int Immunopharmacol* 4: 577-592.

59. Van Belle, A. B., M. de Heusch, M. M. Lemaire, E. Hendrickx, G. Warnier, K. Dunussi-Joannopoulos, L. A. Fouser, J. C. Renauld, and L. Dumoutier. 2011. IL-22 is required for imiquimod-induced psoriasiform skin inflammation in mice. *J Immunol* 188: 462-469.

60. Pickert, G., C. Neufert, M. Leppkes, Y. Zheng, N. Wittkopf, M. Warntjen, H. A. Lehr, S. Hirth, B. Weigmann, S. Wirtz, W. Ouyang, M. F. Neurath, and C. Becker. 2009. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J Exp Med* 206: 1465-1472.

61. Wei, L., A. Laurence, K. M. Elias, and J. J. O'Shea. 2007. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem* 282: 34605-34610.

62. Codarri, L., G. Gyulveszi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter, and B. Becher. 2011. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 12: 560-567.

63. Van Kaer, L., V. V. Parekh, and L. Wu. Invariant NK T cells: potential for immunotherapeutic targeting with glycolipid antigens. *Immunotherapy* 3: 59-75.

64. Walker, J. A., J. L. Barlow, and A. N. McKenzie. 2013. Innate lymphoid cells--how did we miss them? *Nat Rev Immunol* 13: 75-87.

65. Shibata, K., H. Yamada, H. Hara, K. Kishihara, and Y. Yoshikai. 2007. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *J Immunol* 178: 4466-4472.

66. Sutton, C. E., S. J. Lalor, C. M. Sweeney, C. F. Brereton, E. C. Lavelle, and K. H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31: 331-341.

67. Martin, B., K. Hirota, D. J. Cua, B. Stockinger, and M. Veldhoen. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* 31: 321-330.

68. Jensen, K. D., X. Su, S. Shin, L. Li, S. Youssef, S. Yamasaki, L. Steinman, T. Saito, R. M. Locksley, M. M. Davis, N. Baumgarth, and Y. H. Chien. 2008. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity* 29: 90-100.

69. Cai, Y., X. Shen, C. Ding, C. Qi, K. Li, X. Li, V. R. Jala, H. G. Zhang, T. Wang, J. Zheng, and J. Yan. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity* 35: 596-610.

70. Laggner, U., P. Di Meglio, G. K. Perera, C. Hundhausen, K. E. Lacy, N. Ali, C. H. Smith, A. C. Hayday, B. J. Nickoloff, and F. O. Nestle. Identification of a novel proinflammatory human skin-homing Vgamma9Vdelta2 T cell subset with a potential role in psoriasis. *J Immunol* 187: 2783-2793.

71. Strid, J., O. Sobolev, B. Zafirova, B. Polic, and A. Hayday. 2011. The intraepithelial T cell response to NKG2D-ligands links lymphoid stress surveillance to atopy. *Science* 334: 1293-1297.

72. Cai, Y., X. Shen, C. Ding, C. Qi, K. Li, X. Li, V. R. Jala, H. G. Zhang, T. Wang, J. Zheng, and J. Yan. 2011. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity* 35: 596-610.

73. Van Belle, A. B., M. de Heusch, M. M. Lemaire, E. Hendrickx, G. Warnier, K. Dunussi-Joannopoulos, L. A. Fouser, J. C. Renauld, and L. Dumoutier. 2012. IL-22 is required for imiquimod-induced psoriasiform skin inflammation in mice. *J Immunol* 188: 462-469.

74. Yin, Z., C. Chen, S. J. Szabo, L. H. Glimcher, A. Ray, and J. Craft. 2002. T-Bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-gamma by gammadelta T cells. *J Immunol* 168: 1566-1571.

75. Ribot, J. C., A. deBarros, D. J. Pang, J. F. Neves, V. Peperzak, S. J. Roberts, M. Girardi, J. Borst, A. C. Hayday, D. J. Pennington, and B. Silva-Santos. 2009. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat Immunol* 10: 427-436.

76. Dunne, M. R., B. A. Mangan, L. Madrigal-Estebas, and D. G. Doherty. 2011. Preferential Th1 cytokine profile of phosphoantigen-stimulated human Vgamma9Vdelta2 T cells. *Mediators Inflamm* 2010: 704941.

77. Bonneville, M., R. L. O'Brien, and W. K. Born. 2010. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* 10: 467-478.

78. Ismaili, J., V. Olislagers, R. Poupot, J. J. Fournie, and M. Goldman. 2002. Human gamma delta T cells induce dendritic cell maturation. *Clin Immunol* 103: 296-302.

79. Brandes, M., K. Willimann, A. B. Lang, K. H. Nam, C. Jin, M. B. Brenner, C. T. Morita, and B. Moser. 2003. Flexible migration program regulates gamma delta T-cell involvement in humoral immunity. *Blood* 102: 3693-3701.

80. Fournie, J. J., H. Sicard, M. Poupot, C. Bezombes, A. Blanc, F. Romagne, L. Ysebaert, and G. Laurent. 2013. What lessons can be learned from gammadelta T cell-based cancer immunotherapy trials? *Cell Mol Immunol* 10: 35-41.

81. Caccamo, N., C. La Mendola, V. Orlando, S. Meraviglia, M. Todaro, G. Stassi, G. Sireci, J. J. Fournie, and F. Dieli. 2011. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood* 118: 129-138.

82. Chowers, Y., W. Holtmeier, J. Harwood, E. Morzycka-Wroblewska, and M. F. Kagnoff. 1994. The V delta 1 T cell receptor repertoire in human small intestine and colon. *J Exp Med* 180: 183-190.

83. Mangan, B. A., M. R. Dunne, V. P. O'Reilly, P. J. Dunne, M. A. Exley, D. O'Shea, E. Scotet, A. E. Hogan, and D. G. Doherty. 2013. Cutting edge: CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vdelta3 T cells. *J Immunol* 191: 30-34.

84. Lunardi, C., C. Marguerie, M. J. Walport, and A. K. So. 1992. T gamma delta cells and their subsets in blood and synovial fluid from patients with rheumatoid arthritis. *Br J Rheumatol* 31: 527-530.

85. Wucherpfennig, K. W., J. Newcombe, H. Li, C. Keddy, M. L. Cuzner, and D. A. Hafler. 1992. Gamma delta T-cell receptor repertoire in acute multiple sclerosis lesions. *Proc Natl Acad Sci U S A* 89: 4588-4592.

86. Hua, F., N. Kang, Y. A. Gao, L. X. Cui, D. N. Ba, and W. He. 2013. Potential regulatory role of in vitro-expanded Vdelta1 T cells from human peripheral blood. *Immunol Res* 56: 172-180.

87. Sheridan, B. S., P. A. Romagnoli, Q. M. Pham, H. H. Fu, F. Alonzo, 3rd, W. D. Schubert, N. E. Freitag, and L. Lefrancois. 2013. gammadelta T Cells Exhibit Multifunctional and Protective Memory in Intestinal Tissues. *Immunity* 39: 184-195.

88. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science* 169: 1042-1049.

89. Starr, T. K., S. C. Jameson, and K. A. Hogquist. 2003. Positive and negative selection of T cells. *Annu Rev Immunol* 21: 139-176.

90. Hernandez, J. B., R. H. Newton, and C. M. Walsh. Life and death in the thymus--cell death signaling during T cell development. *Curr Opin Cell Biol* 22: 865-871.

91. Mills, K. H. 2004. Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol* 4: 841-855.

92. Clark, L. B., M. W. Appleby, M. E. Brunkow, J. E. Wilkinson, S. F. Ziegler, and F. Ramsdell. 1999. Cellular and molecular characterization of the scurfy mouse mutant. *J Immunol* 162: 2546-2554.

93. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4: 330-336.

94. Chatila, T. A., F. Blaeser, N. Ho, H. M. Lederman, C. Voulgaropoulos, C. Helms, and A. M. Bowcock. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest* 106: R75-81.

95. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198: 1875-1886.

96. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188: 287-296.

97. Kohm, A. P., P. A. Carpentier, H. A. Anger, and S. D. Miller. 2002. Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol* 169: 4712-4716.

98. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30: 636-645.

99. Misra, N., J. Bayry, S. Lacroix-Desmazes, M. D. Kazatchkine, and S. V. Kaveri. 2004. Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J Immunol* 172: 4676-4680.

100. Corthay, A. 2009. How do regulatory T cells work? *Scand J Immunol* 70: 326-336.

101. Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annu Rev Immunol* 27: 485-517.

102. Miossec, P., and J. K. Kolls. 2012. Targeting IL-17 and TH17 cells in chronic inflammation. *Nat Rev Drug Discov* 11: 763-776.

103. Trapp, B. D., and K. A. Nave. 2008. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci* 31: 247-269.

104. Compston, A., and A. Coles. 2008. Multiple sclerosis. *Lancet* 372: 1502-1517.

105. Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annu Rev Immunol* 23: 683-747.

106. Fletcher, J. M., S. J. Lalor, C. M. Sweeney, N. Tubridy, and K. H. Mills. 2010. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol* 162: 1-11.

107. Scholz, C., K. T. Patton, D. E. Anderson, G. J. Freeman, and D. A. Hafler. 1998. Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation. *J Immunol* 160: 1532-1538.

108. Ontaneda, D., M. Hyland, and J. A. Cohen. 2012. Multiple sclerosis: new insights in pathogenesis and novel therapeutics. *Annu Rev Med* 63: 389-404.

109. Lovett-Racke, A. E., J. L. Trotter, J. Lauber, P. J. Perrin, C. H. June, and M. K. Racke. 1998. Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. *J Clin Invest* 101: 725-730.

110. Steinman, L. 1999. Assessment of animal models for MS and demyelinating disease in the design of rational therapy. *Neuron* 24: 511-514.

111. Tzartos, J. S., M. A. Friese, M. J. Craner, J. Palace, J. Newcombe, M. M. Esiri, and L. Fugger. 2008. Interleukin-17 production in central nervous system-infiltrating T cells

and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 172: 146-155.

112. Hafler, D. A., A. Compston, S. Sawcer, E. S. Lander, M. J. Daly, P. L. De Jager, P. I. de Bakker, S. B. Gabriel, D. B. Mirel, A. J. Ivinson, M. A. Pericak-Vance, S. G. Gregory, J. D. Rioux, J. L. McCauley, J. L. Haines, L. F. Barcellos, B. Cree, J. R. Oksenberg, and S. L. Hauser. 2007. Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* 357: 851-862.

113. Maier, L. M., D. E. Anderson, C. A. Severson, C. Baecher-Allan, B. Healy, D. V. Liu, K. D. Wittrup, P. L. De Jager, and D. A. Hafler. 2009. Soluble IL-2RA levels in multiple sclerosis subjects and the effect of soluble IL-2RA on immune responses. *J Immunol* 182: 1541-1547.

114. Stromnes, I. M., and J. M. Goverman. 2006. Active induction of experimental allergic encephalomyelitis. *Nat Protoc* 1: 1810-1819.

115. Miller, S. D., and W. J. Karpus. 2007. Experimental autoimmune encephalomyelitis in the mouse. *Curr Protoc Immunol* Chapter 15: Unit 15 11.

116. Pettinelli, C. B., and D. E. McFarlin. 1981. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *J Immunol* 127: 1420-1423.

117. Bettelli, E., B. Sullivan, S. J. Szabo, R. A. Sobel, L. H. Glimcher, and V. K. Kuchroo. 2004. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med* 200: 79-87.

118. Segal, B. M., B. K. Dwyer, and E. M. Shevach. 1998. An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. *J Exp Med* 187: 537-546.

119. Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S. A. Lira, D. Gorman, R. A. Kastelein, and J. D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421: 744-748.

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

121. Gonzalez-Garcia, I., Y. Zhao, S. Ju, Q. Gu, L. Liu, J. K. Kolls, and B. Lu. 2009. IL-17 signaling-independent central nervous system autoimmunity is negatively regulated by TGF-beta. *J Immunol* 182: 2665-2671.

122. Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177: 566-573.

123. Perera, G. K., P. Di Meglio, and F. O. Nestle. 2012. Psoriasis. *Annu Rev Pathol* 7: 385-422.

124. Schon, M. P., and W. H. Boehncke. 2005. Psoriasis. *N Engl J Med* 352: 1899-1912.

125. Lowes, M. A., A. M. Bowcock, and J. G. Krueger. 2007. Pathogenesis and therapy of psoriasis. *Nature* 445: 866-873.

126. Ellis, C. N., D. C. Gorsulowsky, T. A. Hamilton, J. K. Billings, M. D. Brown, J. T. Headington, K. D. Cooper, O. Baadsgaard, E. A. Duell, T. M. Annesley, and et al. 1986. Cyclosporine improves psoriasis in a double-blind study. *JAMA* 256: 3110-3116.

127. Nickoloff, B. J., and F. O. Nestle. 2004. Recent insights into the immunopathogenesis of psoriasis provide new therapeutic opportunities. *J Clin Invest* 113: 1664-1675.

128. Nestle, F. O., and C. Conrad. 2004. The IL-12 family member p40 chain as a master switch and novel therapeutic target in psoriasis. *J Invest Dermatol* 123: xiv-xv.

129. Blauvelt, A. 2008. T-helper 17 cells in psoriatic plaques and additional genetic links between IL-23 and psoriasis. *J Invest Dermatol* 128: 1064-1067.

130. Wilson, N. J., K. Boniface, J. R. Chan, B. S. McKenzie, W. M. Blumenschein, J. D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel, J. C. Lecron, R. A. Kastelein, D. J. Cua, T. K. McClanahan, E. P. Bowman, and R. de Waal Malefyt. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 8: 950-957.

131. Guttman-Yassky, E., M. A. Lowes, J. Fuentes-Duculan, L. C. Zaba, I. Cardinale, K. E. Nograles, A. Khatcherian, I. Novitskaya, J. A. Carucci, R. Bergman, and J. G. Krueger. 2008. Low expression of the IL-23/Th17 pathway in atopic dermatitis compared to psoriasis. *J Immunol* 181: 7420-7427.

132. Martin, D. A., J. E. Towne, G. Kricorian, P. Klekotka, J. E. Gudjonsson, J. G. Krueger, and C. B. Russell. 2013. The emerging role of IL-17 in the pathogenesis of psoriasis: preclinical and clinical findings. *J Invest Dermatol* 133: 17-26.

133. Papp, K. A., C. Leonardi, A. Menter, J. P. Ortonne, J. G. Krueger, G. Kricorian, G. Aras, J. Li, C. B. Russell, E. H. Thompson, and S. Baumgartner. 2012. Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. *N Engl J Med* 366: 1181-1189.

134. Leonardi, C., R. Matheson, C. Zachariae, G. Cameron, L. Li, E. Edson-Heredia, D. Braun, and S. Banerjee. 2012. Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. *N Engl J Med* 366: 1190-1199.

135. Becher, B., and S. Pantelyushin. 2012. Hiding under the skin: Interleukin-17-producing gammadelta T cells go under the skin? *Nat Med* 18: 1748-1750.

136. Bosma, M. J., and A. M. Carroll. 1991. The SCID mouse mutant: definition, characterization, and potential uses. *Annu Rev Immunol* 9: 323-350.

137. Wrone-Smith, T., and B. J. Nickoloff. 1996. Dermal injection of immunocytes induces psoriasis. *J Clin Invest* 98: 1878-1887.

138. Boehncke, W. H., D. Dressel, T. M. Zollner, and R. Kaufmann. 1996. Pulling the trigger on psoriasis. *Nature* 379: 777.

139. Lee, C. K., D. T. Rao, R. Gertner, R. Gimeno, A. B. Frey, and D. E. Levy. 2000. Distinct requirements for IFNs and STAT1 in NK cell function. *J Immunol* 165: 3571-3577.

140. Boyman, O., H. P. Hefti, C. Conrad, B. J. Nickoloff, M. Suter, and F. O. Nestle. 2004. Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-alpha. *J Exp Med* 199: 731-736.

141. Bissonnette, R., K. Papp, C. Maari, Y. Yao, G. Robbie, W. I. White, C. Le, and B. White. 2010. A randomized, double-blind, placebo-controlled, phase I study of MEDI-545, an anti-interferon-alfa monoclonal antibody, in subjects with chronic psoriasis. *J Am Acad Dermatol* 62: 427-436.

142. van der Fits, L., S. Mourits, J. S. Voerman, M. Kant, L. Boon, J. D. Laman, F. Cornelissen, A. M. Mus, E. Florencia, E. P. Prens, and E. Lubberts. 2009. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* 182: 5836-5845.

143. Szeimies, R. M., M. J. Gerritsen, G. Gupta, J. P. Ortonne, S. Serresi, J. Bichel, J. H. Lee, T. L. Fox, and A. Alomar. 2004. Imiquimod 5% cream for the treatment of actinic keratosis: results from a phase III, randomized, double-blind, vehicle-controlled, clinical trial with histology. *J Am Acad Dermatol* 51: 547-555.

144. Geisse, J. K., P. Rich, A. Pandya, K. Gross, K. Andres, A. Ginkel, and M. Owens. 2002. Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: a double-blind, randomized, vehicle-controlled study. *J Am Acad Dermatol* 47: 390-398.

145. Gilliet, M., C. Conrad, M. Geiges, A. Cozzio, W. Thurlimann, G. Burg, F. O. Nestle, and R. Dummer. 2004. Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors. *Arch Dermatol* 140: 1490-1495.

146. Wu, J. K., G. Siller, and G. Strutton. 2004. Psoriasis induced by topical imiquimod. *Australas J Dermatol* 45: 47-50.

147. Mabuchi, T., T. Takekoshi, and S. T. Hwang. 2011. Epidermal CCR6+ gammadelta T cells are major producers of IL-22 and IL-17 in a murine model of psoriasiform dermatitis. *J Immunol* 187: 5026-5031.

148. Hedrick, M. N., A. S. Lonsdorf, A. K. Shirakawa, C. C. Richard Lee, F. Liao, S. P. Singh, H. H. Zhang, A. Grinberg, P. E. Love, S. T. Hwang, and J. M. Farber. 2009. CCR6 is required for IL-23-induced psoriasis-like inflammation in mice. *J Clin Invest* 119: 2317-2329.

149. Wynn, T. A., R. Morawetz, T. Scharton-Kersten, S. Hieny, H. C. Morse, 3rd, R. Kuhn, W. Muller, A. W. Cheever, and A. Sher. 1997. Analysis of granuloma formation in double cytokine-deficient mice reveals a central role for IL-10 in polarizing both T helper cell 1- and T helper cell 2-type cytokine responses in vivo. *J Immunol* 159: 5014-5023.

150. Chaudhry, A., D. Rudra, P. Treuting, R. M. Samstein, Y. Liang, A. Kas, and A. Y. Rudensky. 2009. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* 326: 986-991.

151. Huber, S., N. Gagliani, E. Esplugues, W. O'Connor, Jr., F. J. Huber, A. Chaudhry, M. Kamanaka, Y. Kobayashi, C. J. Booth, A. Y. Rudensky, M. G. Roncarolo, M. Battaglia, and R. A. Flavell. Th17 cells express interleukin-10 receptor and are controlled by Foxp3 and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity* 34: 554-565.

152. Stumhofer, J. S., A. Laurence, E. H. Wilson, E. Huang, C. M. Tato, L. M. Johnson, A. V. Villarino, Q. Huang, A. Yoshimura, D. Sehy, C. J. Saris, J. J. O'Shea, L. Hennighausen, M. Ernst, and C. A. Hunter. 2006. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. *Nat Immunol* 7: 937-945.

153. Kleinschek, M. A., A. M. Owyang, B. Joyce-Shaikh, C. L. Langrish, Y. Chen, D. M. Gorman, W. M. Blumenschein, T. McClanahan, F. Brombacher, S. D. Hurst, R. A. Kastelein, and D. J. Cua. 2007. IL-25 regulates Th17 function in autoimmune inflammation. *J Exp Med* 204: 161-170.

154. Laurence, A., C. M. Tato, T. S. Davidson, Y. Kanno, Z. Chen, Z. Yao, R. B. Blank, F. Meylan, R. Siegel, L. Hennighausen, E. M. Shevach, and J. O'Shea J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26: 371-381.

155. Bozza, S., T. Zelante, S. Moretti, P. Bonifazi, A. DeLuca, C. D'Angelo, G. Giovannini, C. Garlanda, L. Boon, F. Bistoni, P. Puccetti, A. Mantovani, and L. Romani. 2008. Lack of Toll IL-1R8 exacerbates Th17 cell responses in fungal infection. *J Immunol* 180: 4022-4031.

156. Smith, K. A. 1988. Interleukin-2: inception, impact, and implications. *Science* 240: 1169-1176.

157. Malek, T. R., and I. Castro. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity* 33: 153-165.

158. Wang, X., M. Rickert, and K. C. Garcia. 2005. Structure of the quaternary complex of interleukin-2 with its alpha, beta, and gammac receptors. *Science* 310: 1159-1163.

159. Cheng, G., A. Yu, and T. R. Malek. T-cell tolerance and the multi-functional role of IL-2R signaling in T-regulatory cells. *Immunol Rev* 241: 63-76.

160. Moriggl, R., D. J. Topham, S. Teglund, V. Sexl, C. McKay, D. Wang, A. Hoffmeyer, J. van Deursen, M. Y. Sangster, K. D. Bunting, G. C. Grosveld, and J. N. Ihle. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity* 10: 249-259.

161. Burchill, M. A., C. A. Goetz, M. Prlic, J. J. O'Neil, I. R. Harmon, S. J. Bensinger, L. A. Turka, P. Brennan, S. C. Jameson, and M. A. Farrar. 2003. Distinct effects of STAT5 activation on CD4+ and CD8+ T cell homeostasis: development of CD4+CD25+ regulatory T cells versus CD8+ memory T cells. *J Immunol* 171: 5853-5864.

162. Murawski, M. R., S. A. Litherland, M. J. Clare-Salzler, and A. Davoodi-Semiromi. 2006. Upregulation of Foxp3 expression in mouse and human Treg is IL-2/STAT5 dependent: implications for the NOD STAT5B mutation in diabetes pathogenesis. *Ann N Y Acad Sci* 1079: 198-204.

163. Yang, X. P., K. Ghoreschi, S. M. Steward-Tharp, J. Rodriguez-Canales, J. Zhu, J. R. Grainger, K. Hirahara, H. W. Sun, L. Wei, G. Vahedi, Y. Kanno, J. J. O'Shea, and A. Laurence. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol* 12: 247-254.

164. Chen, Y., C. J. Haines, I. Gutcher, K. Hochweller, W. M. Blumenschein, T. McClanahan, G. Hammerling, M. O. Li, D. J. Cua, and M. J. McGeachy. Foxp3(+) regulatory T cells promote T helper 17 cell development in vivo through regulation of interleukin-2. *Immunity* 34: 409-421.

165. Pandiyan, P., H. R. Conti, L. Zheng, A. C. Peterson, D. R. Mathern, N. Hernandez-Santos, M. Edgerton, S. L. Gaffen, and M. J. Lenardo. CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. *Immunity* 34: 422-434.

166. Rickert, M., X. Wang, M. J. Boulanger, N. Goriatcheva, and K. C. Garcia. 2005. The structure of interleukin-2 complexed with its alpha receptor. *Science* 308: 1477-1480.

167. Rubin, L. A., and D. L. Nelson. 1990. The soluble interleukin-2 receptor: biology, function, and clinical application. *Ann Intern Med* 113: 619-627.

168. Sugaya, S., T. Sakimoto, J. Shoji, and M. Sawa. Regulation of soluble interleukin-6 (IL-6) receptor release from corneal epithelial cells and its role in the ocular surface. *Jpn J Ophthalmol* 55: 277-282.

169. Heaney, M. L., and D. W. Golde. 1998. Soluble receptors in human disease. *J Leukoc Biol* 64: 135-146.

170. Chistiakov, D. A., E. I. Chistiakova, N. V. Voronova, R. I. Turakulov, and K. V. Savost'anov. A Variant of the Il2ra / Cd25 Gene Predisposing to Graves' Disease is Associated with Increased Levels of Soluble Interleukin-2 Receptor. *Scand J Immunol* 74: 496-501.

171. Yang, Z. Z., D. M. Grote, S. C. Ziesmer, M. K. Manske, T. E. Witzig, A. J. Novak, and S. M. Ansell. Soluble IL-2R{alpha} facilitates IL-2-mediated immune responses and predicts reduced survival in follicular B-cell non-Hodgkin lymphoma. *Blood* 118: 2809-2820.

172. O'Neill, L. A. 2008. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol Rev* 226: 10-18.

173. Dinarello, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 27: 519-550.

174. Chung, Y., S. H. Chang, G. J. Martinez, X. O. Yang, R. Nurieva, H. S. Kang, L. Ma, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2009. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 30: 576-587.

175. Gelman, A. E., D. F. LaRosa, J. Zhang, P. T. Walsh, Y. Choi, J. O. Sunyer, and L. A. Turka. 2006. The adaptor molecule MyD88 activates PI-3 kinase signaling in CD4+ T cells and enables CpG oligodeoxynucleotide-mediated costimulation. *Immunity* 25: 783-793.

176. Reynolds, J. M., B. P. Pappu, J. Peng, G. J. Martinez, Y. Zhang, Y. Chung, L. Ma, X. O. Yang, R. I. Nurieva, Q. Tian, and C. Dong. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity* 32: 692-702.

177. Garlanda, C., H. J. Anders, and A. Mantovani. 2009. TIR8/SIGIRR: an IL-1R/TLR family member with regulatory functions in inflammation and T cell polarization. *Trends Immunol* 30: 439-446.

178. Wald, D., J. Qin, Z. Zhao, Y. Qian, M. Naramura, L. Tian, J. Towne, J. E. Sims, G. R. Stark, and X. Li. 2003. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. *Nat Immunol* 4: 920-927.

179. Thomassen, E., B. R. Renshaw, and J. E. Sims. 1999. Identification and characterization of SIGIRR, a molecule representing a novel subtype of the IL-1R superfamily. *Cytokine* 11: 389-399.

180. Mantovani, A., M. Locati, N. Polentarutti, A. Vecchi, and C. Garlanda. 2004. Extracellular and intracellular decoys in the tuning of inflammatory cytokines and Toll-like receptors: the new entry TIR8/SIGIRR. *J Leukoc Biol* 75: 738-742.

181. Gulen, M. F., Z. Kang, K. Bulek, W. Youzhong, T. W. Kim, Y. Chen, C. Z. Altuntas, K. Sass Bak-Jensen, M. J. McGeachy, J. S. Do, H. Xiao, G. M. Delgoffe, B. Min, J. D. Powell, V. K. Tuohy, D. J. Cua, and X. Li. 2010. The receptor SIGIRR suppresses Th17 cell proliferation via inhibition of the interleukin-1 receptor pathway and mTOR kinase activation. *Immunity* 32: 54-66.

182. Bulek, K., S. Swaidani, J. Qin, Y. Lu, M. F. Gulen, T. Herjan, B. Min, R. A. Kastelein, M. Aronica, M. Kosz-Vnenchak, and X. Li. 2009. The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response. *J Immunol* 182: 2601-2609.

183. Qin, J., Y. Qian, J. Yao, C. Grace, and X. Li. 2005. SIGIRR inhibits interleukin-1 receptor- and toll-like receptor 4-mediated signaling through different mechanisms. *J Biol Chem* 280: 25233-25241.

184. Riva, F., E. Bonavita, E. Barbati, M. Muzio, A. Mantovani, and C. Garlanda. 2012. TIR8/SIGIRR is an Interleukin-1 Receptor/Toll Like Receptor Family Member with Regulatory Functions in Inflammation and Immunity. *Front Immunol* 3: 322.

185. Garlanda, C., D. Di Liberto, A. Vecchi, M. P. La Manna, C. Buracchi, N. Caccamo, A. Salerno, F. Dieli, and A. Mantovani. 2007. Damping excessive inflammation and tissue damage in Mycobacterium tuberculosis infection by Toll IL-1 receptor 8/single Ig IL-1-related receptor, a negative regulator of IL-1/TLR signaling. *J Immunol* 179: 3119-3125.

186. Veliz Rodriguez, T., F. Moalli, N. Polentarutti, M. Paroni, E. Bonavita, A. Anselmo, M. Nebuloni, S. Mantero, S. Jaillon, A. Bragonzi, A. Mantovani, F. Riva, and C.

Garlanda. 2012. Role of TIR8/SIGIRR, a Negative Regulator of IL-1R/TLR Signalling, in Resistance to Acute Pseudomonas aeruginosa Lung Infection. *Infect Immun.*

187. Sham, H. P., E. Y. Yu, M. F. Gulen, G. Bhinder, M. Stahl, J. M. Chan, L. Brewster, V. Morampudi, D. L. Gibson, M. R. Hughes, K. M. McNagny, X. Li, and B. A. Vallance. 2013. SIGIRR, a Negative Regulator of TLR/IL-1R Signalling Promotes Microbiota Dependent Resistance to Colonization by Enteric Bacterial Pathogens. *PLoS Pathog* 9: e1003539.

188. Xiao, H., M. F. Gulen, J. Qin, J. Yao, K. Bulek, D. Kish, C. Z. Altuntas, D. Wald, C. Ma, H. Zhou, V. K. Tuohy, R. L. Fairchild, C. de la Motte, D. Cua, B. A. Vallance, and X. Li. 2007. The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. *Immunity* 26: 461-475.

189. Drexler, S. K., S. M. Sacre, and B. M. Foxwell. 2006. Toll-like receptors: a new target in rheumatoid arthritis? *Expert Rev Clin Immunol* 2: 585-599.

190. Sacre, S. M., S. K. Drexler, E. Andreakos, M. Feldmann, F. M. Brennan, and B. M. Foxwell. 2007. Could toll-like receptors provide a missing link in chronic inflammation in rheumatoid arthritis? Lessons from a study on human rheumatoid tissue. *Ann Rheum Dis* 66 Suppl 3: iii81-86.

191. Drexler, S. K., P. Kong, J. Inglis, R. O. Williams, C. Garlanda, A. Mantovani, A. S. Yazdi, F. Brennan, M. Feldmann, and B. M. Foxwell. 2010. SIGIRR/TIR-8 is an inhibitor of Toll-like receptor signaling in primary human cells and regulates inflammation in models of rheumatoid arthritis. *Arthritis Rheum* 62: 2249-2261.

192. Batliwalla, F. M., W. Li, C. T. Ritchlin, X. Xiao, M. Brenner, T. Laragione, T. Shao, R. Durham, S. Kemshetti, E. Schwarz, R. Coe, M. Kern, E. C. Baechler, T. W. Behrens, P. K. Gregersen, and P. S. Gulko. 2005. Microarray analyses of peripheral blood cells identifies unique gene expression signature in psoriatic arthritis. *Mol Med* 11: 21-29.

193. Garlanda, C., F. Riva, N. Polentarutti, C. Buracchi, M. Sironi, M. De Bortoli, M. Muzio, R. Bergottini, E. Scanziani, A. Vecchi, E. Hirsch, and A. Mantovani. 2004. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. *Proc Natl Acad Sci U S A* 101: 3522-3526.

194. Tortola, L., E. Rosenwald, B. Abel, H. Blumberg, M. Schafer, A. J. Coyle, J. C. Renauld, S. Werner, J. Kisielow, and M. Kopf. 2012. Psoriasiform dermatitis is driven by IL-36-mediated DC-keratinocyte crosstalk. *J Clin Invest* 122: 3965-3976.

195. Sadlack, B., H. Merz, H. Schorle, A. Schimpl, A. C. Feller, and I. Horak. 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 75: 253-261.

196. Willerford, D. M., J. Chen, J. A. Ferry, L. Davidson, A. Ma, and F. W. Alt. 1995. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity* 3: 521-530.

197. Suzuki, H., Y. W. Zhou, M. Kato, T. W. Mak, and I. Nakashima. 1999. Normal regulatory alpha/beta T cells effectively eliminate abnormally activated T cells lacking the interleukin 2 receptor beta in vivo. *J Exp Med* 190: 1561-1572.

198. Chistiakov, D. A., E. I. Chistiakova, N. V. Voronova, R. I. Turakulov, and K. V. Savost'anov. 2011. A variant of the Il2ra / Cd25 gene predisposing to graves' disease is associated with increased levels of soluble interleukin-2 receptor. *Scand J Immunol* 74: 496-501.

199. Symons, J. A., N. C. Wood, F. S. Di Giovine, and G. W. Duff. 1988. Soluble IL-2 receptor in rheumatoid arthritis. Correlation with disease activity, IL-1 and IL-2 inhibition. *J Immunol* 141: 2612-2618.

200. Zhou, W., F. Zhang, and T. M. Aune. 2003. Either IL-2 or IL-12 is sufficient to direct Th1 differentiation by nonobese diabetic T cells. *J Immunol* 170: 735-740.

201. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6: 1142-1151.

202. Morgan, D. A., F. W. Ruscetti, and R. Gallo. 1976. Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* 193: 1007-1008.

203. Chen, Y., C. J. Haines, I. Gutcher, K. Hochweller, W. M. Blumenschein, T. McClanahan, G. Hammerling, M. O. Li, D. J. Cua, and M. J. McGeachy. 2011. Foxp3(+) regulatory T cells promote T helper 17 cell development in vivo through regulation of interleukin-2. *Immunity* 34: 409-421.

204. Setoguchi, R., S. Hori, T. Takahashi, and S. Sakaguchi. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 201: 723-735.

205. Turka, L. A., and P. T. Walsh. 2008. IL-2 signaling and CD4+ CD25+ Foxp3+ regulatory T cells. *Front Biosci* 13: 1440-1446.

206. Yang, X. P., K. Ghoreschi, S. M. Steward-Tharp, J. Rodriguez-Canales, J. Zhu, J. R. Grainger, K. Hirahara, H. W. Sun, L. Wei, G. Vahedi, Y. Kanno, J. J. O'Shea, and A. Laurence. 2011. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol* 12: 247-254.

207. Liao, W., J. X. Lin, L. Wang, P. Li, and W. J. Leonard. 2011. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat Immunol* 12: 551-559.

208. Reboldi, A., C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, A. Uccelli, A. Lanzavecchia, B. Engelhardt, and F. Sallusto. 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* 10: 514-523.

209. Rubin, L. A., G. Jay, and D. L. Nelson. 1986. The released interleukin 2 receptor binds interleukin 2 efficiently. *J Immunol* 137: 3841-3844.

210. Martin, R. 2012

Anti-CD25 (daclizumab) monoclonal antibody therapy in relapsing-remitting multiple sclerosis. *Clin Immunol* 142: 9-14.

211. Leonardi, C. L., A. B. Kimball, K. A. Papp, N. Yeilding, C. Guzzo, Y. Wang, S. Li, L. T. Dooley, and K. B. Gordon. 2008. Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). *Lancet* 371: 1665-1674.

212. Debets, R., J. P. Hegmans, P. Croughs, R. J. Troost, J. B. Prins, R. Benner, and E. P. Prens. 1997. The IL-1 system in psoriatic skin: IL-1 antagonist sphere of influence in lesional psoriatic epidermis. *J Immunol* 158: 2955-2963.

213. Mee, J. B., M. J. Cork, F. S. di Giovine, G. W. Duff, and R. W. Groves. 2006. Interleukin-1: a key inflammatory mediator in psoriasis? *Cytokine* 33: 72-78.

214. Uribe-Herranz, M., L. H. Lian, K. M. Hooper, K. A. Milora, and L. E. Jensen. 2013. IL-1R1 Signaling Facilitates Munro's Microabscess Formation in Psoriasiform Imiquimod-Induced Skin Inflammation. *J Invest Dermatol.*

215. Riva, F., E. Bonavita, E. Barbati, M. Muzio, A. Mantovani, and C. Garlanda. 2012. TIR8/SIGIRR is an Interleukin-1 Receptor/Toll Like Receptor Family Member with Regulatory Functions in Inflammation and Immunity. *Front Immunol* 3: 322.

216. Gulen, M. F., Z. Kang, K. Bulek, W. Youzhong, T. W. Kim, Y. Chen, C. Z. Altuntas, K. Sass Bak-Jensen, M. J. McGeachy, J. S. Do, H. Xiao, G. M. Delgoffe, B. Min, J. D. Powell, V. K. Tuohy, D. J. Cua, and X. Li. The receptor SIGIRR suppresses Th17 cell proliferation via inhibition of the interleukin-1 receptor pathway and mTOR kinase activation. *Immunity* 32: 54-66.

217. Lech, M., V. Skuginna, O. P. Kulkarni, J. Gong, T. Wei, R. W. Stark, C. Garlanda, A. Mantovani, and H. J. Anders. 2010. Lack of SIGIRR/TIR8 aggravates hydrocarbon oil-induced lupus nephritis. *J Pathol* 220: 596-607.

218. Gong, J., T. Wei, R. W. Stark, F. Jamitzky, W. M. Heckl, H. J. Anders, M. Lech, and S. C. Rossle. 2010. Inhibition of Toll-like receptors TLR4 and 7 signaling pathways by SIGIRR: a computational approach. *J Struct Biol* 169: 323-330.

219. Mabuchi, T., T. Takekoshi, and S. T. Hwang. 2011. Epidermal CCR6+ {gamma}{delta} T Cells Are Major Producers of IL-22 and IL-17 in a Murine Model of Psoriasiform Dermatitis. *J Immunol.*

220. Cua, D. J., and C. M. Tato. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 10: 479-489.

221. Shepherd, J., M. C. Little, and M. J. Nicklin. 2004. Psoriasis-like cutaneous inflammation in mice lacking interleukin-1 receptor antagonist. *J Invest Dermatol* 122: 665-669.

222. Wang, Y., H. Gao, C. M. Loyd, W. Fu, D. Diaconu, S. Liu, K. D. Cooper, T. S. McCormick, D. I. Simon, and N. L. Ward. 2010. Chronic skin-specific inflammation promotes vascular inflammation and thrombosis. *J Invest Dermatol* 132: 2067-2075.

223. Van Belle, A. B., M. de Heusch, M. M. Lemaire, E. Hendrickx, G. Warnier, K. Dunussi-Joannopoulos, L. A. Fouser, J. C. Renauld, and L. Dumoutier. 2010. IL-22 is required for imiquimod-induced psoriasiform skin inflammation in mice. *J Immunol* 188: 462-469.

224. Johnston, A., Y. Fritz, S. M. Dawes, D. Diaconu, P. M. Al-Attar, A. M. Guzman, C. S. Chen, W. Fu, J. E. Gudjonsson, T. S. McCormick, and N. L. Ward. 2013. Keratinocyte Overexpression of IL-17C Promotes Psoriasiform Skin Inflammation. *J Immunol* 190: 2252-2262.

225. El Malki, K., S. H. Karbach, J. Huppert, M. Zayoud, S. Reissig, R. Schuler, A. Nikolaev, K. Karram, T. Munzel, C. R. Kuhlmann, H. J. Luhmann, E. von Stebut, S. Wortge, F. C. Kurschus, and A. Waisman. 2013. An alternative pathway of imiquimod-induced psoriasis-like skin inflammation in the absence of interleukin-17 receptor a signaling. *J Invest Dermatol* 133: 441-451.

226. Benham, H., P. Norris, J. Goodall, M. D. Wechalekar, O. FitzGerald, A. Szentpetery, M. Smith, R. Thomas, and H. Gaston. 2013. Th17 and Th22 cells in psoriatic arthritis and psoriasis. *Arthritis Res Ther* 15: R136.

227. Russell, S. E., A. C. Moore, P. G. Fallon, and P. T. Walsh. 2012. Soluble IL-2Ralpha (sCD25) exacerbates autoimmunity and enhances the development of Th17 responses in mice. *PLoS One* 7: e47748.

228. Russell, S. E., A. M. Stefanska, M. Kubica, R. M. Horan, A. Mantovani, C. Garlanda, P. G. Fallon, and P. T. Walsh. 2013. Toll IL-1R8/Single Ig IL-1-Related Receptor Regulates Psoriasiform Inflammation through Direct Inhibition of Innate IL-17A Expression by gammadelta T Cells. *J Immunol.*

229. Murphy, C. A., C. L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R. A. Kastelein, J. D. Sedgwick, and D. J. Cua. 2003. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 198: 1951-1957.

230. Tesmer, L. A., S. K. Lundy, S. Sarkar, and D. A. Fox. 2008. Th17 cells in human disease. *Immunol Rev* 223: 87-113.

231. Hueber, W., D. D. Patel, T. Dryja, A. M. Wright, I. Koroleva, G. Bruin, C. Antoni, Z. Draelos, M. H. Gold, P. Durez, P. P. Tak, J. J. Gomez-Reino, C. S. Foster, R. Y. Kim, C. M. Samson, N. S. Falk, D. S. Chu, D. Callanan, Q. D. Nguyen, K. Rose, A. Haider, and F. Di Padova. 2010. Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. *Sci Transl Med* 2: 52ra72.

232. Hueber, W., B. E. Sands, S. Lewitzky, M. Vandemeulebroecke, W. Reinisch, P. D. Higgins, J. Wehkamp, B. G. Feagan, M. D. Yao, M. Karczewski, J. Karczewski, N. Pezous, S. Bek, G. Bruin, B. Mellgard, C. Berger, M. Londei, A. P. Bertolino, G. Tougas, and S. P. Travis. 2012. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* 61: 1693-1700.

233. Symons, A., A. L. Budelsky, and J. E. Towne. 2012. Are Th17 cells in the gut pathogenic or protective? *Mucosal Immunol* 5: 4-6.

234. Meelu, P., A. Awasthi, V. K. Kuchroo, and V. Yajnik. 2011. Deficiency in IL23R Accelerates Wound Healing in Experimental Colitis. *Gastroenterology* 140: S-142-S-143.

235. Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M. A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D. J. Cua, R. A. Kastelein, and D. Rennick. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 116: 1310-1316.

236. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8: 275-283.

237. Batten, M., J. Li, S. Yi, N. M. Kljavin, D. M. Danilenko, S. Lucas, J. Lee, F. J. de Sauvage, and N. Ghilardi. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. *Nat Immunol* 7: 929-936.

238. Young, A., E. Linehan, E. Hams, A. C. O'Hara Hall, A. McClurg, J. A. Johnston, C. A. Hunter, P. G. Fallon, and D. C. Fitzgerald. 2012. Cutting edge: suppression of GM-CSF expression in murine and human T cells by IL-27. *J Immunol* 189: 2079-2083.

239. Ponomarev, E. D., L. P. Shriver, K. Maresz, J. Pedras-Vasconcelos, D. Verthelyi, and B. N. Dittel. 2007. GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. *J Immunol* 178: 39-48.

240. El-Behi, M., B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G. X. Zhang, B. N. Dittel, and A. Rostami. 2011. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* 12: 568-575.

241. Stumhofer, J. S., J. S. Silver, A. Laurence, P. M. Porrett, T. H. Harris, L. A. Turka, M. Ernst, C. J. Saris, J. J. O'Shea, and C. A. Hunter. 2007. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 8: 1363-1371.

242. Awasthi, A., Y. Carrier, J. P. Peron, E. Bettelli, M. Kamanaka, R. A. Flavell, V. K. Kuchroo, M. Oukka, and H. L. Weiner. 2007. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* 8: 1380-1389.

243. Fitzgerald, D. C., G. X. Zhang, M. El-Behi, Z. Fonseca-Kelly, H. Li, S. Yu, C. J. Saris, B. Gran, B. Ciric, and A. Rostami. 2007. Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. *Nat Immunol* 8: 1372-1379.

244. Kleinewietfeld, M., A. Manzel, J. Titze, H. Kvakan, N. Yosef, R. A. Linker, D. N. Muller, and D. A. Hafler. 2013. Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature* 496: 518-522.

245. Wu, C., N. Yosef, T. Thalhamer, C. Zhu, S. Xiao, Y. Kishi, A. Regev, and V. K. Kuchroo. 2013. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature* 496: 513-517.

246. Buonocore, S., P. P. Ahern, H. H. Uhlig, Ivanov, II, D. R. Littman, K. J. Maloy, and F. Powrie. 2010. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* 464: 1371-1375.

247. Miossec, P., T. Korn, and V. K. Kuchroo. 2009. Interleukin-17 and type 17 helper T cells. *N Engl J Med* 361: 888-898.

248. Bettelli, E., T. Korn, M. Oukka, and V. K. Kuchroo. 2008. Induction and effector functions of T(H)17 cells. *Nature* 453: 1051-1057.

249. Feinen, B., and M. W. Russell. 2009. Contrasting Roles of IL-22 and IL-17 in Murine Genital Tract Infection by Neisseria gonorrhoeae. *Front Immunol* 3: 11.

250. Kreymborg, K., R. Etzensperger, L. Dumoutier, S. Haak, A. Rebollo, T. Buch, F. L. Heppner, J. C. Renauld, and B. Becher. 2007. IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. *J Immunol* 179: 8098-8104.

251. Boraschi, D., and A. Tagliabue. 2013. The interleukin-1 receptor family. *Semin Immunol* 25: 394-407.

252. van de Veerdonk, F. L., A. K. Stoeckman, G. Wu, A. N. Boeckermann, T. Azam, M. G. Netea, L. A. Joosten, J. W. van der Meer, R. Hao, V. Kalabokis, and C. A. Dinarello. 2012. IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist. *Proc Natl Acad Sci U S A* 109: 3001-3005.

Chapter 7: Appendix





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Toll IL-1R8/Single Ig IL-1–Related Receptor Regulates Psoriasiform Inflammation through Direct Inhibition of Innate IL-17A Expression by γδ T Cells

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Toll IL-1R8/Single Ig IL-1–Related Receptor Regulates Psoriasiform Inflammation through Direct Inhibition of Innate IL-17A Expression by γδ T Cells

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Expression of the orphan receptor Toll IL-1R8/single Ig IL-1–related receptor has been reported to be reduced in the peripheral blood of psoriatic arthritis patients. However whether TIR8/SIGIRR activity plays a specific role in regulating psoriatic inflammation is unknown. We report that *Tir8/Sigirr*-deficient mice develop more severe psoriatic inflammation in both the chemical (Aldara)- and cytokine (rIL-23)-induced models of psoriasis. Increased disease severity was associated with enhanced infiltration of Vγ4$^+$ γδ T cells that express significantly elevated levels of IL-17A. Critically, we also demonstrate that TIR8/SIGIRR activity directly suppressed innate IL-17A expression by γδ T cells in vitro and in vivo. Importantly, treatment of *Tir8/Sigirr*$^{-/-}$ mice with an IL-17A neutralization Ab reversed the enhanced disease severity observed in these mice. This study identifies TIR8/SIGIRR as a novel intrinsic negative regulator of innate IL-17A expression and characterizes a novel mechanism involved in the regulation of psoriatic inflammation. *The Journal of Immunology*, 2013, 191: 3337–3346.

Psoriasis is a common chronic immune-driven disease of the skin with incidences of between 1 and 3% in the population of western countries. Although the disease rarely proves fatal, patients suffer significant physical discomfort from the appearance of inflamed psoriatic skin lesions, which can also be associated with an added psychological burden. The pathogenesis of psoriasis involves a complex interplay of immunological, genetic, and environmental factors, all of which can contribute in varying degrees to the specific disease phenotype in humans (1, 2). The condition is commonly characterized by rapid and dysregulated keratinocyte proliferation leading to a thickened epidermis (acantosis). This aberrant keratinocyte behavior often leads to significant retention of nucleated cells within the granular layer (parakeratosis) and a very apparent erythema (redness) because of increasingly dilated blood vessels in the dermis. These manifestations occur in conjunction with a marked immune cell infiltrate composed primarily of dense concentrations of T cells along with dendritic cells, neutrophils, and macrophages (3). Classically, psoriasis has come to be viewed as a T cell–driven autoimmune disorder due at least in part to its responsiveness to cyclosporine treatment (4) and also as Th1 cells, expressing IFN-γ, were seen as the key drivers of disease pathogenesis (5, 6). However, more recently a deeper characterization of both psoriatic lesional infiltrates and the peripheral blood of patients have uncovered a prominent role for inflammatory cells driven by the IL-23/IL-17 signaling axis as central mediators of disease (7, 8). Such cells include subsets of innate γδ T cells and lymphoid cells that express abundant amounts of proinflammatory IL-17A, IL-17F, and IL-22 at key skin barrier sites (9, 10). The importance of these cells, along with CD4$^+$ Th17 cells that express a similar cytokine repertoire, in psoriasis pathogenesis has underscored the development of a range of mAbs targeting the IL-23/IL-17 signaling axis and, more specifically, IL-17R A (Brodalumab) and one of its ligands, IL-17A (Ixekizumab) (11, 12). These targeted therapies have met with considerable early success in the clinic and will most likely form the next range of front line therapies for psoriasis treatment. However, despite these exciting advances in drug development, significant gaps remain in our understanding of the regulatory mechanisms that underpin the IL-23/IL-17 signaling axis and the cellular and molecular interactions at play in the context of dermal inflammation in psoriasis.

IL-23 is a myeloid-derived cytokine that acts as a key growth factor inducing the expression of a number of type 17 cytokines including IL-17A, IL-17F, and IL-22 from both Th17 cells and innate γδ cells, which a number of recent studies have implicated as central drivers of psoriatic inflammation (7, 10, 13, 14). IL-23 shares its p40 subunit with the Th1-instructive cytokine IL-12, and an anti-p40 mAb (Ustekinumab) has met with some successes for the treatment of psoriasis in the clinic (15). Although the role IL-23 plays in the development of type 17 responses and associated autoimmunity is well established, a number of other cytokines are also known to contribute to, and indeed compliment, the actions of IL-23 in similar settings. One such example is IL-1, a cytokine that exists in two distinct forms, IL-1β and IL-1α. These signaling mediators both share the same receptor, IL-1R1 type 1 (IL-1R1), and elicit remarkably similar effects despite significant differences

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S.E.R. and P.T.W. conceived and designed the research; S.E.R., A.M.S., M.K., and R.M.H. performed the experiments; S.E.R., A.M.S., M.K., P.G.F., and P.T.W. analyzed the data; C.G., A.M., and P.G.F. contributed reagents/materials/analysis tools; and S.E.R. and P.T.W. wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: IL-1R1, IL-1R1 type 1; mTOR, mammalian target of rapamycin; SIGIRR, single Ig IL-1–related receptor; TIR8, Toll IL-1R8; WT, wild-type.

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in their expression and activity (16). It has long been surmised that IL-1R1 signaling is dysregulated in psoriasis; however, its specific role has proved controversial (17, 18). Recently reports have demonstrated that IL-1R1 signaling is indispensable for acanthosis, neutrophil recruitment, and Munro's abscess formation in the Aldara-induced model of psoriasis (19). These findings coupled with its established role in complimenting the proinflammatory actions of IL-23 highlight the need to further examine the role IL-1R1 signaling plays in psoriasis pathogenesis and in particular investigate the action of signaling mediators that are known to regulate its effects.

One such mediator is Toll IL-1R8 also known as single Ig IL-1–related receptor (TIR8/SIGIRR), which serves as a negative regulator of inflammation driven by the Toll/IL-1R superfamily members (20, 21). TIR8/SIGIRR is currently designated as an orphan receptor for reasons attributed to its unique structure within the Toll/IL-1R superfamily. It possesses a single extracellular Ig domain, distinct from the characteristic three domains of other family members such as the IL-1R1 and IL-18R, and current consensus suggests the small size of this single extracellular portion precludes binding of a cognate extracellular ligand. Furthermore, TIR8/SIGIRR possesses two amino acid substitutions (Ser^{447} and Tyr^{536} replaced by Cys^{222} and Le^{u305}) in its common intracellular TIR signaling domain. Although the functional consequences, if any, of these substitutions are unknown, the observation is considered suggestive of impaired signal transduction ability (21-23). Recently, TIR8/SIGIRR has been identified as a modulator of autoimmune disease through its ability to inhibit the IL-1R1–driven proliferation of Th17 cells (24). Little is known of the impact TIR8/SIGIRR may elicit in human disease; however, it is noteworthy that its expression is significantly downregulated in the peripheral blood of psoriatic arthritis patients (25). This observation coupled with the results of murine studies implicating an important role in the regulation of IL-23/IL-17 signaling strongly indicates a potential role in the regulation of psoriatic inflammation.

In this study, we define a role for TIR8/SIGIRR as a modulator of psoriatic inflammation through direct suppression of innate IL-17A expression by γδ T cells. *Tir8/Sigirr*$^{-/-}$ mice display an exacerbated disease phenotype in two murine models of psoriasiform inflammation. These events are associated with enhanced infiltration of $V\gamma4^{+}$ γδ T cells with significantly increased expression of IL-17A. Critically, we also demonstrate that TIR8/SIGIRR activity suppressed innate IL-17A expression by γδ T cells in vitro and in vivo. Importantly, the increased disease severity observed in the absence of TIR8/SIGIRR is reversible through treatment with an anti–IL-17A neutralization Ab. These data identify a previously unappreciated role for TIR8/SIGIRR in the regulation of innate IL-17A expression by γδ T cells and as a novel regulator of dermal inflammation in the context of psoriasis.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice were purchased from Harlan UK. *Tir8/Sigirr*$^{-/-}$ mice on a C57BL/6 background were provided by A. Mantovani (University of Milan). All mice used were between 6 and 8 wk and were housed under specific pathogen-free conditions at Institute for Molecular Medicine, St. James Hospital (Dublin, Ireland). All animal experiments were performed in compliance with Irish Department of Health regulations (license number B100/4272) and approved by the institutional ethical review board.

Materials

ELISA kits for mouse IL-17A, IL-17F, IL-17A/F, and IL-22 were purchased from eBioscience (Hatfield, U.K.) and were carried out according to manufacturer's instructions. Recombinant mouse IL-1β and IL-23 were purchased from eBioscience. The following Abs were used for surface staining CD45 (30-F11), CD3 (145-2C11), γδ TCR (Ebio GL3), Vγ4 (UC3-10A6) (BioLegend), and CD4 (RM4-5). Intracellular staining was performed for IL-17A (17B7), IL-22 (IL22JOP), IL-17F (eBio18F10), IFN-γ (XMG 1.2), and BrdU (3D4). All Abs were purchased from eBioscience unless otherwise stated. The JNK inhibitor SP600125 and mammalian target of rapamycin (mTOR) inhibitor Temsirolimus were purchased from Sigma-Aldrich.

Aldara-induced model of psoriasiform inflammation and anti–IL-17A treatment

Mice were shaved on their back before hair removal using a depilatory cream. Two days later, imiquimod (5% cream; Aldara MEDA Pharmaceuticals, Dublin, Ireland) or control cream was applied to the back (3.125 mg/day) or ears (1 mg/day) of mice for 1–7 d, depending on experimental requirements. Ear thickness was measured using a thickness gauge (Hitec) before the initiation of the experiment on day 0 and every day thereafter until the termination of the experiment. Cells from skin draining lymph nodes were restimulated with IL-23 (50 ng/ml) for 12 h before further analysis by flow cytometry. Ears from WT and *Tir8/Sigirr*$^{-/-}$ mice were also taken and homogenized in PBS containing a protease inhibitor mixture (Sigma-Aldrich). Protein concentrations were equalized, and homogenate was analyzed for expression of IL-1β and IL-23 by ELISA. For IL-17A neutralization experiments, mice were treated on days 0 and 2 with 250 μg/mouse anti–IL-17A Ab (17F3) or mouse IgG1 isotype control (MOPC-21) (BioXCell, West Lebanon, NH), and disease was monitored as described previously (9).

IL-23 intradermal injection model

WT and *Tir8/Sigirr*$^{-/-}$ mice were anesthetized and injected intradermally with either 200 ng IL-23 or PBS in a 20-μl volume every day using a 30-gauge needle. Ear thickness was measured using a thickness guage (Hitec) before the initiation of the experiment on day 0 and every day thereafter for a period of 7 d. Cells from psoriatic skin were restimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 2 h in the presence of brefeldin A (5 μg/ml) before further analysis by flow cytometry.

Cell preparation

Ears were cut into small pieces and digested for 1 h at 37°C in complete RPMI 1640 medium supplemented with 1 mg/ml collagenase D and 100 mg/ml DNase I before passing through a 40 μM filter to form a single-cell suspension. Lymph nodes or spleens were processed into single-cell suspensions by dissociation of tissue and passing through a 40 μM filter.

Flow cytometry

Cells were analyzed for surface and intracellular protein expression using an LSR/Fortessa (BD Biosciences) before further analysis using FlowJo software (Tree Star). For all experiments, initial gating was performed on live lymphocytes from forward versus side scatter plots. Subsequent gating for γδ T cells and subsets were performed for CD45, γδ TCR, and Vγ4 positivity as appropriate. Other unconventional cell subsets were gated on CD45-positive and CD3-negative staining. CD4 T cells were gated on CD4-positive cells from the live lymphocyte gate. For intracellular staining, fixation and permeabilization of cells were performed using a Fix/Perm Kit (DakoCytomation), according to the manufacturer's instructions. Appropriate isotype control Abs were used for setting positive gates in intracellular staining experiments.

In vivo footpad model

Mice were injected with 50 ng IL-1β, IL-23, or a combination of both or with the indicated TLR ligands into each hind footpad in a total volume of 20 μl as described previously (26). PBS injection was used as a vehicle control. After 4 h, the popliteal lymph nodes were harvested and processed to single-cell suspension before culture in the presence of brefeldin A (5 μg/ml) for an additional 4 h. Cells were subsequently analyzed for surface and intracellular protein by FACS.

BrdU flow kit (BD Pharmingen) was used according to the manufacturer's instructions for in vivo BrdU labeling of cells. Briefly, mice were injected i.p. with BrdU (1 mg/mouse) 48 and 24 h prior to initiation of the footpad model as described above. Upon isolation, cells were stained for surface protein before fixation and permeabilization with BD Cytofix/Cytoperm Buffer. Cells were subsequently treated with DNaseI to expose intracellular BrdU and stained with anti-BrdU.

RT-PCR and quantification

Extraction of RNA from whole tissue was performed using an Isolate RNA mini kit (Bioline), and RNA levels were equalized before performing RT-

PCR using a High Capacity cDNA kit (Applied Biosystems), all according to the manufacturer's instructions. Real-time PCR was performed using SensiFAST Probe Hi-ROX kit (Bioline) incorporating predesigned gene expression assays for IL-17A, IL-22, IL-1β, IL-23, and tir8 (Applied Biosystems). Expression was normalized to 18s rRNA. Samples were assayed on an Applied Biosystems 7900HT Fast Real-Time PCR System.

Histopathological analysis of tissue

Tissue was fixed overnight in 10% formalin before dehydration and embedding into paraffin blocks. Eight-micrometer sections were subsequently cut and stained with H&E. Sections were scored blindly for pathological manifestations of psoriatic inflammation on a scale of 0–4 (0, no observable difference over control; 1, mild; 2, moderate; 3, marked; and 4, severe) for the following parameters: acantosis, desquamation, parakeratosis, and infiltration. Scores for each parameter were combined for an overall histological severity score.

In vitro cell stimulation

WT and *Tir8/Sigirr*$^{-/-}$ splenocytes were cultured in the presence of IL-23 (10 ng/ml) and IL-1β (0.01 ng–10 ng/ml) in the absence of plate bound Ab stimulation for 72 h before analysis of the cell culture supernatants by ELISA. For inhibition of IL-1R1 signaling pathways, cells were preinclubated for 2 h at 37°C at the indicated concentrations prior to washing and stimulation. WT and *Tir8/Sigirr*$^{-/-}$ γδ T cells were purified (>98%) by FACS sorting using a MoFlo sorter (Beckman Coulter) and cultured in the same manner as above or in combination with rIL-6 (10 ng/ml) and TGF-β (5 ng/ml) or immiquimod (5 μg/ml) before performing intracellular cytokine staining and analysis of cell culture supernatants by ELISA. Identical experiments were also performed in the presence (1:1 ratio) of APCs (T-depleted splenocytes), which had been prestimulated overnight with immiquimod (5 μg/ml)

Results

Enhanced severity of Aldara-induced psoriasiform inflammation in Tir8/Sigirr$^{-/-}$ *mice*

Innate γδ T cells that express IL-17A in response to IL-1R1 stimulation are thought to play a key pathological role in a variety of autoimmune disorders with recent emerging evidence, suggesting a critical role in the progression of psoriasis (10, 27). Interestingly, it has been observed that TIR8/SIGIRR expression is significantly downregulated in the peripheral blood of psoriatic patients; however, despite these observations, the role of TIR8/SIGIRR in the

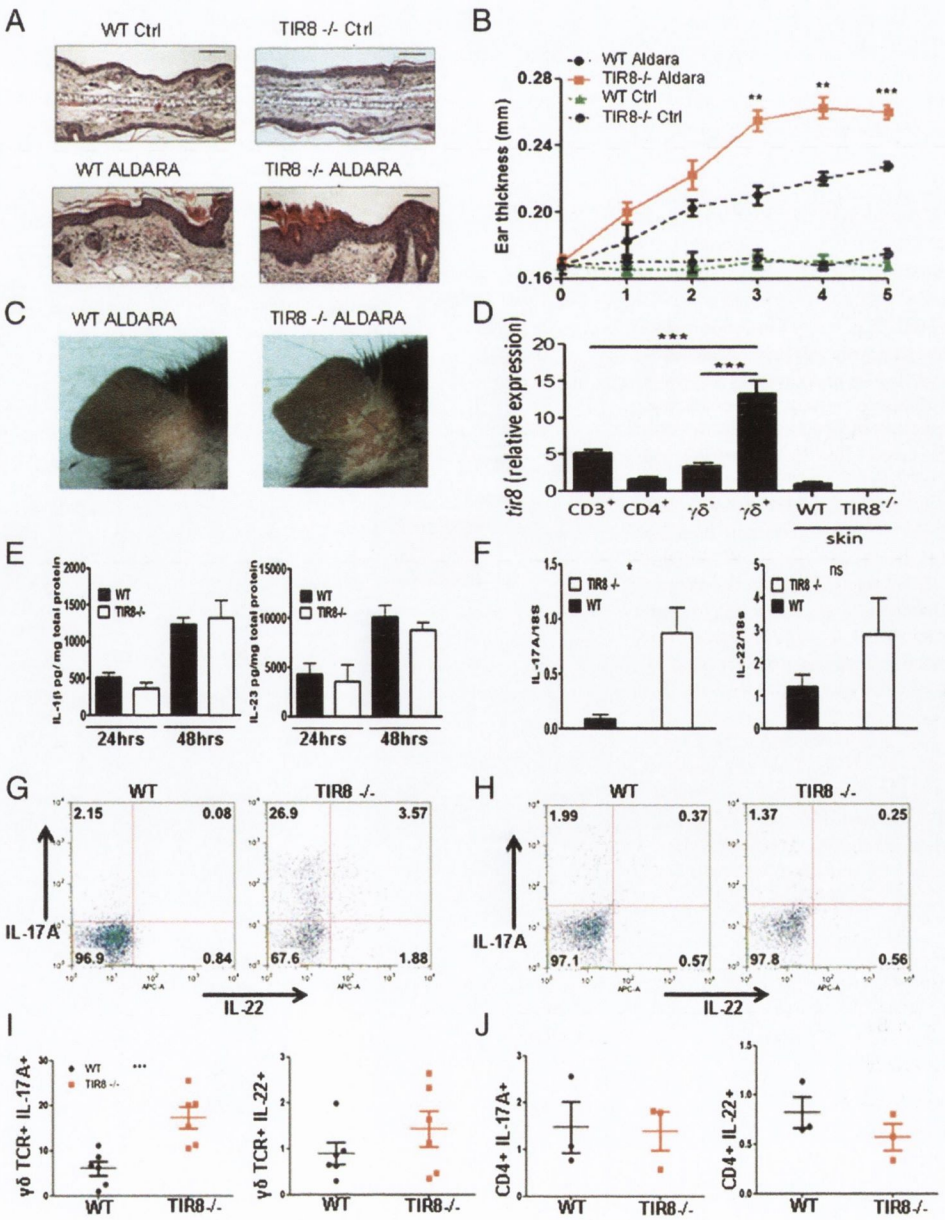


FIGURE 1. Enhanced severity of Aldara-induced psoriasiform inflammation in *Tir8/Sigirr*$^{-/-}$ mice. (**A**) H&E-stained sections from ears of WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara (1 mg/day) or control cream for 5 d (scale bar, 50 μm). (**B**) Increased epidermal thickening in *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara. (**C**) Representative macroscopic photographs of ears from WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara. (**D**) Real-time PCR analysis for basal expression of *Tir8* in the indicated cells and tissues. WT unless stated otherwise. (**E**) ELISAs for IL-1β and IL-23 were performed on tissue lysates of ears from WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara for the indicated times. (**F**) Real-time PCR analysis of IL-17A and IL-22 from psoriatic skin of WT and TIR8$^{-/-}$ mice after 5 d of treatment with Aldara. Cells from skin draining lymph nodes of WT and *Tir8/Sigirr*$^{-/-}$ mice were examined by flow cytometry for intracellular IL-17A and IL-22 expression from γδ T cells (**G**, **I**) and CD4 T cells (**H**, **J**). These data are representative of at least three independent experiments with similar results. Statistical significance determined by unpaired Student *t* test; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

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regulation γδ T cell–driven pathology and more specifically its role in the regulation of dermal inflammation in psoriasis have yet to be addressed (25). To answer these questions, we used a murine model of psoriasiform inflammation where γδ T cells are known to play a key role in disease progression. This model, which is IL-23 dependent, involves daily topical application of the TLR7 agonist imiquimod formulated in a commercially available cream, Aldara (28). Aldara has been used for some time in the treatment of a variety of dermal malignancies such as actinic keratoses and superficial basal cell carcinomas (29, 30); however, a side effect of such treatment is the exacerbation of psoriatic lesions in some patients, even those with well controlled psoriasis (31, 32). On the basis of this observation, use of Aldara in murine studies as an inducer of psoriasiform inflammation has come to be accepted as a clinically faithful model of psoriasis, particularly as the pathological manifestations induced bear striking similarities to human psoriasis but also because it is responsive to a range of the current frontline psoriasis therapies (9, 10, 28).

Aldara cream applied to the ears of WT control and *Tir8/Sigirr*$^{-/-}$ mice over a period of 5–7 d resulted in an exacerbated inflammatory phenotype in *Tir8/Sigirr*$^{-/-}$ mice. Analysis of H&E-stained sections of treated ears revealed enhanced acantosis, desquamation (scaling and outer layer shedding), and dermal infiltration of nucleated cells in *Tir8/Sigirr*$^{-/-}$ mice (Fig. 1A). Also, measurement of the overall thickness of treated ears revealed significantly enhanced epidermal thickening in *Tir8/Sigirr*$^{-/-}$ mice (Fig. 1B). This enhanced disease phenotype is also clearly visible macroscopically with enhanced desquamation and erythema on the ears of *Tir8/Sigirr*$^{-/-}$ mice (Fig. 1C). To investigate which cell types TIR8/SIGIRR activity may play a role in the context of dermal inflammation, we first examined levels of gene expression on relevant cell subsets and tissues by real-time PCR analysis. Interestingly, basal expression of the *Tir8/Sigirr* gene was found to be significantly higher in γδ T cells when compared with CD3$^+$, CD4$^+$, and CD3$^+$γδ$^-$ cell subsets and murine skin (Fig. 1D). Application of Aldara is thought, in part, to drive psoriasiform inflammation through induction of IL-23 and IL-1β from dendritic cells and keratinocytes (19, 28). To investigate the possibility that an enhancement of either, or both, of these cytokines in the *Tir8/Sigirr*$^{-/-}$ mouse was

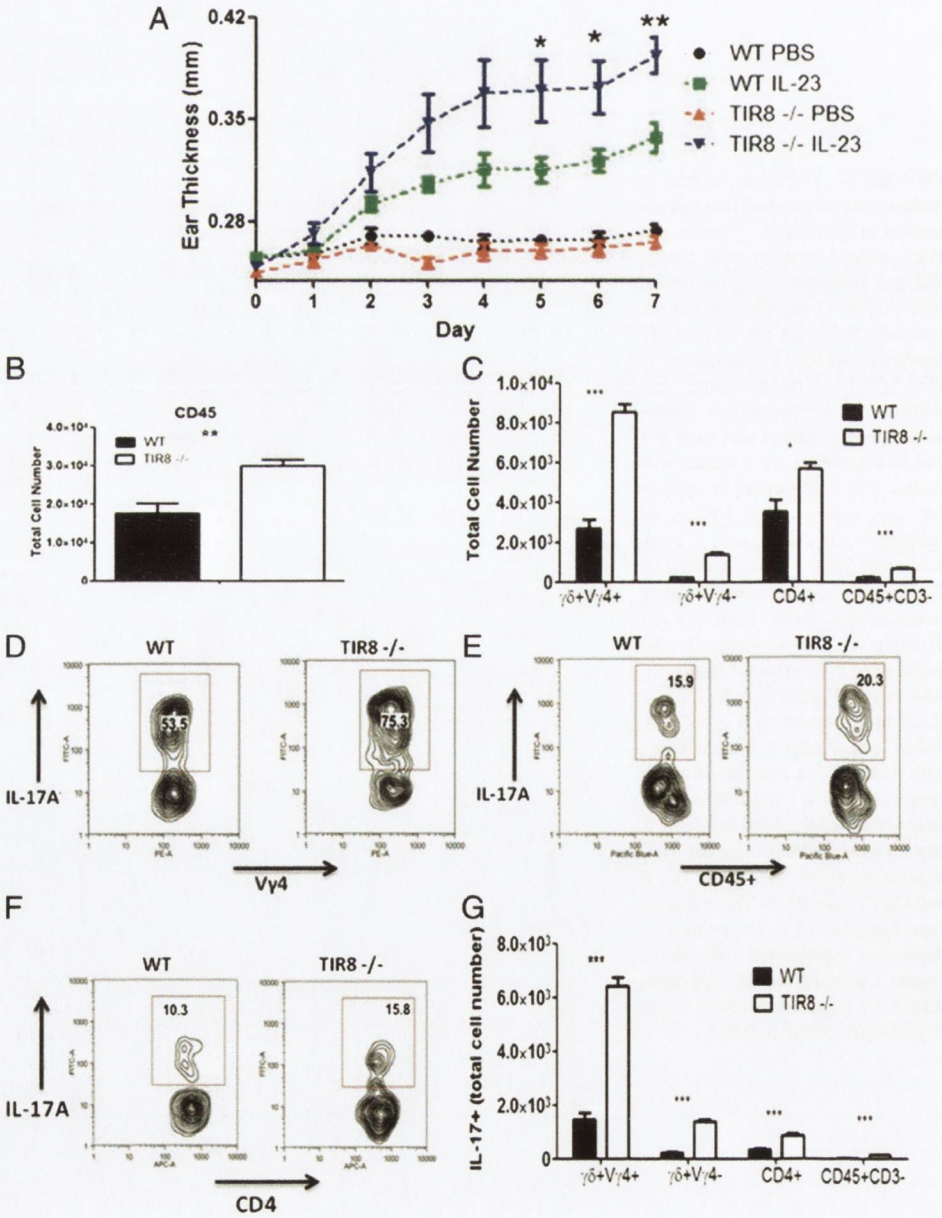


FIGURE 2. IL-23 injection results in significantly enhanced inflammation and IL-17A expression in tissues of *Tir8/Sigirr*$^{-/-}$ mice. (**A**) Intradermal injection of IL-23 (200 ng) or vehicle control into the ears of WT and *Tir8/Sigirr*$^{-/-}$ mice resulted in enhanced epidermal thickening in *Tir8/Sigirr*$^{-/-}$ mice. Increased numbers of total (**B**) and specific (**C**) immune cell subsets infiltrating the skin of *Tir8/Sigirr*$^{-/-}$ mice after rIL-23 treatment for 7 d. Skin-infiltrating immune cell subsets were analyzed by flow cytometry for intracellular IL-17A expression by Vγ4 cells (**D**), CD4$^+$ T cells (**E**), and CD3$^-$CD45$^+$ cells (**F**). (**G**) IL-17A$^+$ cell numbers of skin infiltrating cell subsets from IL-23–treated WT and *Tir8/Sigirr*$^{-/-}$ mice. Statistical significance determined by unpaired Student *t* test; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

accounting for the disease phenotype observed, skin biopsies from treated mice were homogenized and analyzed for gene and protein expression levels of both IL-1β and IL-23. Although no significant differences were observed in the levels of expression of IL-1β or IL-23, we did observe significantly increased levels of IL-17A gene expression in the skin of *Tir8/Sigirr*$^{-/-}$ mice when compared with controls (Fig. 1E, 1F). Furthermore, analysis of γδ T cells in the skin-draining lymph nodes of Aldara-treated mice revealed significantly enhanced levels of IL-17A, but not IL-22, expression by *Tir8/Sigirr*$^{-/-}$ mice (Fig. 1G, 1I). γδ T cells, rather than Th17 cells, appear to be the primary source of IL-17A in this setting because limited expression of IL-17A by CD4$^+$ T cells was observed (Fig. 1H, 1J). These data demonstrate that in the absence of TIR8/SIGIRR there is increased Aldara-induced psoriasiform dermal inflammation that occurs in association with increased IL-17A expression by γδ T cells.

Intradermal IL-23 injection results in significantly enhanced dermal inflammation in Tir8/Sigirr$^{-/-}$ *mice*

Intradermal injection of IL-23 is another widely used model of psoriasiform inflammation, which is robustly comparable to the Aldara model in terms of the key cellular mechanisms involved such as the induction of an IL-1R1–dependent type 17 response through direct stimulation of T cell subsets (10, 33). Because TIR8 /SIGIRR has been characterized as a negative regulator of TLR7 signaling (34, 35), it is possible that the enhanced dermal inflammatory phenotype observed in *Tir8/Sigirr*$^{-/-}$ mice in the Aldara model described above is dependent on this activity. To address this possibility, WT and *Tir8/Sigirr*$^{-/-}$ mice were injected intradermally with rIL-23 or vehicle control daily for up to 7 d. Consistent with previous reports, IL-23 injection induced epidermal thickening in WT mice (Fig. 2A) (33, 36). Similar to the Aldara model, *Tir8/Sigirr*$^{-/-}$ mice display significantly enhanced epidermal thickening compared with WT controls in this model (Fig. 2A). To determine the composition of dermal infiltrating cells and assess any differences in populations between WT and *Tir8/Sigirr*$^{-/-}$ mice, CD45$^+$ cells purified from skin tissue were analyzed by flow cytometry. The total numbers of skin-infiltrating cells were significantly increased in the absence of TIR8/SIGIRR activity (Fig. 2B) with the most significant increase observed in the Vγ4 subset of γδ T cells, which have previously been implicated as important mediators of dermal inflammation in this model

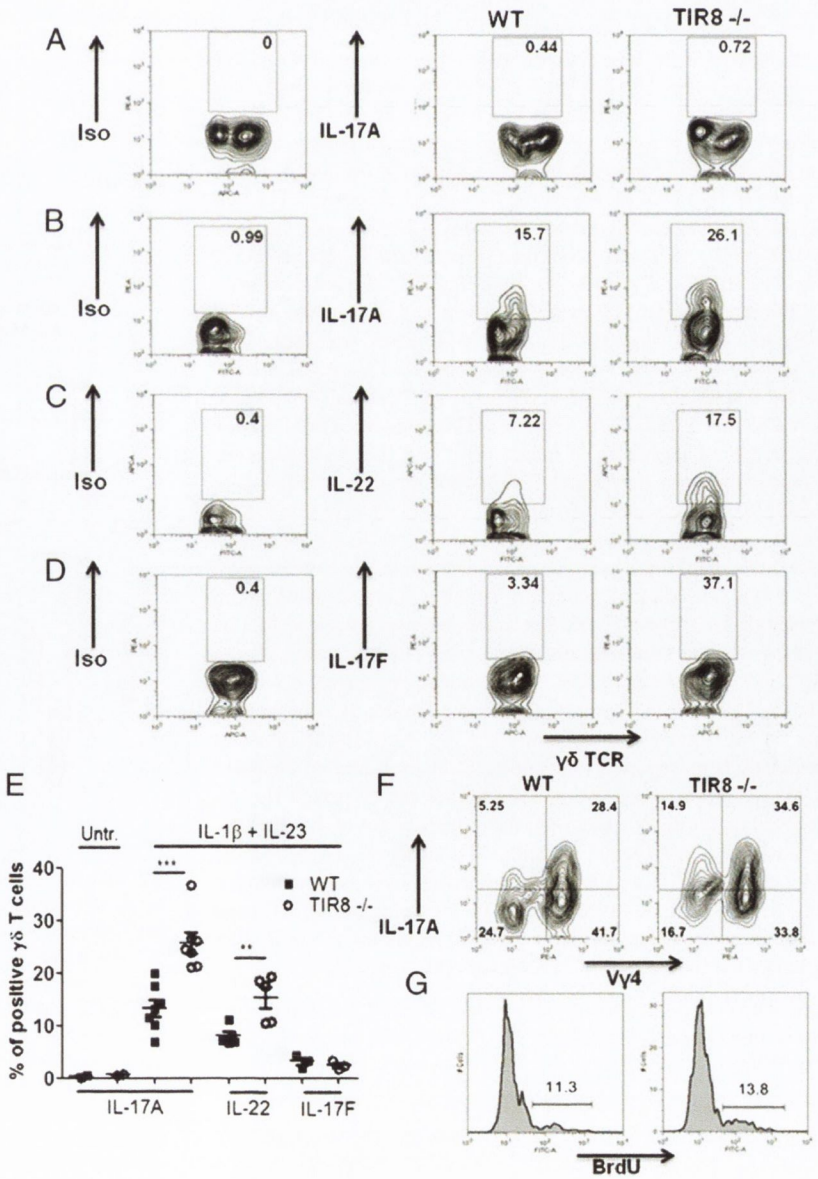


FIGURE 3. Innate IL-1β and IL-23 drive enhanced IL-17A and IL-22 expression from *Tir8/Sigirr*$^{-/-}$ γδ T cell subsets in vivo. WT and *Tir8/Sigirr*$^{-/-}$ mice injected in the hind footpad with a combination of IL-1β (100 ng) and IL-23 (100 ng). After 4 h, popliteal lymph nodes were harvested from treated mice and untreated controls. γδ T cells were subsequently analyzed by flow cytometry for intracellular expression of the following cytokines. IL-17A from untreated mice (**A**), IL-17A from treated mice (**B**), IL-22 from treated mice (**C**), and IL-17F from treated mice (**D**). (**E**) Expression of cytokines by γδ T cells from untreated and treated mice. Each dot indicates one mouse. (**F**) Expression of innate IL-17A by the Vγ4 subset of γδ T cells. (**G**) Analysis of BrdU incorporation by the Vγ4 subset after stimulation with IL-1β and IL-23. These data are representative of at least three independent experiments with similar results. Statistical significance determined by unpaired Student *t* test; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

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(Fig. 2C) (10). Importantly, as well as increased numbers, expression levels of IL-17A on a per cell basis were also significantly enhanced among *Tir8/Sigirr*$^{-/-}$ Vγ4 cells, with these cells accounting for the vast majority of IL-17A–expressing cells in this setting (Fig. 2D). Expression of IL-17A by Vγ4$^-$ γδ T cell subsets and a small population of CD45$^+$ non-T cells were also significantly enhanced in *Tir8/Sigirr*$^{-/-}$ mice (Fig. 2E, 2G). Finally, although increased numbers of CD4$^+$ T cells were also observed in inflamed skin, their overall contribution to IL-17A expression when compared with that observed from innate subsets was limited (Fig. 2F, 2G). These data demonstrate that intradermal administration of IL-23 also leads to enhanced psoriasiform inflammation in *Tir8/Sigirr*$^{-/-}$ mice, and this occurs in association with increased skin infiltration of Vγ4 γδ T cells expressing elevated levels of IL-17A.

IL-1β and IL-23 induce enhanced innate IL-17A and IL-22 expression from Tir8/Sigirr$^{-/-}$ *γδ T cell subsets in vivo*

In support of the role IL-23 plays in the progression of IL-17A–driven dermal inflammation, we were interested to assess the potential role TIR8/SIGIRR plays in regulating complementary IL-1 and IL-23 signaling in the context of innate IL-17A expression by γδ T cell subsets. To address these questions, we used an in vivo innate stimulation system whereby WT and *Tir8/Sigirr*$^{-/-}$ mice were injected in the hind footpad with a combination of IL-1β and IL-23 as described previously (26). After 4 h, popliteal lymph node cells were harvested from treated mice and untreated controls and cultured in the presence of brefeldin A, without further ex vivo stimulation, before analysis by flow cytometry. Although no expression of IL-17A was detected in the untreated controls (Fig. 3A, 3E), significantly elevated expression of IL-17A was observed from *Tir8/Sigirr*$^{-/-}$ γδ T cells from mice treated with IL-1β and IL-23 (Fig. 3B, 3E). IL-22 was also elevated (Fig. 3C, 3E), but there were no differences in IL-17F expression, which was detected at relatively low levels (Fig. 3D, 3E). The Vγ4 subset once again was the predominant subset expressing elevated IL-17A, but it is noteworthy that another population of Vγ4$^-$ γδ T cells also expressed increased levels of IL-17A (Fig. 3F). This suggests TIR8/SIGIRR acts as a broad regulator of innate γδ T cell IL-17A expression. Interestingly, both IL-1β and IL-23 were absolutely required to induce IL-17A expression from γδ *T* cells in vivo in this setting because mice injected singly with either IL-1 or IL-23 yielded no discernible cytokine expression. Furthermore, innate expression of IFN-γ was not detected under any stimulus (data not shown). Interestingly *Tir8/Sigirr*$^{-/-}$ γδ T cells also express enhanced IL-17A in response to in vivo administration of the TLR4 agonist LPS and the TLR9 agonist CpG ODN in this model, suggesting a broader role for TIR8/SIGIRR in the regulation of innate IL-17A expression by γδ T cells (Supplemental Fig. 1). As TIR8/SIGIRR has previously been reported to suppress Th17 proliferation mediated by IL-1β, we assessed the possibility that the enhanced IL-17A observed in this assay system was as a consequence of dysregulated expansion of *Tir8/Sigirr*$^{-/-}$ cells (24). However, no detectable differences in γδ T cell BrdU incorporation over normal homeostatic levels were observed (Fig. 3G). This observation indicates a direct effect of TIR8/SIGIRR activity in regulating innate IL-17A expression without altering cell expansion in vivo.

Enhanced IL-1β–dependent expression of IL-17A following innate stimulation of Tir8/Sigirr$^{-/-}$ *splenocytes is dependent on mTOR*

TIR8/SIGIRR has previously been reported to inhibit IL-1R1 signaling (20). On the basis of our findings from the in vivo footpad model, we hypothesized that enhanced innate IL-17A expression by γδ T cells in *Tir8/Sigirr*$^{-/-}$ mice occurred as a consequence of enhanced IL-1R1–dependent signaling. To address this possibility, we carried out an innate stimulation of WT and *Tir8/Sigirr*$^{-/-}$ splenocytes in vitro with increasing levels of IL-1β in the presence of IL-23. These conditions resulted in significantly elevated expression of IL-17A, IL-22, and IL-17A/F heterodimer in an IL-1β–dependent manner after 72 h (Fig. 4A-C). Levels of expression of IL-17F were not altered (Fig. 4D). To examine the molecular mechanisms at play in the dysregulated expression of these cytokines in the absence of TIR8/SIGIRR activity, we stimulated splenocytes in the presence of inhibitors of two established IL-1R1–dependent signaling pathways mediated by JNK and mTOR. Although no effects on IL-17A expression were observed in the presence of the JNK-specific inhibitor SP600125, inhibition of mTOR-dependent signaling with Temsirolimus resulted in a significantly decreased innate IL-17A expression by both WT and *Tir8/Sigirr*$^{-/-}$ cells, with a complete loss of the enhanced IL-17A expression in the absence of TIR8/SIGIRR (Fig. 4E), These data indicate a role for TIR8/SIGIRR in the modulation of mTOR-mediated IL-1R signaling in γδ T cells.

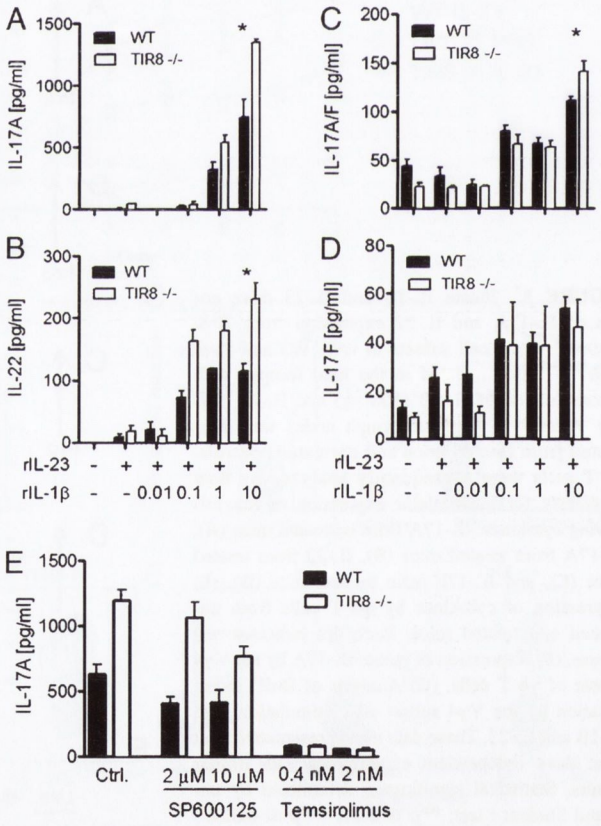


FIGURE 4. Enhanced IL-1β–dependent expression of IL-17A following innate stimulation of *Tir8/Sigirr*$^{-/-}$ splenocytes is mTOR dependent. WT and *Tir8/Sigirr*$^{-/-}$ splenocytes were innately stimulated with IL-23 (10 ng/ml) and increasing concentrations of IL-1β for 72 h. Cell culture supernatants were analyzed by ELISA for levels of IL-17A (**A**), IL-22 (**B**), IL-17A/F (**C**), and IL-17F (**D**). (**E**) WT and *Tir8/Sigirr*$^{-/-}$ splenocytes were pretreated for 2 h with the indicated doses of SP600125 or Temsirolimus before washing and culturing in the presence of IL-1β and IL-23 for an additional 72 h. Cell culture supernatants were subsequently analyzed for levels of IL-17A. These data are representative of at least two independent experiments with similar results Statistical significance determined by unpaired Student *t* test; $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Purified γδ T cells express enhanced IL-17A and IL-22 in response to IL-1β and IL-23 in vitro

γδ T cells have been previously demonstrated as capable of expressing IL-17A directly in response to IL-1β and IL-23 stimulation in vitro (27). Similar to the splenocyte experiments described above, purified γδ T cells from *Tir8/Sigirr*$^{-/-}$ mice expressed enhanced IL-17A when stimulated with IL-1β and IL-23 (Fig. 5A, 5B). We also extended this analysis to examine other cytokines known to induce type 17 responses in CD4$^+$ T cells. Purified WT and *Tir8/Sigirr*$^{-/-}$ γδ T cells were also stimulated with different combinations of IL-1β, IL-23, IL-6, and TGF-β or with the TLR7 agonist immiquimod. However, only IL-1β and IL-23 together were found to induce innate IL-17A expression even in the presence of TLR stimulated APCs (Fig. 5D, 5E). These data confirm the dependency of innate IL-17A from γδ T cells on IL-1β and IL-23 and further confirms the ability of TIR8/SIGIRR to regulate this signaling axis.

Enhanced severity of psoriasiform inflammation in Tir8/Sigirr$^{-/-}$ *mice is dependent on IL-17A*

Because we have identified a novel mechanism through which TIR8/SIGIRR regulates innate IL-17A expression, we next sought to determine whether the observed exacerbation of psoriasiform dermatitis in *Tir8/Sigirr*$^{-/-}$ mice was mediated by increased IL-17A expression. To achieve this, we used an IL-17A neutralization Ab in the Aldara disease model. Similar to our earlier observations, *Tir8/Sigirr*$^{-/-}$ mice treated with isotype control Ab displayed enhanced disease characterized by enhanced acantosis, desquamation, and infiltration of nucleated cells into the dermal layers when compared with isotype control Ab–treated WT mice. Strikingly, treatment of *Tir8/Sigirr*$^{-/-}$ mice with an anti–IL-17A neutralizing Ab reduced disease severity to levels comparable with those observed in WT mice (Fig. 6A, 6B). This reduction in disease severity was evident from histological scoring performed on H&E-stained tissue sections from diseased skin and also through analysis of epidermal skin thickening in treated mice (Fig. 6C, 6D). Taken together, these data confirm that the enhanced dermal inflammation observed in *Tir8/Sigirr*-deficient mice is mediated by dysregulated expression of IL-17A.

Discussion

Psoriasis is a common autoimmune disease of the skin which continues to pose a significant socioeconomic burden. Despite major advances in our understanding of the specific mechanisms that contribute toward disease pathogenesis significant questions remain to be answered (1). In recent times it has become evident that innate cell subsets provide a major source of IL-17A expression in settings of autoimmunity and host defense, particularly during the early stages of the response (37). A number of studies in murine models of psoriasiform inflammation have defined a role for innate cells

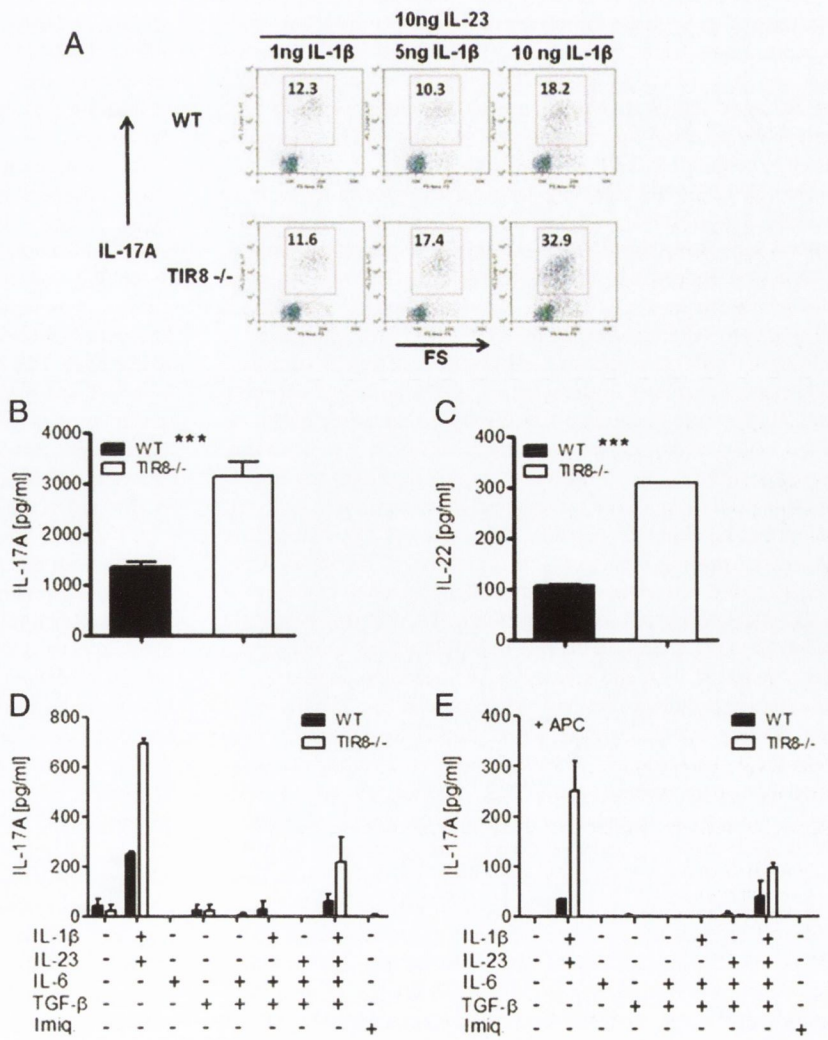


FIGURE 5. FACS purified γδ T cells express enhanced IL-17A and IL-22 in response to IL-1β and IL-23 treatment. (**A**) FACS purified WT and *Tir8/Sigirr*$^{-/-}$ γδ T cells were innately stimulated with IL-23 (10 ng/ml) and increasing concentrations of IL-1β for 72 h before analysis of IL-17A expression by intracellular staining. Cell culture supernatants were also analyzed for expression of IL-17A (**B**) and IL-22 (**C**) after 72 h. Auto-MACS–purified γδ T cells were cultured in the absence (**D**) or presence (**E**) of TLR-activated APCs along with the indicated combinations of cytokines for 72 h before analysis of the cell culture supernatants for IL-17A.

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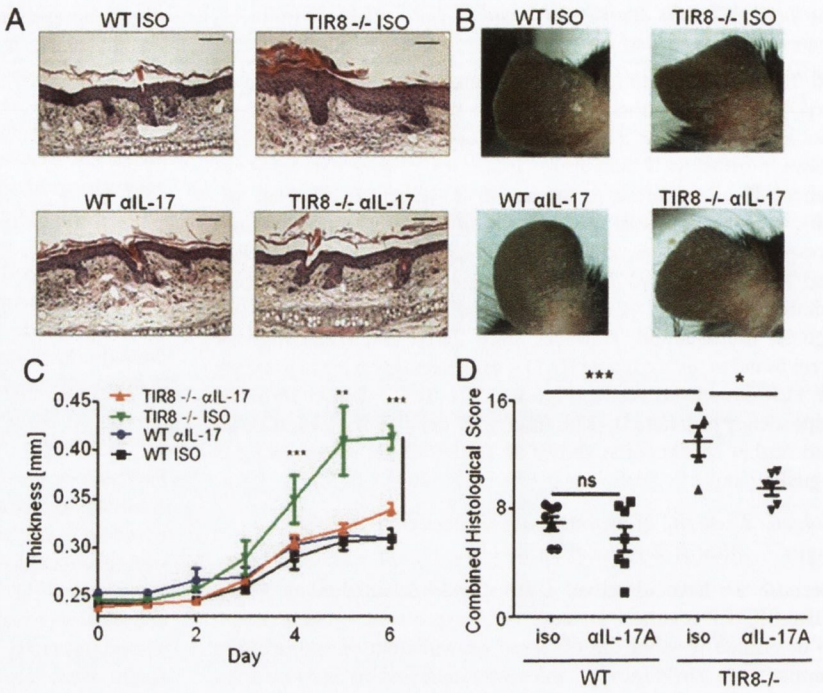


FIGURE 6. Enhanced Aldara-induced psoriasiform inflammation in *Tir8/Sigirr*$^{-/-}$ mice is reversed by neutralization of IL-17A. (**A**) Representative H&E-stained skin sections of WT and *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara and either isotype control or anti–IL-17A neutralization Ab, illustrating the reversal of increased epidermal thickening and inflammatory cell infiltration with anti–IL-17A treatment (scale bar, 50 μm). (**B**) Representative macroscopic pictures of ears from treated mice. (**C**) Increased ear thickness from *Tir8/Sigirr*$^{-/-}$ mice treated with Aldara is reversed by treatment with anti–IL-17A. (**D**) Combined histological scoring of the H&E-stained sections capturing scoring for acantosis, parakeratosis, desquamation, and infiltration. These data are representative of at least three independent experiments with similar results. Statistical significance determined by unpaired Student *t* test; $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

governed by the IL-23/IL-17 signaling axis as central mediators of disease (9, 10, 33, 36). However, specific pathways that regulate the responses of these innate subsets remain to be identified. This study uncovers a novel role for the receptor TIR8/SIGIRR as a regulator of innate IL-17A–driven dermal inflammation in psoriasis, which further underscores its importance as signaling mediator with therapeutic potential for interventions focusing on IL-1R1 and its interplay with the IL-23/IL-17 signaling axis.

The role of IL-1R1 signaling has received renewed focus in the context of psoriasiform inflammation as a consequence of observations implicating the IL-23/IL-17 axis as a central mediator of disease (19). Interestingly mice deficient in IL-1R1 antagonist, an endogenous suppressor of IL-1R1 signaling, develop spontaneous autoimmune inflammation in the skin (38). This phenotype is strikingly similar to common manifestations of human psoriasis, with marked acantosis, hyperkeratosis, and a nucleated cell infiltrate. Furthermore these mice also display severe aortic inflammation, a serious cardiovascular complication observed in some chronic psoriasis suffers (39). These observations highlight the importance of examining IL-1R1–mediated inflammation in psoriasis and further highlight the potential importance of TIR8/SIGIRR as a critical signaling mediator in this context. TIR8/SIGIRR has been described as a negative regulator of inflammation in settings of fungal infection and murine autoimmunity (24, 40). However, these studies have not addressed the role of TIR8/SIGIRR in the regulation of innate IL-17A and how this may impact the progression of autoimmune pathology. We have demonstrated that *Tir8/Sigirr*$^{-/-}$ mice develop enhanced inflammation, displaying a number of the histological characteristics of human psoriasis upon topical application of Aldara containing the TLR7 agonist imiquimod. *Tir8/Sigirr*$^{-/-}$ mice display enhanced acantosis, desquamation, infiltration of nucleated cells, and also ear thickening compared with WT mice. This model has been described as IL-23 and IL-1 dependent, with innate γδ T cells, expressing IL-17A, established as critical mediators of disease pathogenesis. Protein expression levels of IL-1β and IL-23 in the skin while upregulated in response to Aldara treatment were unaltered between WT and *Tir8/Sigirr*$^{-/-}$ mice. This lack of difference in protein expression levels is suggestive of a less prominent role for dysregulated stromal cell or myeloid cell involvement in the progression of dermal inflammation in the absence of TIR8/SIGIRR. Furthermore the overall higher expression of TIR8/SIGIRR on γδ T cells compared with CD4 T cells and skin underscores the importance TIR8/SIGIRR plays in the regulation of inflammation driven by these cells in such settings of dermal inflammation.

Examination of the skin draining lymph nodes revealed significantly enhanced expression of IL-17A by γδ T cells from *Tir8/Sigirr*$^{-/-}$ mice. IL-22, another type 17 cytokine important for driving inflammation in the Aldara model (41), was unaltered between WT and *Tir8/Sigirr*$^{-/-}$ mice. Both IL-17A and IL-22 expression were virtually undetectable from CD4^{+} T cells, which is in line with previous reports implicating γδ T cells as the primary source of IL-17A in this setting (9). The low expression of IL-22 in both WT and *Tir8/Sigirr*$^{-/-}$ mice is somewhat surprising in particular as it is clear from our in vivo and in vitro stimulation experiments that TIR8/SIGIRR activity can also inhibit IL-1R1 driven IL-22 expression. However, the levels of IL-22 detected in vitro are in the order of 5- to 10-fold less than the levels of IL-17A detected, which may explain our failure to detect any such difference in the Aldara model. It is also noteworthy that non-T cells have previously been implicated as important sources of IL-22 in this model (9, 41). Imiquimod stimulation of TLR7 on myeloid cells is thought to drive IL-23 expression in the Aldara-induced model of psoriasiform inflammation (10, 28). TIR8/SIGIRR has also been described as a negative regulator of TLR7 signaling (34, 35) and as such, the observed enhancement of inflammation in *Tir8/Sigirr*$^{-/-}$ mice, could be as a consequence of this activity. Therefore, we also used an established model of intradermal injection of IL-23 to assess the impact of TIR8/SIGIRR activity on dermal inflammation. In this model, *Tir8/Sigirr*$^{-/-}$ mice also displayed an enhanced disease phenotype compared with their WT counterparts confirming that TIR8/SIGIRR activity more broadly regulates dermal inflammation independently of its role as an inhibitor of TLR7 signaling. The IL-23 model is also primarily dependent on the direct stimulation of proinflammatory T cell subsets strongly indicating that TIR8/SIGIRR activity plays a prominent

regulatory role on these cells in the context of psoriasiform inflammation. In line with previous reports, we observed the Vγ4 subset of γδ T cells as the primary source of innate IL-17A expression. Notably, these cells are also present in the inflamed skin in significantly elevated numbers. TIR8/SIGIRR has previously been described as a negative regulator of IL-1–driven proliferation of Th17 cells, which could account for this observed expansion of infiltrating cells (24). These elevated cell numbers could also be as a consequence of a feed-forward mechanism in what are remarkably inflamed tissues, which is facilitative of further uncontrolled infiltration of proinflammatory cells. Interestingly, we have also demonstrated that TIR8/SIGIRR can directly negatively regulate innate IL-1–driven IL-17A expression in vivo without altering expansion. As well as γδ T cells we have also identified $CD4^+$ T cells and a population of $CD45^+CD3^-$ non–T cells as sources of elevated IL-17A in the inflamed skin of *Tir8/Sigirr*$^{-/-}$ mice. These cells have also been identified in a number of similar studies as sources of IL-17A in this setting (9). However their relative contribution toward disease progression especially given their low numbers warrants further investigation. These data confirm dysregulated activity in dermal infiltrating hematopoietic cells in the absence of TIR8/SIGIRR activity and identify a novel role for TIR8/SIGIRR as a suppressive signaling mediator of the innate arm of the IL-23/IL-17 pathway. We have also demonstrated enhanced expression of IL-17A, IL-22 and IL-17A/F but not IL-17F from *Tir8/Sigirr*$^{-/-}$ splenocytes in vitro and in vivo in an IL-1 dependant manner. This enhanced expression appears to be dependent on dysregulated mTOR activity, in agreement with a previous report describing the role TIR8/SIGIRR plays in the regulation of IL-1R1 mediated mTOR activity (24). Despite the unaltered expression of IL-23 and IL-1β in the inflamed skin of WT and *Tir8/Sigirr*$^{-/-}$ mice, the enhanced sensitivity of the *Tir8/Sigirr*$^{-/-}$ cells to IL-1R1 stimulation coupled with the established role aberrant IL-1R1 signaling plays in the progression of dermal inflammation provides a potential mechanistic explanation for the enhanced disease observed in these mice.

IL-17A expression is established as a key cytokine driving inflammation in humans and murine models of psoriasis (7). *Tir8/Sigirr*$^{-/-}$ mice display an enhanced psoriatic disease phenotype associated with enhanced IL-17A expression from γδ T cells in both the skin draining lymph nodes and tissue-infiltrating immune cells. Treatment of *Tir8/Sigirr*$^{-/-}$ mice with an IL-17A neutralization Ab reverses the enhanced disease to levels observed in WT mice. These observations in *Tir8/Sigirr*$^{-/-}$ mice are broadly in line with observations in the clinic where neutralization of elevated IL-17A in human patients results in amelioration of disease symptoms (12). Interestingly in this study, WT mice treated with anti–IL-17A displayed a mild but not significant reduction in disease severity after histological examination (Fig. 5D). This lack of efficacy in WT mice is potentially explained by compensatory mechanisms associated with other proinflammatory IL-17 family members such as IL-17C, which is significantly upregulated in psoriatic plaques and utilizes IL-17A receptor for its signaling activities (42). Also pathways independent of IL-17A and IL-17 receptor A have recently been identified as playing an important role in the progression of Aldara- induced disease in WT mice (43). Because anti–IL-17A is demonstrating considerable promise in clinical trials among psoriasis patients, and decreased TIR8/SIGIRR expression/activity has previously been reported in PBMCs of psoriatic patients, these data identify an important mechanism that may have direct relevance in human disease. These observations also highlight the requirement for greater understanding of the molecular and cellular mechanisms at play in such settings.

Taken together, to our knowledge, this study has for the first time identified TIR8/SIGIRR as a central regulator of innate type 17 responses with particular significance in the context of psoriasiform inflammation where its role as a critical mediator of IL-1R1 signaling identifies it as a potential target for future therapeutic interventions.

Disclosures

The authors have no financial conflicts of interest.

References

1. Perera, G. K., P. Di Meglio, and F. O. Nestle. 2012. Psoriasis. *Annu. Rev. Pathol.* 7: 385–422.
2. Schön, M. P., and W. H. Boehncke. 2005. Psoriasis. *N. Engl. J. Med.* 352: 1899–1912.
3. Lowes, M. A., A. M. Bowcock, and J. G. Krueger. 2007. Pathogenesis and therapy of psoriasis. *Nature* 445: 866–873.
4. Ellis, C. N., D. C. Gorsulowsky, T. A. Hamilton, J. K. Billings, M. D. Brown, J. T. Headington, K. D. Cooper, O. Baadsgaard, E. A. Duell, T. M. Annesley, et al. 1986. Cyclosporine improves psoriasis in a double-blind study. *JAMA* 256: 3110–3116.
5. Nickoloff, B. J., and F. O. Nestle. 2004. Recent insights into the immunopathogenesis of psoriasis provide new therapeutic opportunities. *J. Clin. Invest.* 113: 1664–1675.
6. Nestle, F. O., and C. Conrad. 2004. The IL-12 family member p40 chain as a master switch and novel therapeutic target in psoriasis. *J. Invest. Dermatol.* 123: xiv–xv.
7. Blauvelt, A. 2008. T-helper 17 cells in psoriatic plaques and additional genetic links between IL-23 and psoriasis. *J. Invest. Dermatol.* 128: 1064–1067.
8. Wilson, N. J., K. Boniface, J. R. Chan, B. S. McKenzie, W. M. Blumenschein, J. D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel, et al. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat. Immunol.* 8: 950–957.
9. Pantelyushin, S., S. Haak, B. Ingold, P. Kulig, F. L. Heppner, A. A. Navarini, and B. Becher. 2012. Rorγt+ innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *J. Clin. Invest.* 122: 2252–2256.
10. Cai, Y., X. Shen, C. Ding, C. Qi, K. Li, X. Li, V. R. Jala, H. G. Zhang, T. Wang, J. Zheng, and J. Yan. 2011. Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* 35: 596–610.
11. Papp, K. A., C. Leonardi, A. Menter, J. P. Ortonne, J. G. Krueger, G. Kricorian, G. Aras, J. Li, C. B. Russell, E. H. Thompson, and S. Baumgartner. 2012. Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. *N. Engl. J. Med.* 366: 1181–1189.
12. Leonardi, C., R. Matheson, C. Zachariae, G. Cameron, L. Li, E. Edson-Heredia, D. Braun, and S. Banerjee. 2012. Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. *N. Engl. J. Med.* 366: 1190–1199.
13. Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, et al. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421: 744–748.
14. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
15. Leonardi, C. L., A. B. Kimball, K. A. Papp, N. Yeilding, C. Guzzo, Y. Wang, S. Li, L. T. Dooley, and K. B. Gordon; PHOENIX 1 study investigators. 2008. Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). *Lancet* 371: 1665–1674.
16. Dinarello, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 27: 519–550.
17. Debets, R., J. P. Hegmans, P. Croughs, R. J. Troost, J. B. Prins, R. Benner, and E. P. Prens. 1997. The IL-1 system in psoriatic skin: IL-1 antagonist sphere of influence in lesional psoriatic epidermis. *J. Immunol.* 158: 2955–2963.
18. Mee, J. B., M. J. Cork, F. S. di Giovine, G. W. Duff, and R. W. Groves. 2006. Interleukin-1: a key inflammatory mediator in psoriasis? *Cytokine* 33: 72–78.
19. Uribe-Herranz, M., L. H. Lian, K. M. Hooper, K. A. Milora, and L. E. Jensen. 2013. IL-1R1 signaling facilitates Munro's microabscess formation in psoriasiform imiquimod-induced skin inflammation. *J. Invest. Dermatol.* 133: 1541–1549.
20. Wald, D., J. Qin, Z. Zhao, Y. Qian, M. Naramura, L. Tian, J. Towne, J. E. Sims, G. R. Stark, and X. Li. 2003. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. *Nat. Immunol.* 4: 920–927.
21. Riva, F., E. Bonavita, E. Barbati, M. Muzio, A. Mantovani, and C. Garlanda. 2012. TIR8/SIGIRR is an interleukin-1 receptor/Toll like receptor family member with regulatory functions in inflammation and immunity. *Front. Immunol.* 3: 322.
22. Qin, J., Y. Qian, J. Yao, C. Grace, and X. Li. 2005. SIGIRR inhibits interleukin-1 receptor- and Toll-like receptor 4-mediated signaling through different mechanisms. *J. Biol. Chem.* 280: 25233–25241.

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23. Thomassen, E., B. R. Renshaw, and J. E. Sims. 1999. Identification and characterization of SIGIRR, a molecule representing a novel subtype of the IL-1R superfamily. *Cytokine* 11: 389–399.
24. Gulen, M. F., Z. Kang, K. Bulek, W. Youzhong, T. W. Kim, Y. Chen, C. Z. Altuntas, K. Sass Bak-Jensen, M. J. McGeachy, J. S. Do, et al. 2010. The receptor SIGIRR suppresses Th17 cell proliferation via inhibition of the interleukin-1 receptor pathway and mTOR kinase activation. *Immunity* 32: 54–66.
25. Batliwalla, F. M., W. Li, C. T. Ritchlin, X. Xiao, M. Brenner, T. Laragione, T. Shao, R. Durham, S. Kemshetti, E. Schwarz, et al. 2005. Microarray analyses of peripheral blood cells identifies unique gene expression signature in psoriatic arthritis. *Mol. Med.* 11: 21–29.
26. Russell, S. E., and P. T. Walsh. 2012. Sterile inflammation—do innate lymphoid cell subsets play a role? *Front Immunol* 3: 246.
27. Sutton, C. E., S. J. Lalor, C. M. Sweeney, C. F. Brereton, E. C. Lavelle, and K. H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31: 331–341.
28. van der Fits, L., S. Mourits, J. S. Voerman, M. Kant, L. Boon, J. D. Laman, F. Cornelissen, A. M. Mus, E. Florencia, E. P. Prens, and E. Lubberts. 2009. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J. Immunol.* 182: 5836–5845.
29. Szeimies, R. M., M. J. Gerritsen, G. Gupta, J. P. Ortonne, S. Serresi, J. Bichel, J. H. Lee, T. L. Fox, and A. Alomar. 2004. Imiquimod 5% cream for the treatment of actinic keratosis: results from a phase III, randomized, double-blind, vehicle-controlled, clinical trial with histology. *J. Am. Acad. Dermatol.* 51: 547–555.
30. Geisse, J. K., P. Rich, A. Pandya, K. Gross, K. Andres, A. Ginkel, and M. Owens. 2002. Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: a double-blind, randomized, vehicle-controlled study. *J. Am. Acad. Dermatol.* 47: 390–398.
31. Gilliet, M., C. Conrad, M. Geiges, A. Cozzio, W. Thürlimann, G. Burg, F. O. Nestle, and R. Dummer. 2004. Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors. *Arch. Dermatol.* 140: 1490–1495.
32. Wu, J. K., G. Siller, and G. Strutton. 2004. Psoriasis induced by topical imiquimod. *Australas. J. Dermatol.* 45: 47–50.
33. Hedrick, M. N., A. S. Lonsdorf, A. K. Shirakawa, C. C. Richard Lee, F. Liao, S. P. Singh, H. H. Zhang, A. Grinberg, P. E. Love, S. T. Hwang, and J. M. Farber. 2009. CCR6 is required for IL-23-induced psoriasis-like inflammation in mice. *J. Clin. Invest.* 119: 2317–2329.
34. Lech, M., V. Skuginna, O. P. Kulkarni, J. Gong, T. Wei, R. W. Stark, C. Garlanda, A. Mantovani, and H. J. Anders. 2010. Lack of SIGIRR/TIR8 aggravates hydrocarbon oil-induced lupus nephritis. *J. Pathol.* 220: 596–607.
35. Gong, J., T. Wei, R. W. Stark, F. Jamitzky, W. M. Heckl, H. J. Anders, M. Lech, and S. C. Rössle. 2010. Inhibition of Toll-like receptors TLR4 and 7 signaling pathways by SIGIRR: a computational approach. *J. Struct. Biol.* 169: 323–330.
36. Mabuchi, T., T. Takekoshi, and S. T. Hwang. 2011. Epidermal CCR6$^+$ γδ T cells are major producers of IL-22 and IL-17 in a murine model of psoriasiform dermatitis. *J. Immunol.* 187: 5026-5031.
37. Cua, D. J., and C. M. Tato. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nat. Rev. Immunol.* 10: 479–489.
38. Shepherd, J., M. C. Little, and M. J. Nicklin. 2004. Psoriasis-like cutaneous inflammation in mice lacking interleukin-1 receptor antagonist. *J. Invest. Dermatol.* 122: 665–669.
39. Wang, Y., H. Gao, C. M. Loyd, W. Fu, D. Diaconu, S. Liu, K. D. Cooper, T. S. McCormick, D. I. Simon, and N. L. Ward. 2012. Chronic skin-specific inflammation promotes vascular inflammation and thrombosis. *J. Invest. Dermatol.* 132: 2067–2075.
40. Bozza, S., T. Zelante, S. Moretti, P. Bonifazi, A. DeLuca, C. D'Angelo, G. Giovannini, C. Garlanda, L. Boon, F. Bistoni, et al. 2008. Lack of Toll IL-1R8 exacerbates Th17 cell responses in fungal infection. *J. Immunol.* 180: 4022–4031.
41. Van Belle, A. B., M. de Heusch, M. M. Lemaire, E. Hendrickx, G. Warnier, K. Dunussi-Joannopoulos, L. A. Fouser, J. C. Renauld, and L. Dumoutier. 2012. IL-22 is required for imiquimod-induced psoriasiform skin inflammation in mice. *J. Immunol.* 188: 462–469.
42. Johnston, A., Y. Fritz, S. M. Dawes, D. Diaconu, P. M. Al-Attar, A. M. Guzman, C. S. Chen, W. Fu, J. E. Gudjonsson, T. S. McCormick, and N. L. Ward. 2013. Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *J. Immunol.* 190: 2252–2262.
43. El Malki, K., S. H. Karbach, J. Huppert, M. Zayoud, S. Reissig, R. Schüler, A. Nikolaev, K. Karram, T. Münzel, C. R. Kuhlmann, et al. 2013. An alternative pathway of imiquimod-induced psoriasis-like skin inflammation in the absence of interleukin-17 receptor a signaling. *J. Invest. Dermatol.* 133: 441–451.

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Soluble IL-2Rα (sCD25) Exacerbates Autoimmunity and Enhances the Development of Th17 Responses in Mice

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Abstract

A strong association exists between mutations at the *IL2 receptor alpha chain (CD25)* gene locus and susceptibility to a number of T cell driven autoimmune diseases. Interestingly, the presence of certain *CD25* susceptibility alleles has been correlated with significantly increased levels of the soluble form of CD25 (sCD25) in the serum of patients. However, the functional consequences, if any, of this observation are unknown. We have demonstrated that elevated levels of sCD25 *in vivo* resulted in exacerbated experimental autoimmune encephalomyelitis (EAE) and enhanced antigen-specific Th17 responses in the periphery. sCD25 exerted its effects early during the Th17 developmental programme *in vitro*, through inhibiting signalling downstream of the IL-2R. Although, sCD25 did not interact with the T cell surface, it specifically bound to secreted IL-2 demonstrating its ability to act as a decoy receptor for IL-2 in the T cell microenvironment. These data identify the ability of sCD25 to promote autoimmune disease pathogenesis and enhance Th17 responses through its ability to sequester local IL-2.

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Introduction

The primary non-redundant function of interleukin-2 (IL-2) is as a mediator of peripheral T cell tolerance [1]. As well as the maintenance of peripheral tolerance, IL-2 has also been somewhat paradoxically described play an important role in driving the proliferative response of activated T cells and as a critical factor in the generation of an appropriate memory T cell response [2]. IL-2 exerts these pleiotropic effects through interacting with the heterotrimeric IL-2 receptor (IL-2R) complex comprised of α (CD25), β (CD122) and common γ (CD132) chains expressed on the surface of activated T cells [3]. The critical role for IL-2 in the maintenance of T cell tolerance is evident from studies on transgenic mice deficient for either the cytokine itself or its receptor [4,5]. These mice develop profound autoimmunity characterized by an uncontrolled expansion of auto-reactive T cells. Further analysis has revealed the mechanistic basis for these observations, and uncovered the critical role of IL-2R signalling in maintaining the competitive fitness of peripheral regulatory T cells (Tregs) [6], and in the direct inhibition of Th17 cell responses [7–9]. These observations are further underscored by the strong genetic linkage between mutations at both the *IL-2* and *IL2RA/CD25* gene loci and several T cell mediated autoimmune diseases in humans [10]. However, precisely how such polymorphisms confer susceptibility to autoimmunity remains incompletely understood.

A number of specific *CD25* alleles associated with autoimmune susceptibility occur in association with enhanced levels of the soluble form of the IL-2R alpha chain (sCD25) in the serum of patients [10,11]. However, the functional consequences of these observations are unknown. Numerous examples of soluble cytokine receptors have been described to exert immunomodulatory effects *in vivo*. These range from antagonistic (IL-1RII) or agonistic (IL-6R) effects on receptor signalling, to acting as ligand chaperones or carrier proteins (IL-4R) [12]. Although there have been several descriptions of sCD25 acting as an inhibitor of IL-2 induced T cell responses *in vitro* [10,13], whether it plays a similar role *in vivo* has not been determined. sCD25 is known to be generated as a result of proteolytic cleavage, largely from the surface of activated T cells and levels of CD25 'shedding' are directly related to the rate of proliferation of activated T cells [14]. The levels of systemic sCD25 in the steady state are known to be remarkably stable and as a consequence sCD25 has been used extensively as a biomarker reflecting inflammatory diseases and tumours characterized by T cell expansion [14]. However, whether these increased levels of sCD25 play any direct role in modulating disease has not been fully investigated.

In this study we demonstrate for the first time that sCD25 exacerbates experimental autoimmune encephalomyelitis (EAE). These effects are associated with the enhanced generation of Th17 type responses in the periphery and increased infiltration of both CD4+ Th1 and Th17 cell subsets into the central nervous system (CNS). Similar to monoclonal antibody mediated IL-2 neutralization, sCD25 also enhances Th17 responses *in vitro* and acts early during the Th17 developmental programme by inhibiting signalling downstream of the IL-2R through its ability to sequester local IL-2. These data identify a previously unappreciated role for sCD25 in the pathogenesis of autoimmune disease.

Materials and Methods

Mice

Female C57BL/6J mice (Charles River, Ireland) and IL-17AeGFP mice, on a C57BL/6 background, (Biocytogen, Worcester, MA, USA) aged between 6–8 weeks were utilised in experiments. Mice were housed under SPF conditions at the Institute for Molecular Medicine, St. James Hospital Dublin. All animal experiments were performed in compliance with Irish Department of Health regulations (license number B100/4272) and approved by the institutional ethical review board.

Materials

ELISA kits for mouse IL-17A, IFNγ, IL-2 and IL-22 were purchased from ebioscience (Hatfield UK). ELISA kit for sCD25 was purchased from R&D systems (Abingdon, UK) Recombinant murine sCD25His was purchased from R&D systems. Endotoxin levels in sCD25 were determined by LAL assay and found to be lower than 0.05 EU/μg of protein. These levels were found to exert no detectable levels of immune stimulation on primary macrophages *in vitro*. All antibodies used in this study from ebioscience unless otherwise stated. Recombinant mouse IL-6 was purchased from Becton Dickinson (Oxford, UK), IL-12p70 and TGFβ from ebioscience. Anti-IL-2 neutralizing antibody (JES6-1A12) was purchased from ebioscience.

Flow Cytometry

Cells were analysed for surface and intracellular protein expression using an LSR/Fortessa (BD). For intracellular staining, cells were stimulated with PMA 10 ng/ml and ionomycin 1 μg/ml for 6 h in the presence of Brefeldin A (Sigma) 5 μg/ml for the final 4 hrs. Fixation and permeabilisation was performed using FIX/PERM Kit (Dako) according to manufacturer's instructions. Intracellular staining was performed for IL-17A (17B7), IL-17F (eBio18F10), IFN-γ (XMG 1.2) and FoxP3 (FJK-16s) and RORγT (AFKJS-9) (ebioscience, UK). pSTAT5 (pY694) and pSTAT3 (pY705) staining was performed with Phosflow kit according to the manufacturer's instructions (Becton Dickinson, UK). Surface staining was performed for CD4 and CCR6. Cell surface staining for sCD25 was performed using anti-HIS (GG11-8F3.5.1) (Miltenyi Biotec).

EAE Induction and sCD25 treatment

EAE was induced according to manufacturer's instructions using active EAE induction kit EK-0113 (Hooke Labs MA. U.S.A). Mice were monitored daily for signs of disease with disease severity recorded as follows 0. Normal, 1. Limp tail, 2. Wobbly gait, 3. Severe hind limb weakness 4. Complete hind limb paralysis 5. Moribund or death. Recombinant sCD25 was administered immediately prior to immunization and every 12 hours thereafter for the first 3 days. Control mice were treated with PBS.

T cell isolation and differentiation

Naive CD4+CD62L+T cells from spleens of 8 week old mice were purified by magnetic bead separation (Miltenyi Biotec). Cells were activated with plate bound anti-CD3 (145-2C11) and anti-CD28 (37.51) both 5 μg/ml. For Th17 differentiation cells were cultured in the presence of TGF-β 5ng/ml, IL-6 10 ng/ml, anti-IFN-γ 10 μg/ml (XMG 1.2) and anti-IL-4 10 μg/ml (11B11). For Th1 differentiation cells were cultured with IL-12 10 ng/ml and anti-IL-4 10 μg/ml. iTreg cells were induced in the presence of TGF-β 5 ng/ml and rIL-2 (10 U/ml). After 72–96 hours supernatants were analysed by ELISA and cells were examined for intracellular cytokine/transcription factor expression by flow cytometry.

Results

sCD25 leads to exacerbated autoimmune disease and increased antigen-specific peripheral Th17 responses

Specific alleles at the *CD25* gene locus, known to be associated with susceptibility to autoimmune diseases such as Multiple Sclerosis (MS), lead to increased levels of soluble CD25 in patient's serum [10]. Although such observations implicate sCD25 as having an important mechanistic role in disease pathogenesis, it is not clear how sCD25 may contribute to a loss of self tolerance. To determine what role, if any, sCD25 may play in autoimmunity we induced experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, in the presence of exogenous recombinant sCD25 administered immediately prior to, and during the first 3 days after immunization. Increased levels of sCD25 during the early stages of antigen-specific T cell priming led to a significant exacerbation of disease symptoms during the onset and induction phase of the disease from day 10 through to the peak of disease after 18 days ($P>0.01$) (Fig. 1A). To examine the cells infiltrating into the CNS during EAE, IL-17-eGFP reporter mice were immunized with MOG, in the presence or absence of sCD25, and the expression of IL-17A or IFNγ was assessed at 15 days after induction of EAE. Although the relative percentages of infiltrating Th1 versus Th17 type cells was not altered (Fig. 1B), administration of sCD25 resulted in significantly increased numbers of both subsets in the spinal cords of treated mice at day 15 during disease induction (Fig. 1C). We also examined the effects of sCD25 administration on the generation of peripheral antigen specific T cell responses *in vivo*. Significantly, increased levels of sCD25 were found to result in increased antigen-specific T cell expression of IL-17A upon MOG antigen restimulation *ex vivo* 7 days after immunization ($p>0.05$) (Fig. 2A &B). Expression of IFNγ was not significantly affected (Fig. 2A). Furthermore, administration of sCD25 did not affect the levels or relative numbers of CD4+Foxp3+ regulatory T cells in immunized mice after 7 days, indicating that increased severity of EAE did not occur in association with any effects on Treg homeostasis (Fig. 2C&D). Together these data demonstrate that increased levels of sCD25 *in vivo* led to increased severity of EAE that occurs in association with enhanced generation of antigen specific Th17 responses in the periphery and increased numbers of both of CD4+ Th1 and Th17 cell subsets in the CNS. These observations are consistent with previous reports which demonstrate that administration of an anti-IL-2 neutralizing antibody leads to the spontaneous development of EAE-like symptoms in mice and also that treatment with recombinant IL-2 during the early stages of disease can offer significant protection from EAE [15–16].

sCD25 enhances the development of Th17 cell responses

Conflicting studies have demonstrated both antagonistic and agonistic roles for sCD25 in the context of IL-2R signalling indicating that sCD25 could either promote or inhibit Treg responses and inhibit IL-2 mediated activation induced cell death *in vitro* [10] [17]. As sCD25 enhances the generation of peripheral autoimmune antigen-specific Th17 responses *in vivo*, we sought to determine how sCD25 might regulate these events by investigating the effects of sCD25 on the generation of Th17, Th1 and Treg responses *in vitro*.

sCD25 significantly enhanced the generation of Th17 type responses after 96 hours *in vitro* in a dose dependant manner and to a similar extent to an anti-IL-2 neutralizing antibody

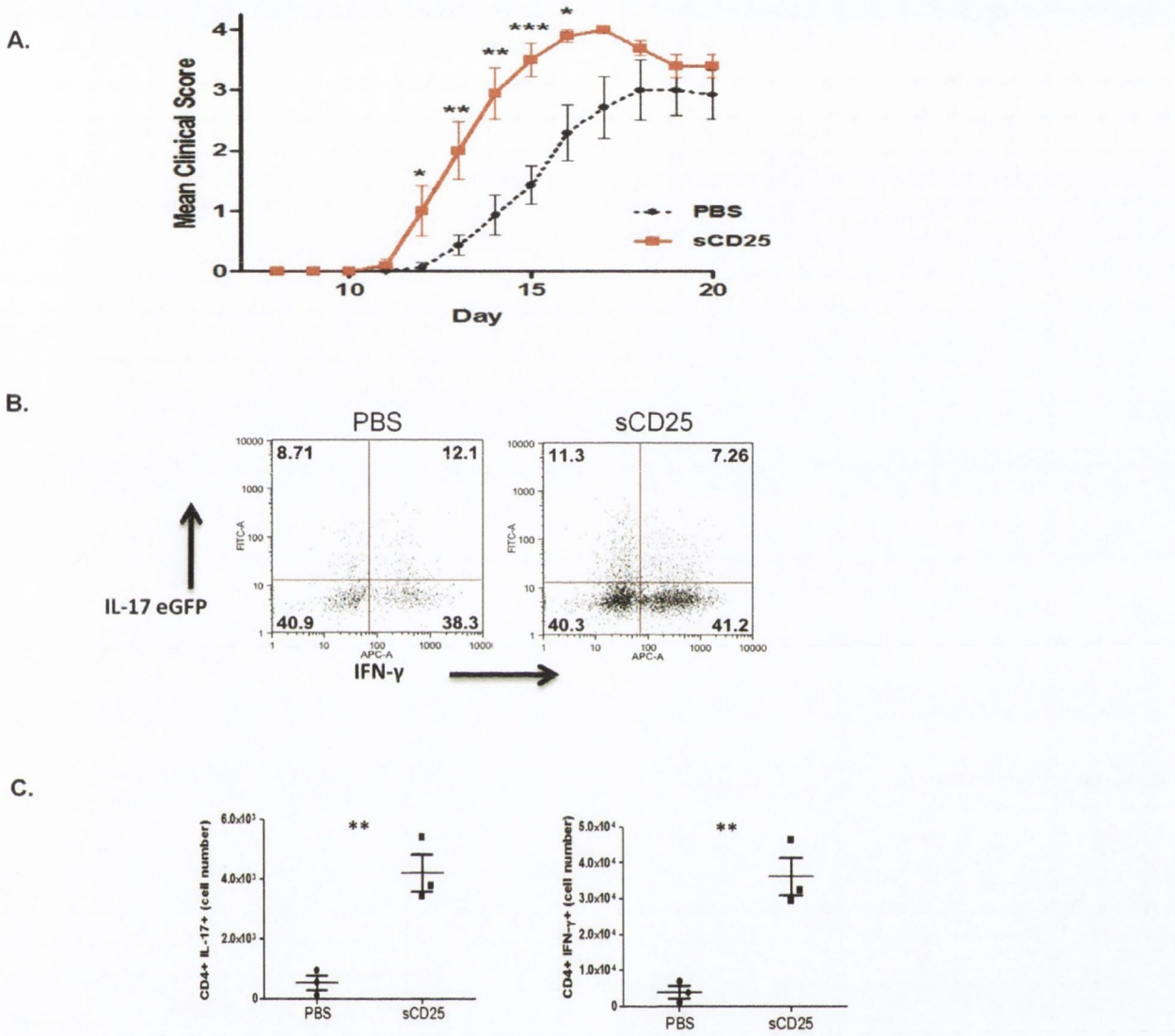


Figure 1. Exogenous sCD25 exacerbates autoimmunity. (**A**) MOG_{33-55} immunized C57BL/6 mice developed clinical symptoms of EAE from day 12 after immunization with a peak of disease severity observed from day 19. Subcutaneous administration of recombinant sCD25 (25 μg/mouse) immediately prior to immunization and every 12 hours thereafter for 72 hrs resulted in a significant exacerbation in severity of symptoms during disease onset and induction. 6–7 mice used per group. (**B**) Mononuclear cells harvested from spinal cords of control (PBS) and sCD25 treated IL-17A-eGFP reporter mice (3 per group) on day 15 after immunization and analysed for expression of IL-17 and IFN-γ by CD4+ cells. (**C**) Cell numbers of CD4+IL-17+ and CD4+ IFNγ+ cells in spinal cords of IL-17A-eGFP reporter mice at day 15. Data representative of mean +/− std dev of 3 mice per group and 2 independent experiments.
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(Figure 3A). In contrast, no effects of sCD25 were observed on the generation of either Th1 or inducible Treg subsets (Figure 3 A, B & C). As Th17 cells are considered critical in driving the pathogenesis of EAE, these data are consistent with our earlier *in vivo* data (Figure 1). We also examined what influence sCD25 may have on Th17 cell proliferation and survival as IL-2 is a well established T cell growth factor and we observed an increase in the overall numbers of infiltrating CD4+ T cells in the spinal cords of treated mice (Figure 1C), Although sCD25 resulted in an increase in the proportion of cells expressing IL-17A, it did not affect either the rate of cell division as determined by levels of CFSE dilution, or levels of survival/cell death, as measured by incorporation of 7-AAD, under these conditions (Figure 3D). However, consistent with the enhanced generation of a Th17 response, we also observed increased levels of phosphorylation of STAT3 (Figure 3E). sCD25 also led to a marginal but reproducible increased expression of both IL-17F and the Th17 associated chemokine receptor CCR6, while levels of IL-22 expression were not affected (data not shown). These data clearly demonstrate that sCD25 can enhance Th17 cell development *in vitro* and suggest a mechanism through which sCD25 may increase autoimmune disease severity.

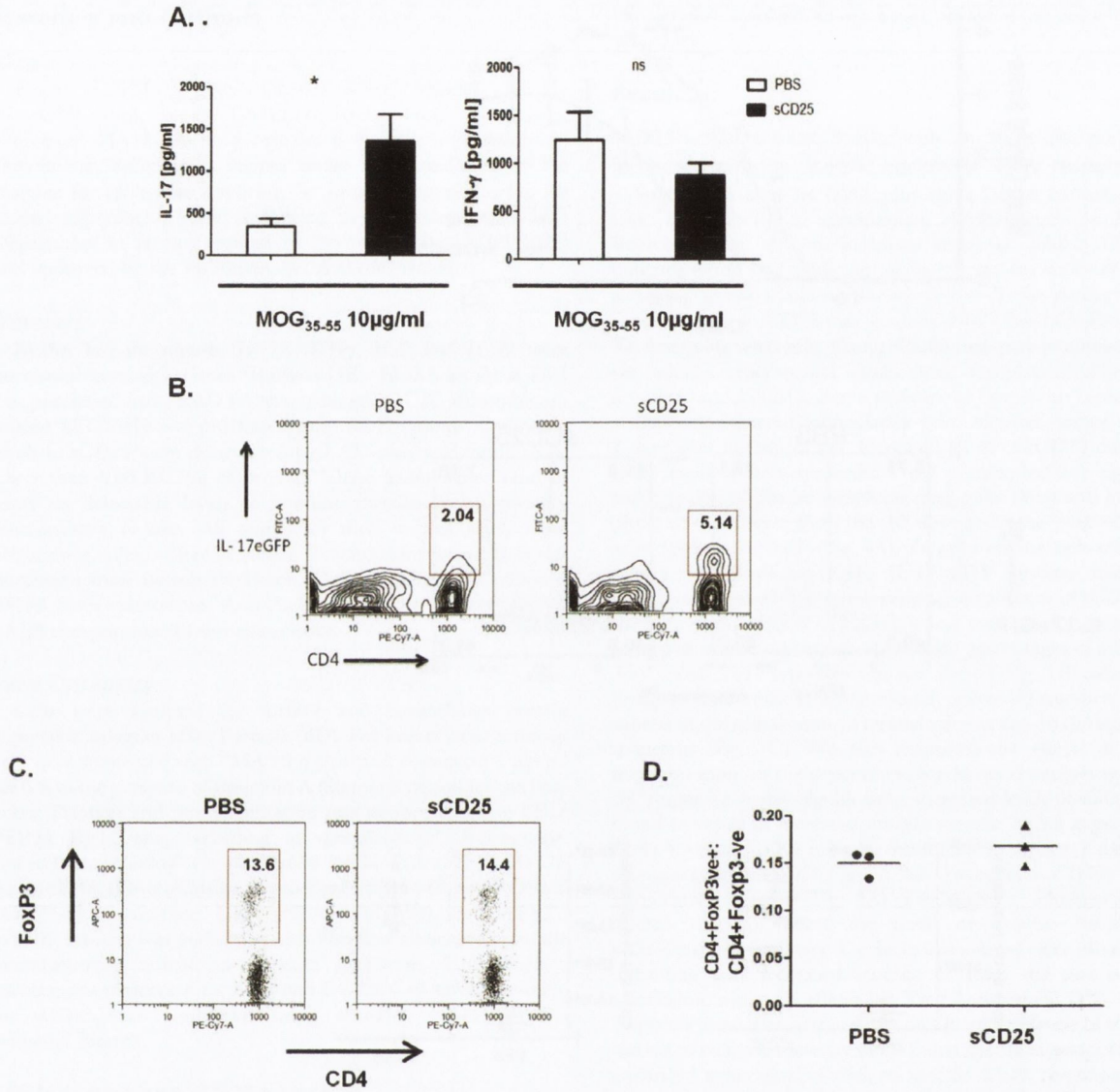


Figure 2. sCD25 enhances peripheral antigen specific Th17 responses. (**A**) Levels of IL-17A and IFNγ secreted by draining lymph node cells of mice immunized and treated as above (3 per group), isolated after 9 days, and restimulated *ex vivo* with MOG_{33-55} (10 µg/ml) for 72 hours. (**B**) Percentage of CD4+ IL-17A-eGFP+ve cells after treatment as above and *ex vivo* restimulation for 72 hours. (**C**) Percentage and (**D**) relative number ratios of CD4+FoxP3+:CD4+FoxP3- T cells in the draining lymph nodes of both sCD25 and control treated immunized mice. 3 mice per group were analysed. Data in D & G is representative of mean +/− standard deviation. Statistical Significance determined by unpaired student's t-test, * $p \leq 0.05$, ** $p \leq 0.01$.

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sCD25 inhibits IL-2R signalling on Th17 cells through sequestering extracellular IL-2

Recent reports have demonstrated that CD4+ FoxP3+ve Tregs can enhance the generation of Th17 type responses early during the developmental programme by limiting the bioavailability of IL-2 through constitutive expression of CD25 on their cell surface [18,19]. Given these observations, we investigated whether sCD25 could act in a similar way. Strikingly, the presence of sCD25 led to a significant enhancement of IL-17A (11% vs 2%) expression early during Th17 cell differentiation (48 hours) indicating that sCD25 also mediated its effects early during Th17 development (Figure 4A). Consistent with this observation, sCD25 also led to increased expression of the Th17 instructive transcription factor RORγT (Figure 4B).

As the effects of sCD25 were similar to those observed for IL-2 neutralization [9], we examined whether signalling downstream of the IL-2R was affected in these cells. IL-2 is expressed early during the first 24 hours after TCR stimulation of CD4+ T cells and activation of Jak3-STAT5 dependent signal pathways in T cells during this time is considered to be largely driven by the autocrine effects IL-2. sCD25 significantly decreased levels of STAT5 activation in Th17 cells demonstrating its ability to inhibit signalling downstream of the IL-2R (Figure 5A). IL-2 dependent activation of STAT5 signalling is known to directly inhibit early

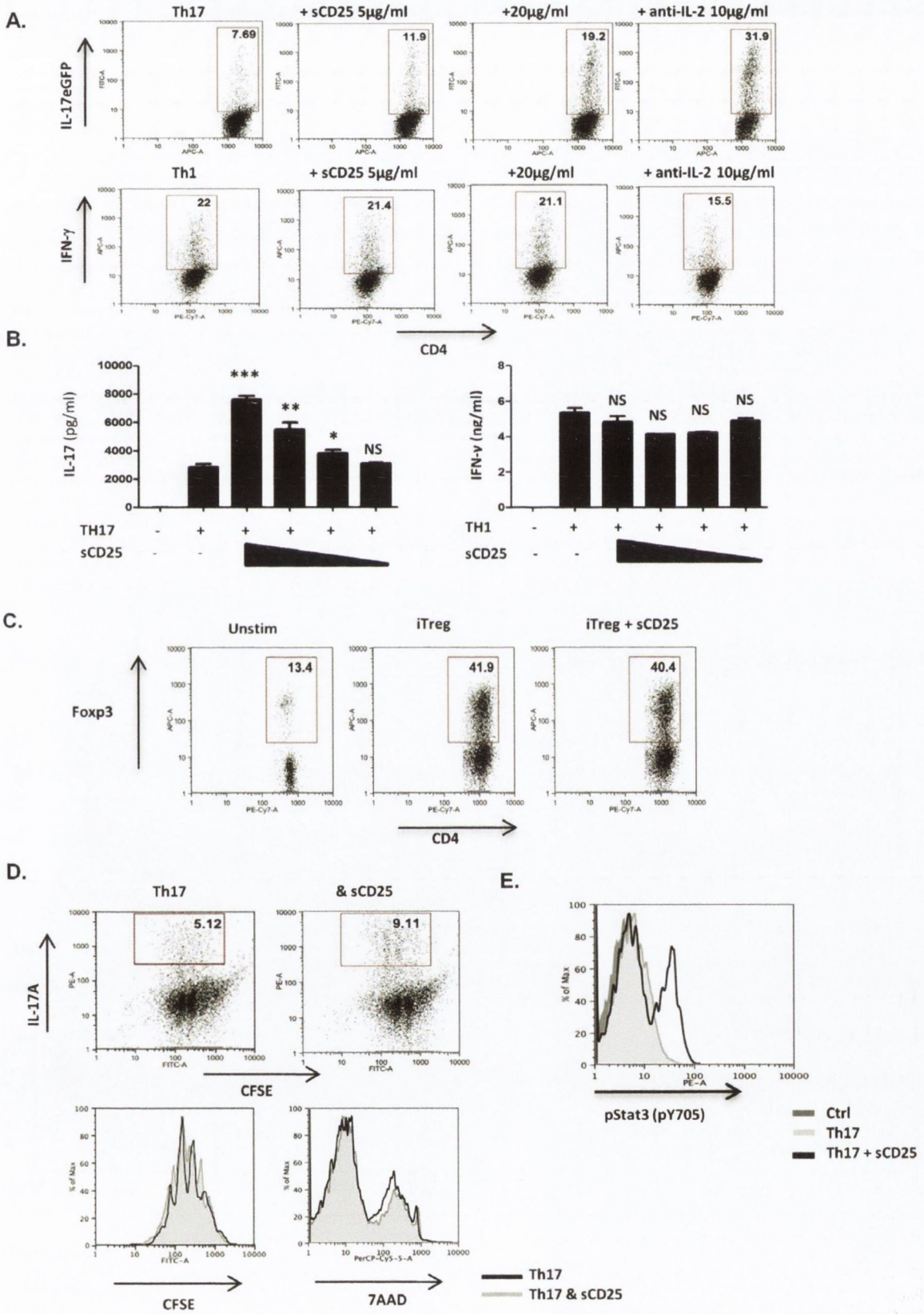

A.
Th17
+ sCD25 5μg/ml
+20μg/ml
+ anti-IL-2 10μg/ml
7.69
11.9
19.2
31.9
IL-17eGFP
Th1
+ sCD25 5μg/ml
+20μg/ml
+ anti-IL-2 10μg/ml
22
21.4
21.1
15.5
IFN-γ
CD4
B.
IL-17 (pg/ml)
***
**
*
NS
TH17
sCD25
IFN-γ (ng/ml)
NS
NS
NS
NS
TH1
sCD25
C.
Unstim
iTreg
iTreg + sCD25
13.4
41.9
40.4
Foxp3
CD4
D.
Th17
& sCD25
5.12
9.11
IL-17A
CFSE
CFSE
7AAD
Th17
Th17 & sCD25
E.
pStat3 (pY705)
Ctrl
Th17
Th17 + sCD25

Figure 3. sCD25 enhances Th17 cell responses *in vitro*. (**A&B**) Purified naive CD4+ T cells were activated under either Th17 or Th1 inducing conditions (as described in methods) in the presence of a range of concentrations of sCD25 (20, 10, 5 or 1 μg/ml) or anti-IL-2 (10 μg/ml). Levels of IL-17A or IFNγ expression were determined after 96 hrs by (**A**) FACS and (**B**) ELISA. (**C**) Purified naive CD4+ T cells were activated under Treg inducing conditions, as described in methods, in the presence or absence of sCD25 (20μg/ml) and FoxP3 expression determined by FACS. (**D**) Naive CD4+ T cells were stained with CFSE (2.5 μM) prior to activation under Th17 conditions in presence or absence of sCD25 (20 μg/ml). After 96 hours, levels of intracellular IL-17A expression and CFSE dilution or 7AAD incorporation were determined by FACS. (**E**) Purified naive CD4+ T cells were activated under Th0, Th17 and Th17& sCD25 (20 μg/ml) conditions for 72 hours and levels of P-Stat3 (pY705) determined by FACS. All data are representative of 3 independent experiments. Statistical Significance determined by unpaired student's t-test, $p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.
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programming events in the development of a Th17 response by blocking the induction of RORγT expression [9]. These data identify a novel mechanism whereby sCD25 enhanced the generation and development of proinflammatory Th17 responses through inhibiting the protolerogenic effects of IL-2R signalling.

To determine the precise mechanism through which sCD25 was mediating this inhibition we considered a number of possibilities. First, sCD25 may inhibit the levels of IL-2 expressed upon T cell activation (although IL-2 neutralization by monoclonal antibodies has previously been found to enhance IL-2 expression by inhibiting an auto-regulatory negative feedback loop [20]). We observed no differences between the levels of IL-2 expressed on a per cell basis either in the presence or absence of sCD25 after 24 hours (Figure 5B). Second, sCD25 may exert its effects at the cell surface by acting to either inhibit appropriate assembly of the heterotrimeric receptor complex or inhibit IL-2 binding. To examine this possibility we used a His-tag on the soluble form of the receptor to discriminate between soluble and surface expressed forms of CD25. However, we were not able to detect any binding of sCD25 to the cell surface during the first 24 hours after activation (Figure 5C). In contrast, the presence of sCD25 did significantly inhibit the upregulation of endogenous surface CD25 expression (Figure 5D). This observation further indicated a role for sCD25 in inhibiting IL-2R signalling as IL-2 is recognised as an important mediator in driving surface CD25 expression early during T cell activation. Third, we investigated the possibility that sCD25 may act to sequester secreted IL-2 in the T cell microenvironment. Significantly, sCD25 inhibited the detection of secreted IL-2 by ELISA in a dose dependent fashion suggesting its ability to sequester secreted IL-2 (Figure 5E). The specific interaction between sCD25 and IL-2 was also demonstrated using a mixed ELISA approach of capture with an anti-IL-2 antibody followed by detection with an anti-CD25 antibody. Using this approach, significant levels of the IL-2/sCD25 complex were detected in the supernatants of CD4+ T cells activated under Th17 conditions in the presence of sCD25 (Figure 5F).

Together these data demonstrate the immunomodulatory activity of the soluble form of the IL-2R alpha chain *in vivo* for

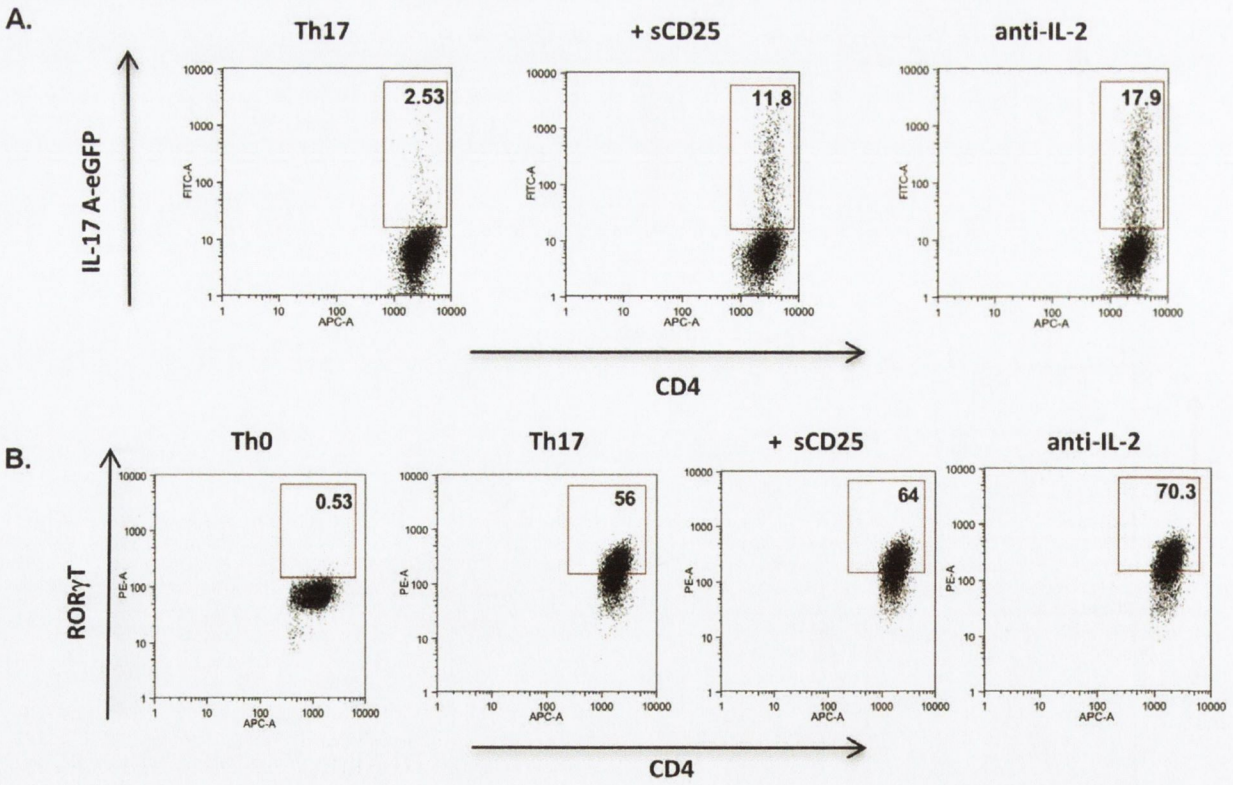


Figure 4. sCD25 acts early during Th17 development. Purified naive CD4+ T cells from IL-17AeGFP reporter mice activated under Th17 inducing conditions in the presence of sCD25 (20 μg/ml) or anti-IL-2 (10 μg/ml) for 48 hrs and examined for levels of (**A**) IL-17A and (**B**) RORγT expression by FACS. All data are representative of at least 3 independent experiments.
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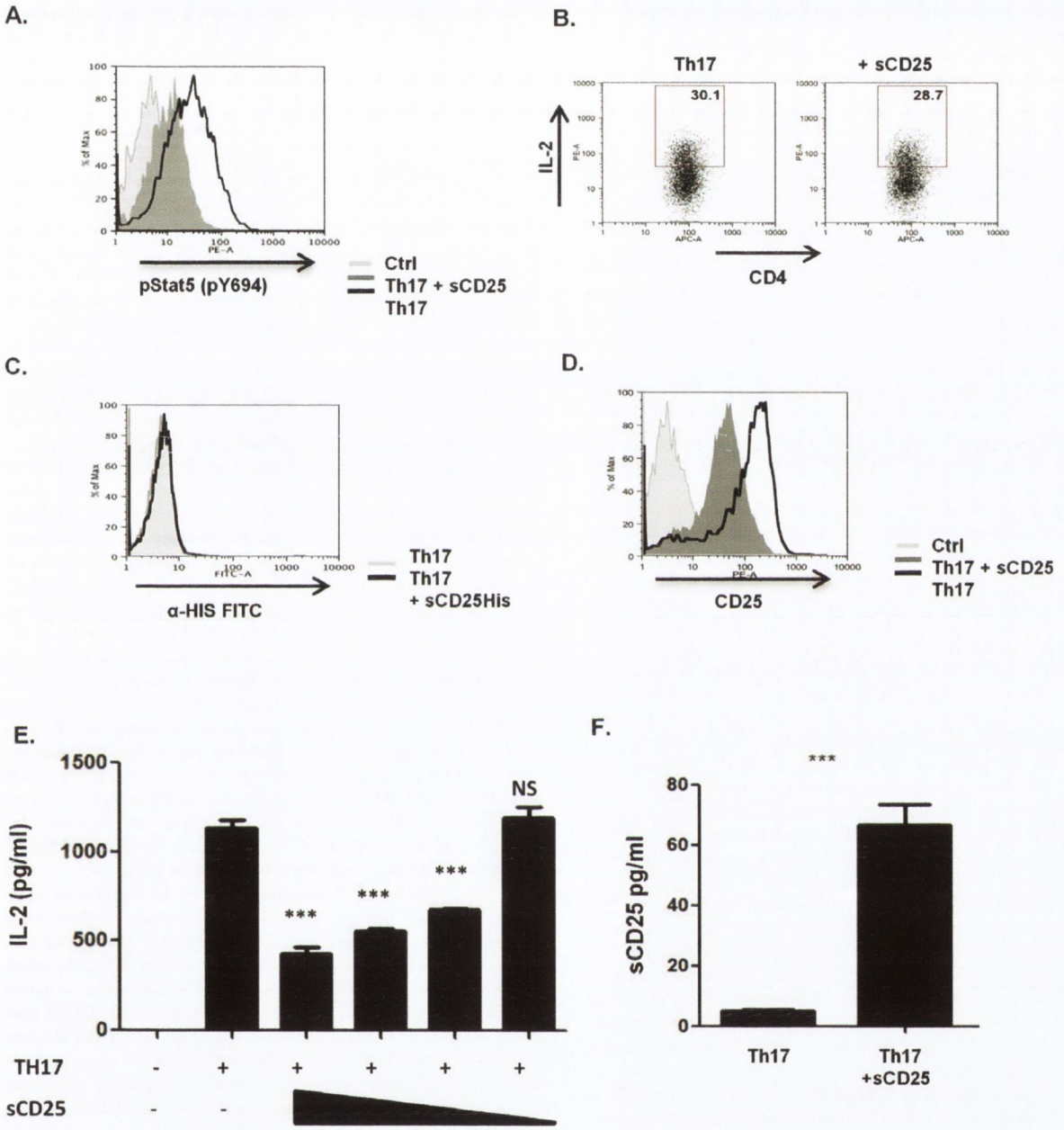


Figure 5. sCD25 inhibits IL-2R signalling by sequestering secreted IL-2. (**A–D**) Purified naive CD4+ T cells activated for 24 hrs under Th17 inducing conditions in the presence or absence of sCD25 and examined for levels of (**A**) p-Stat5 pY694, (**B**) intracellular IL-2 expression (**C**) surface binding of sCD25-His and (**D**) surface expression of endogenous CD25 by FACS analysis. (**E**) Levels of secreted IL-2 detected by ELISA 24 hours after Th17 activation in the presence of decreasing doses of sCD25 (20,10,5,1 μg/ml). (**F**) Levels of IL-2/sCD25 complex in the supernatants of Th17 cells activated for 24 hours in the presence or absence of sCD25 (20 μg/ml). Data shown as mean +/− standard deviation from triplicate experiments. All data are representative of at least 3 independent experiments. Statistical Significance determined by unpaired student's t-test, ***p≤0.001.
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the first time and indicate that these effects are mediated by its capacity to sequester secreted IL-2.

Discussion

The expression of the heterotrimeric IL-2 receptor on the surface of T cells plays a pleiotropic role in directing T cell responses. One such critical non-redundant role is the maintenance of peripheral T cell tolerance. This occurs through its promotion of the induction and persistence of regulatory T cell subsets while also acting directly to inhibit the generation of Th17 type responses which are considered to be critical in driving autoimmune disease [1]. Such observations are thought to form the mechanistic basis for the close association between mutations

at the *CD25* gene locus and enhanced susceptibility to a number of autoimmune diseases in humans.

Similar to other cytokine receptors expressed at the cell surface, the individual chains of the IL-2R are also known to exist in soluble form in serum [21,22]. In particular, stable expression levels of soluble CD25 observed in healthy adults has underscored its clinical use as a biomarker for a variety of inflammatory conditions [23]. Unlike other soluble cytokine receptors, no evidence exists for an alternative splice form at the *CD25* gene locus which encodes a specific soluble CD25 protein. Consequently, the generation of the soluble form of this receptor is thought to occur through proteolytic cleavage at the cell surface by as yet unidentified proteases [24]. Levels of sCD25 generation increase upon T cell activation *in vitro* and enhanced levels observed *in vivo* are thought to be directly related to the magnitude T cell mediated inflammatory responses. However, recent analysis of specific autoimmune susceptibility alleles at the *CD25* gene locus has uncovered a direct association between increased disease susceptibility, disease severity and increased levels of sCD25 [10,11]. These studies indicate that sCD25 may play an important mechanistic role in driving disease pathogenesis.

As expression of all three chains of the IL-2R signalling complex on the cell surface are known to be required for efficient IL-2 binding and the subsequent activation of downstream signalling events [25], whether sCD25 has any physiological relevance or is a mere by-product of T cell activation and expansion has remained controversial. Despite the lower affinity of CD25 for IL-2 when compared to the heterotrimeric IL-2R complex, sCD25 has been found to bind IL-2 efficiently and have immunomodulatory effects *in vitro* [10,26]. It is also possible that sCD25 may interact with an as yet unidentified accessory protein(s) *in vivo* to enhance its affinity for IL-2. Along those lines, it is noteworthy that soluble IL-1RII is known to have its affinity for IL-1α/β enhanced almost 100 fold through its interaction with soluble IL-1R Accessory protein [27]. Although monomeric sCD25 has a molecular weight in the region of 40 kDa, it has previously been found to be present as part of a protein complex with a molecular weight in the region of 100 kDa in the synovial fluid of rheumatoid arthritis patients [28]. Although the accessory proteins involved in this complex were not identified, it was found to efficiently inhibit IL-2 mediated responses *in vitro*. Furthermore, sCD25 has been demonstrated to exist in homodimeric form, although whether this alters its relative affinity for IL-2 is unknown [29]. Studies are ongoing to determine whether sCD25 exerts its immunomodulatory effects in EAE through either oligomerization or binding accessory proteins *in vivo*.

Numerous studies have previously investigated the role of sCD25 in modulating T cell responses *in vitro*. These reports have often led to conflicting results with sCD25 having been variously described to both inhibit and enhance T cell responses. To our knowledge, no previous studies have examined the role of increased sCD25 in the clinical severity of an auto-immune disease. As sCD25 has been previously examined with respect to multiple sclerosis in humans, we chose a murine model of this disease to examine *in vivo* effects of sCD25. While a number of groups have demonstrated the capacity of sCD25 to inhibit IL-2 mediated proliferation of CD8+ cytotoxic T cell lines [28,30], it is noteworthy that Maier et al. also demonstrated that sCD25 could inhibit IL-2 mediated STAT5 phosphorylation in primary CD4+ T cells while enhancing responses through the inhibition of activation induced cell death [10]. Our study further extends these *in vitro* findings and demonstrates that sCD25-mediated blockade of IL-2 signalling modulates T cell responses towards a Th17 phenotype.

Given the established role of IL-2 in mediating Treg homeostasis *in vivo* [3], it is surprising that we did not observe any effects on Treg subsets in the presence of sCD25 in this study. Although we did not specifically examine whether sCD25 affected the suppressive function of Tregs, levels of Foxp3 expression both *in vitro* and *in vivo* clearly indicate that sCD25 did not impact Treg survival or persistence. Similarly, previous reports have found that IL-2 can play a role in enhancing Th1 mediated responses but these were not affected by sCD25 at the concentrations used in this study [31] (Figure 2A, 3B). As Th17 responses are clearly elevated under these conditions, these data may reflect differing levels of sensitivity among pathogenic versus regulatory CD4+ T cell subsets towards the effects of IL-2 signalling. Alternatively, it is possible that Tregs, which express constitutively high levels of surface CD25 (and the heterotrimeric IL-2R) in comparison to Th17 cells, may be competitively less sensitive to sequestration of circulating IL-2 by sCD25. Studies are ongoing to determine how limiting doses of IL-2 may differentially impact CD4+ T cell responses and how sCD25 might influence these events.

It is clear from our *in vivo* studies that the ability of sCD25 to enhance Th17 responses on a per cell basis is only observed in the periphery and not at the site of inflammation where although percentages of both Th17 and Th1 cells are remarkably unaltered upon sCD25 treatment, both are present in significantly increased numbers (Figure 1). Although this may reflect the effects of sCD25 on T cell expansion, this seems unlikely given that we observed no such effects *in vitro* (Figure 3D). A further possible explanation for this is an increased plasticity or inter-conversion between both subsets at the site of inflammation. However, this is unlikely given that the IL-17A eGFP mouse used in these studies allows the identification of cells which also have a legacy of IL-17A expression. As such, increased plasticity would be evident as an increase in the percentage of GFP+ve cells expressing IFNγ which was not detected. More likely, these data indicate that enhanced antigen-specific Th17 cells in the periphery can facilitate the infiltration of both pathogenic Th1 and Th17 cells to the site of inflammation as has been previously reported [32].

In direct relevance to this study, the use of humanized anti-CD25 antibodies is showing considerable promise as a potential therapeutic for Multiple Sclerosis [33]. Although efficacy for this approach has been demonstrated in early clinical trials, the exact mechanism through which these antibodies inhibit disease remains obscure. It is noteworthy that these antibodies bind sCD25 and block its ability to sequester IL-2 [34]. As levels sCD25 are elevated among MS patients [10], blockade of its immunomodulatory effects with anti-CD25 could conceivably play an important part in the mechanism of action of Anti-CD25.

Together these data demonstrate the immunomodulatory activity of the soluble form of the IL-2R alpha chain *in vivo* for the first time and indicate that these effects are mediated by its capacity to act as a decoy receptor for secreted IL-2. Although biochemical studies indicate that CD25 in isolation has a significantly lower affinity for IL-2 when compared to the heterotrimeric IL-2R complex, it has been demonstrated to bind IL-2 efficiently and its ability to suppress IL-2 mediated responses *in vitro* has been extensively reported [10,13,26,30]. The association between elevated levels of sCD25 found in the sera of autoimmune patients and the presence of specific susceptibility alleles at the *CD25* gene locus offer perhaps the clearest indication that sCD25 plays a role in autoimmune pathogenesis [10]. Although whether elevated levels of sCD25 are causally linked to the pathogenesis of human autoimmune disease remains to be determined, our studies demonstrate that sCD25 can act to enhance Th17 cell responses

and provide a novel mechanism which may explain these observations.

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Author Contributions

Conceived and designed the experiments: PTW. Performed the experiments: SER. Analyzed the data: SER ACM PGF PTW. Contributed reagents/materials/analysis tools: PGF. Wrote the paper: SER PTW.

References

1. Malek TR, Castro I (2010) Interleukin-2 receptor signaling: at the interface between tolerance and immunity. Immunity 33: 153–165.
2. Boyman O, Sprent J (2012) The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol 12: 180–190.
3. Turka LA, Walsh PT (2008) IL-2 signaling and CD4+ CD25+ Foxp3+ regulatory T cells. Front Biosci 13: 1440–1446.
4. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, et al. (1993) Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75: 253–261.
5. Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, et al. (1995) Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity 3: 521–530.
6. Suzuki H, Zhou YW, Kato M, Mak TW, Nakashima I (1999) Normal regulatory alpha/beta T cells effectively eliminate abnormally activated T cells lacking the interleukin 2 receptor beta in vivo. J Exp Med 190: 1561–1572.
7. Kryczek I, Wei S, Zou L, Altuwaijri S, Szeliga W, et al. (2007) Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. J Immunol 178: 6730–6733.
8. Kryczek I, Wei S, Vatan L, Escara-Wilke J, Szeliga W, et al. (2007) Cutting edge: opposite effects of IL-1 and IL-2 on the regulation of IL-17+ T cell pool IL-1 subverts IL-2-mediated suppression. J Immunol 179: 1423–1426.
9. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, et al. (2007) Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity 26: 371–381.
10. Maier LM, Anderson DE, Severson CA, Baecher-Allan C, Healy B, et al. (2009) Soluble IL-2RA levels in multiple sclerosis subjects and the effect of soluble IL-2RA on immune responses. J Immunol 182: 1541–1547.
11. Chistiakov DA, Chistiakova EI, Voronova NV, Turakulov RI, Savost'anov KV (2011) A variant of the Il2ra/Cd25 gene predisposing to graves' disease is associated with increased levels of soluble interleukin-2 receptor. Scand J Immunol 74: 496–501.
12. Heaney ML, Golde DW (1998) Soluble receptors in human disease. J Leukoc Biol 64: 135–146.
13. Zorn U, Dallmann I, Grosse J, Kirchner H, Poliwoda H, et al. (1994) Soluble interleukin 2 receptors abrogate IL-2 induced activation of peripheral mononuclear cells. Cytokine 6: 358–364.
14. Rubin LA, Nelson DL (1990) The soluble interleukin-2 receptor: biology, function, and clinical application. Ann Intern Med 113: 619–627.
15. Setoguchi R, Hori S, Takahashi T, Sakaguchi S (2005) Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J Exp Med 201: 723–735.
16. Rouse M, Nagarkatti M, Nagarkatti PS (2012) The role of IL-2 in the activation and expansion of regulatory T-cells and the development of experimental autoimmune encephalomyelitis. Immunobiology.
17. Yang ZZ, Grote DM, Ziesmer SC, Manske MK, Witzig TE, et al. (2012) Soluble IL-2R{alpha} facilitates IL-2-mediated immune responses and predicts reduced survival in follicular B-cell non-Hodgkin lymphoma. Blood 118: 2809–2820.
18. Chen Y, Haines CJ, Gutcher I, Hochweller K, Blumenschein WM, et al. (2011) Foxp3(+) regulatory T cells promote T helper 17 cell development in vivo through regulation of interleukin-2. Immunity 34: 409–421.
19. Pandiyan P, Conti HR, Zheng L, Peterson AC, Mathern DR, et al. (2011) CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. Immunity 34: 422–434.
20. Villarino AV, Tato CM, Stumhofer JS, Yao Z, Cui YK, et al. (2007) Helper T cell IL-2 production is limited by negative feedback and STAT-dependent cytokine signals. J Exp Med 204: 65–71.
21. Osawa H, Josimovic-Alasevic O, Diamantstein T (1986) Interleukin 2 receptors are released by cells in vitro and in vivo. I. Detection of soluble IL 2 receptors in cell culture supernatants and in the serum of mice by an immunoradiometric assay. Eur J Immunol 16: 467–469.
22. Dummer R, Posseckert G, Sugamura K, Grundmann H, Burg G (1996) Circulating interleukin-2 receptors are a group of multimeric proteins with immunoreactivity for interleukin-2 receptor alpha, beta, and gamma chains. J Interferon Cytokine Res 16: 315–320.
23. Witkowska AM (2005) On the role of sIL-2R measurements in rheumatoid arthritis and cancers. Mediators Inflamm 2005: 121–130.
24. Rubin LA, Galli F, Greene WC, Nelson DL, Jay G (1990) The molecular basis for the generation of the human soluble interleukin 2 receptor. Cytokine 2: 330–336.
25. Wang X, Rickert M, Garcia KC (2005) Structure of the quaternary complex of interleukin-2 with its alpha, beta, and gammac receptors. Science 310: 1159–1163.
26. Rubin LA, Jay G, Nelson DL (1986) The released interleukin 2 receptor binds interleukin 2 efficiently. J Immunol 137: 3841–3844.
27. Smith DE, Hanna R, Della F, Moore H, Chen H, et al. (2003) The soluble form of IL-1 receptor accessory protein enhances the ability of soluble type II IL-1 receptor to inhibit IL-1 action. Immunity 18: 87–96.
28. Symons JA, Wood NC, Di Giovine FS, Duff GW (1988) Soluble IL-2 receptor in rheumatoid arthritis. Correlation with disease activity, IL-1 and IL-2 inhibition. J Immunol 141: 2612–2618.
29. Jacques Y, Le Mauff B, Godard A, Naulet J, Concino M, et al. (1990) Biochemical study of a recombinant soluble interleukin-2 receptor. Evidence for a homodimeric structure. J Biol Chem 265: 20252–20258.
30. Rubinstein MP, Kovar M, Purton JF, Cho JH, Boyman O, et al. (2006) Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}. Proc Natl Acad Sci U S A 103: 9166–9171.
31. Liao W, Lin JX, Wang L, Li P, Leonard WJ (2011) Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat Immunol 12: 551–559.
32. Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, et al. (2009) C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nat Immunol 10: 514–523.
33. Martin R (2012) Anti-CD25 (daclizumab) monoclonal antibody therapy in relapsing-remitting multiple sclerosis. Clin Immunol 142: 9–14.
34. Junghans RP, Carrasquillo JA, Waldmann TA (1998) Impact of antigenemia on the bioactivity of infused anti-Tac antibody: implications for dose selection in antibody immunotherapies. Proc Natl Acad Sci U S A 95: 1752–1757.

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Sterile inflammation – do innate lymphoid cell subsets play a role?

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The recent identification of several novel innate lymphoid cell (iLC) subsets has increased our understanding of the mechanisms which link the innate and adaptive immune systems. While the contribution of these subsets toward the pathogenesis of human disease remains largely to be determined, it seems likely that they will play a particularly important role in sterile inflammatory settings where the innate response is seen as a critical mediator of inflammation. Several recent studies have highlighted the role of endogenous damage-associated molecular patterns such as IL-33, IL-1α, and IL-1β in promoting lymphoid cell responses. This review discusses the influence of such endogenous danger signals on novel iLCs such as lymphoid tissue-inducer cells, innate type 2 helper cells, and γδ T cells and explores how these responses may contribute to the development of an inflammatory response in a sterile setting.

Keywords: sterile inflammation, innate lymphoid cell, γδ T cell, type 1 response, type 2 response

INTRODUCTION

Acute inflammation directed by the innate immune response has evolved to efficiently combat infection and is critical to host defense. However, in the absence of overt infection, such innate mechanisms may also be activated as a result of tissue injury resulting from metabolic or exogenous sources (Rock et al., 2010). These events lead to what is termed a sterile inflammatory response which, if not appropriately resolved, can lead to the development of chronic inflammation which underlies or exacerbates a variety of human diseases.

The driving signals of sterile inflammation are known as damage-associated molecular patterns (DAMPs) and while distinct from their pathogen-derived counterparts, they elicit their effects through many of the same signaling pathways and networks (Bianchi, 2007). As a result, much of what we know about the mechanisms involved in sterile inflammation has stemmed from investigations of the innate inflammatory response to infection. Indeed, as a result of collateral damage often observed during the innate response to infection, DAMPs can also contribute to inflammation in non-sterile settings (Yang and Oppenheim, 2009; Yang et al., 2009; Andersson and Tracey, 2011). Most studies on the sterile inflammatory response have focused on the sources of these endogenous DAMPs, such as necrotic cells (Krysko et al., 2011). However, there is a growing awareness of the influence DAMPs play in orchestrating sterile inflammation through activating different cell types (Nace et al., 2012). This review will focus on the effects of specific DAMP activity on recently defined innate lymphoid cell (iLC) populations and how these distinct populations of cells play a role in orchestrating subsequent inflammatory events relevant to human disease.

DAMAGE-ASSOCIATED MOLECULAR PATTERNS

Damage-associated molecular patterns (DAMPs) are proinflammatory mediators largely activated under conditions of cellular stress or injury which may ultimately result in cell death. Stimuli which elicit these responses can range from sterile particulates derived from either aberrant metabolism, e.g., cholesterol crystals in atherosclerosis (Duewell et al., 2010) or external toxins, e.g., asbestos (Dostert et al., 2008). Similarly, unprogramed cell death, such as that observed under ischemic conditions or chemotherapeutic treatment strategies, leads to a loss of cellular integrity which can result in the exposure of DAMPs and the development of sterile inflammation (Arslan et al., 2011; Awong et al., 2012). In recent times, the identity of several DAMPs has been revealed and their effects on the generation of the sterile immune response described. As this review will focus on how these signals specifically influence iLCs responses, a number of the most relevant DAMPs are described below.

IL-1 α/β

The proinflammatory effects of both IL-1α and IL-1β have long been recognized. Although both DAMPs share the same IL-1 receptor, and thus appear to elicit very similar effects, their expression and activity are distinctly regulated (Dinarello, 2009). IL-1β gene expression is induced upon stimulation by numerous inflammatory signals but is normally translated in its inactive proform. Prior to its release from cells IL-1β undergoes maturation through proteolytic cleavage by caspase-1 in events mediated by an inflammasome involving the proteins NLRP3 and ASC (Li et al., 1995; Lamkanfi and Dixit, 2009). Critically, activation of the signaling events required for the appropriate assembly and activation of this NLRP3 inflammasome are known to be mediated by a wide range of sterile particulates and metabolites implicating IL-1β as central to numerous sterile inflammatory conditions (Martinon et al., 2006; Dostert et al., 2008; Masters et al., 2010). Unlike IL-1β, IL-1α appears to be constitutively expressed by a wider range of cell types and although it also undergoes proteolytic cleavage, is active in its unprocessed form. Although IL-1α

does not appear to require inflammasome activity for its activation it is known to be released in large amounts upon cell death through necrosis and has been found play a central role in driving neutrophil recruitment as part of the sterile inflammatory response (Chen et al., 2007).

The effects of IL-1 activity on both the innate and adaptive immune responses have been extensively characterized (Dinarello, 2009), and there is accumulating evidence that these DAMPs play an important role in driving iLC subset responses. These events are likely to play a significant role in driving neutrophil recruitment under sterile conditions and are discussed in greater detail below.

IL-33

IL-33 is another member of the IL-1 family of cytokines which is exposed upon cellular injury and acts upon its cognate receptor ST2 in complex with the IL-1RAcP (Schmitz et al., 2005). Although originally thought to be post transcriptionally regulated by caspase-1-mediated cleavage, in a manner similar to IL-1β, it is now apparent that IL-33 exhibits biological activity in its full-length uncleaved form and is released upon necrotic cell death to exert its biological activity in a similar manner to IL-1α (Liew et al., 2010). As well as acting as a classical "alarmin," intracellular IL-33 can also act as a transcriptional repressor although the specific genes and pathways which it targets in this role are not well-defined (Carriere et al., 2007; Ali et al., 2011). Release of IL-33 is strongly implicated as an instructive signal in the development of type 2 immune responses and as such it is thought to play an important role in the allergic response (Schmitz et al., 2005). More recently it has been demonstrated that IL-33 has profound effects on innate type 2 helper cells and nuocytes and may play a central role in driving type 2 immunity under sterile settings (Kim et al., 2012; Mirchandani et al., 2012). The influence of IL-33 on iLC populations in the context of sterile inflammation is discussed in further detail below.

EFFECTS OF DAMPs ON INNATE LYMPHOID CELL SUBSETS

The effects of DAMP signaling on cells of the innate immune system such as dendritic cells and macrophages have been extensively described elsewhere (Chen and Nunez, 2010). Similarly, since their earliest identification there have been numerous studies detailing how endogenous DAMPs can directly influence the adaptive T cell response. For example, HMGB1, CpG containing DNA motifs, and hsp60 have all been described to directly influence activated T cell responses (Dumitriu et al., 2005; Zanin-Zhorov et al., 2006; LaRosa et al., 2007). In more recent times there has been an intense interest in identifying novel lymphoid cell subsets which are thought to play an important role in bridging the gap between the innate and adaptive immune responses. These subsets are largely segregated by their ability to rapidly express effector cytokines more commonly associated with adaptive T helper cell responses such as IL-17 related cytokines or IL-13 and IL-4. Perhaps unsurprisingly, this has led to significant advances in understanding the role of these subsets in both autoimmune and allergic disease settings (Cai et al., 2011; Barlow et al., 2012; Pantelyushin et al., 2012). However there is significant evidence to suggest that such subsets may also play a central role in inflammation under sterile settings. In particular, the observation that proinflammatory cytokines normally associated with adaptive T cell responses are elevated in certain auto-inflammatory disease settings, in the absence of any identified sources of antigenic stimulation, indicates that iLCs may play an important role (Lasiglie et al., 2011).

INNATE LYMPHOID CELL SUBSETS IMPLICATED IN STERILE INFLAMMATION

γδ T CELLS

Arguably the most extensively described iLC populations are γδ T cells, which comprise about 5% of the overall T cell population. They differ from conventional αβ T cells in that they express invariant γ and δ chains as part of their T cell receptor and are resident predominantly at mucosal sites (Cua and Tato, 2010). γδ T cells appear to lack the requirement for conventional antigen presentation and this has contributed to the hypothesis that these cells act as tissue-resident immune sentinel cells. Of particular relevance to sterile inflammatory conditions are the recent observations from both mouse and human studies that specific subsets of γδ T cells respond to IL-R1 stimulation by expressing significant levels of IL-17A (Sutton et al., 2009; Ness-Schwickerath et al., 2010; Caccamo et al., 2011). These cells which constitutively express CCR6 and the transcription factor RORγT may act as an important instructive signal to promote the generation of adaptive Th17 type responses but have also been implicated as important mediators of disease in their own right (Sutton et al., 2009). In particular, γδ T cells which express IL-17A in response to IL-1α/β play an important role in driving neutrophilia and dermal inflammation in psoriasis in both mouse models and human patients (Cai et al., 2011; Laggner et al., 2011; Pantelyushin et al., 2012). Specifically, human $V\gamma9V\delta2^+$ cells, which are induced to express IL-17A in the presence of IL-1β, have been implicated as playing a prominent role in psoriasis (Laggner et al., 2011). As neutrophilic skin inflammation is a prominent feature of cryopyrin-associated inflammatory syndrome (CAPS), which are autoinflammatory disorders, associated with inflammasome hyperactivity and increased IL-1β processing, it is tempting to speculate that $V\gamma9V\delta2^+$ T cells may play a role in mediating these events (Kolivras et al., 2011). Indeed, in a recent study, significantly elevated levels of IL-17A was found in the serum of CAPS patients and although $CD4^+$ Th17 cells were implicated as the source of IL-17A, the status of γδ T cell subsets was not examined (Lasiglie et al., 2011). Similarly, characterizations of Nlrp3 gene-targeted mice harboring mutations which mimic those causing disease in humans have demonstrated significant skewing toward a Th17 type response. Of particular interest in this regard, Meng et al. (2009) have demonstrated that mice expressing a R258W mutation (corresponding to the R260W mutation found in Muckle–Wells disease patients) exhibit spontaneous skin inflammation associated with neutrophil inflammation and a Th17 dominated response. As this phenotype bears a remarkable similarity to psoriasis, in which γδ T cells are a prominent source of IL-17 related cytokines, it raises the possibility that γδ T cells may also play an important role in skin manifestations among CAPS patients.

While it has been established that caspase-1-dependent IL-1β activity can promote expression of IL-17A by γδ T cells in autoimmune disease settings (Lalor et al., 2011), whether this

occurs under sterile settings has not been investigated. We have found that the presence of IL-1β with IL-23 can induce significant rapid expression of IL-17A by murine γδ T cells *in vivo* under sterile settings and in the absence of exogenous antigen. In contrast, $CD3^+$ γδ TCR^- cells do not express significant levels of IL-17A under these conditions (**Figure 1**). As both IL-1β and IL-23 have been shown to be expressed at higher levels by monocyte derived dendritic cells from CAPS patients (Lasiglie et al., 2011), this further indicates that γδ T cell subsets may provide an important innate source of IL-17 related cytokines in autoinflammatory disease.

LYMPHOID TISSUE-INDUCER CELLS

Another prominent innate lymphoid subset characterized by their ability to quickly and efficiently express IL-17 and/or IL-22 are lymphoid tissue-inducer cells (LTi; Takatori et al., 2009). LTi cells require the activity of the RORγT transcription factor for their development and were initially identified for their role in driving lymphoid tissue development during embryogenesis. More recently, LTi have been found to play important roles in innate immune responses at mucosal surface such as the intestine and the skin where they have been implicated as playing an important role in responding to both pathogenic and commensal bacteria (Ivanov et al., 2006; Pantelyushin et al., 2012). However there is also emerging data indicating that these cells can play a prominent role in sterile inflammatory settings.

As well as the autoinflammatory syndromes referred to above, which result from genetic abnormalities, "sterile" inflammation can also occur as a result of therapeutic regimens designed to target specific cell populations for depletion. Treatments such as chemotherapy and irradiation often result in undesirable effects on the development of a healthy immune system with serious consequences for the patient. Recently, it has been demonstrated that LTi cells play a central role in promoting appropriate thymic

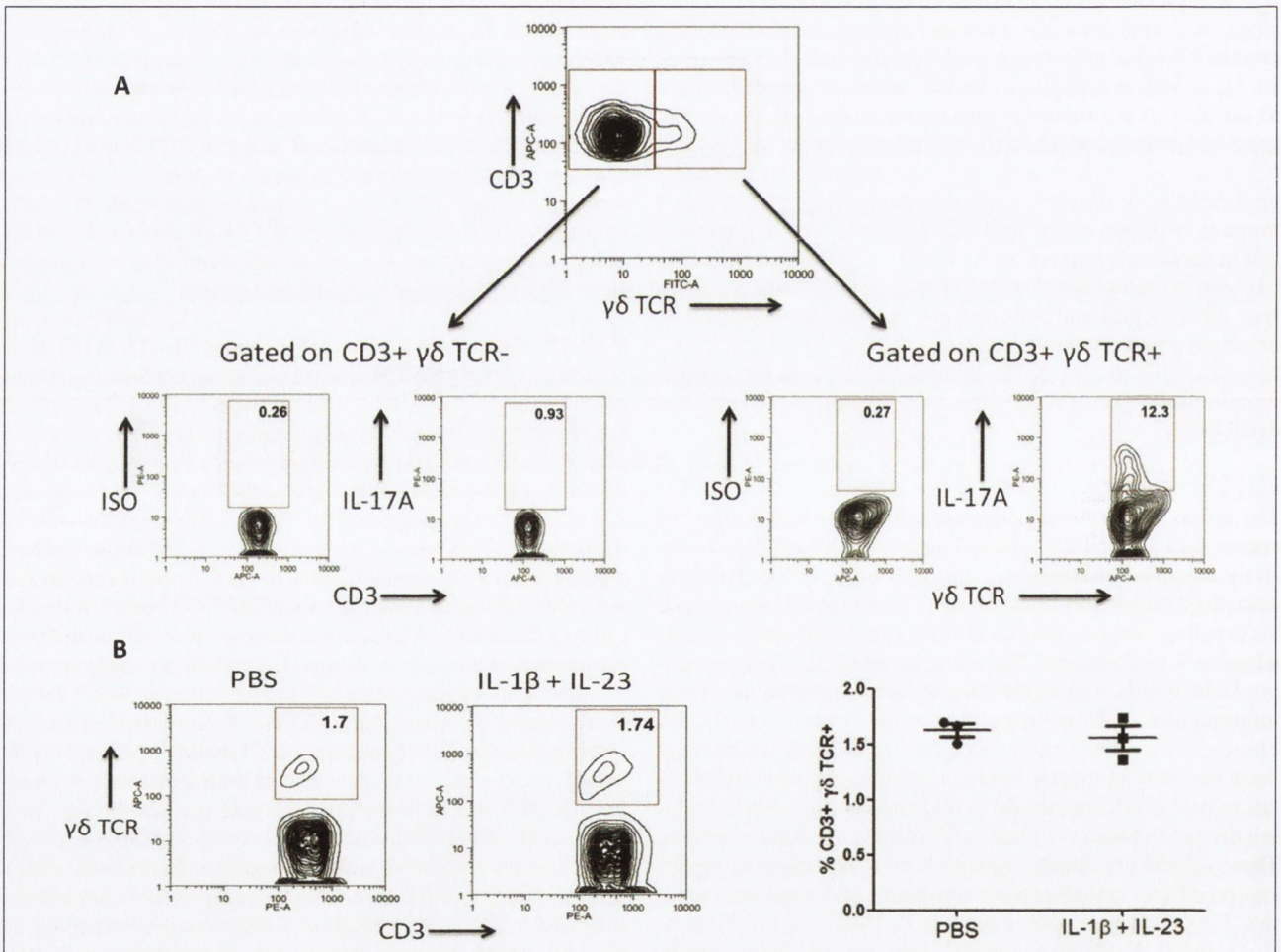


FIGURE 1 | IL-1β and IL-23 induce rapid expression of IL-17A by γδ T cells *in vivo*. C57Bl/6 mice were injected into the hind footpad with IL-1β (50 ng) and IL-23 (50 ng) or PBS. After 4 h cells were harvested from the popliteal lymph nodes and treated for a further 4 h with brefeldin A without *ex vivo* stimulation. Cells were stained for expression of CD3, γδ TCR and intracellular IL-17A or isotype control and examined by flow cytometry. **(A)** Data shown as IL-17A expression by live $CD3^+γδTCR^-$ and $CD3^+γδTCR^+$ cells is representative of four separate experiments. **(B)** The percentage frequency of $CD3^+γδTCR^+$ cells is unaltered after injection with IL-1β and IL-23. Data representative of three individual mice.

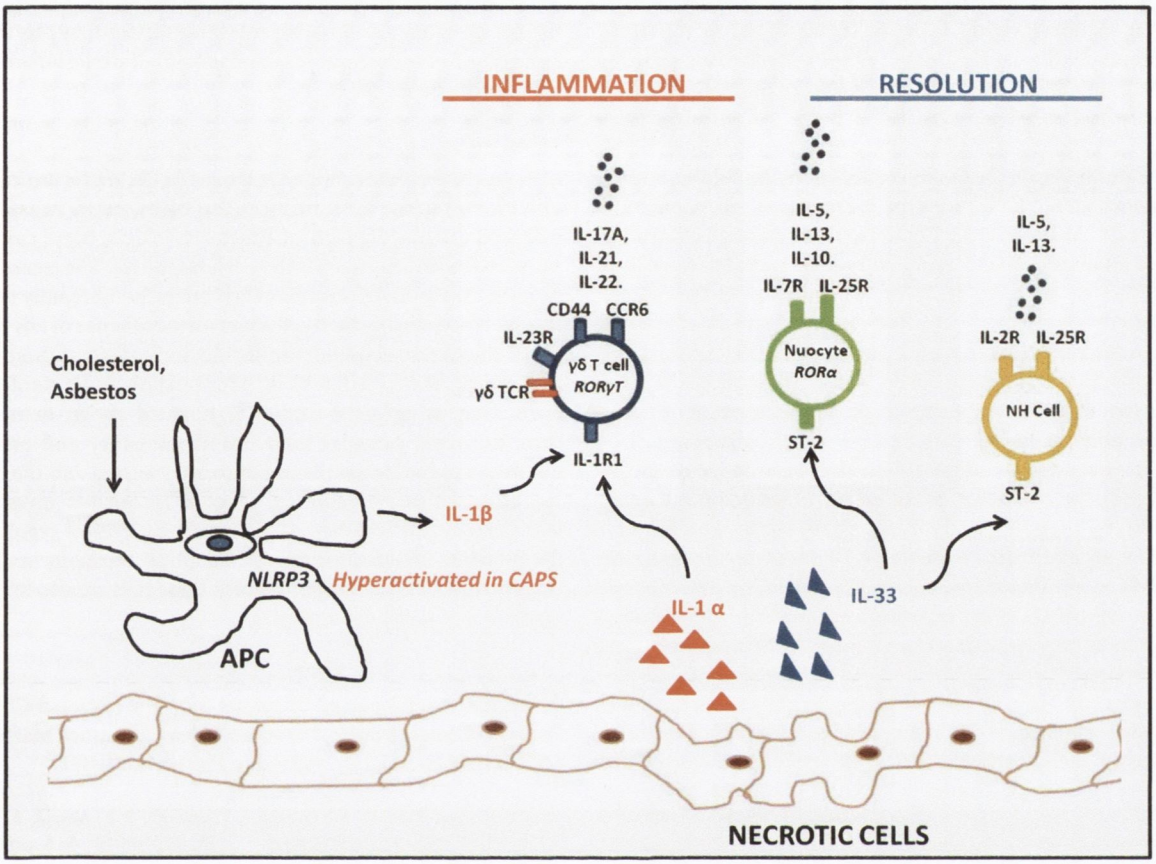


FIGURE 2 | Innate lymphoid subsets responding to sterile inflammation. Innate lymphoid subsets such as γδ T cells and LTi cells may respond to increased IL-1β expression as a result of either genetic- or particulate-driven inflammasome activation or IL-1α exposed upon tissue damage. These DAMPs drive the innate expression of IL-17 family members which may contribute to sterile inflammation or IL-22 which can promote tissue regeneration. On the other hand, IL-33 exposed upon tissue damage, drives type 2 innate responses by nuocytes and NH cells which can have a proresolving influence and dampen sterile inflammatory responses observed in obesity and atherosclerotic plaques.

regeneration in such sterile inflammatory settings (Dudakov et al., 2012). These effects are mediated largely through expression of IL-22 which promotes epithelial repair and tissue regeneration and highlight the role of iLCs in promoting the resolution of "sterile" inflammation (Dudakov et al., 2012). As an important source of IL-17A at mucosal sites, LTi cells have also been found play an important role in driving acute inflammatory responses to an array of both infectious and chemical stimuli (Ivanov et al., 2006; Pantelyushin et al., 2012). Although whether these cells can specifically respond to direct stimulation with DAMPs such as IL-1α/β (or indeed IL-33) has yet to be fully investigated, it is possible that they may also play an important role in bridging innate and adaptive immunity under sterile settings.

INNATE TYPE-2 CELLS

The recent discovery of a number of subsets of innate immune cells expressing ST-2 and responding to IL-33 has advanced our understanding of this cytokine and further expanded its emerging role as an alarmin (Liew et al., 2010). One such cell type, the nuocyte, is an innate type 2 cell which was originally identified as an IL-25 responsive non-T/non-B, γ-common chain-dependent cell which provided IL-4, IL-5, and abundant amounts of IL-13 at the onset of helminth infection (Neill et al., 2010). Interestingly, nuocytes are also expanded upon treatment with IL-33 and cells treated in this manner also secreted IL-6, IL-10, and GM-CSF (Moro et al., 2010).

The ability of nuocytes to respond to IL-33 in this manner implicates a role for these cells in the immune response to cellular stress. It is established that tissue damage caused by factors including high free fatty acids and oxidative stress commonly leads to necrosis and release of IL-33 (Moussion et al., 2008; Cayrol and Girard, 2009). Whilst no direct role for nuocytes has been described under such conditions, it is tempting to speculate that these cells could potentially provide an important source for at least some of the cytokines expressed in these settings, including IL-5, IL-10, and most notably IL-13 (Miller et al., 2010). For example, in atherosclerotic lesions, where severe tissue damage and necrosis are involved, IL-33 is thought to play a protective role through its ability to switch what is a type 1 dominated response toward a less inflammatory type 2 response. IL-33 increases the

levels of IL-4, IL-5, and IL-13 and reduces incidence of F4/80 positive macrophages and CD3 positive cells found in atherosclerotic lesions (Miller et al., 2008). These observations raise the interesting possibility that nuocytes could potentially provide a source of these cytokines and play a protective role in cardiac disease.

The natural helper (NH) cell is another recent addition to the expanding number of innate type 2 lymphoid cell populations (Koyasu and Moro, 2011). These cells are capable of producing large amounts of IL-5 and IL-13 when stimulated by IL-33 in the presence of IL-2, IL-7, or thymic stromal lymphopoietin (TSLP; Halim et al., 2012). Interestingly, and in contrast to the other described innate type 2 populations, NH cells are predominantly found in fat-associated lymphoid clusters where it is thought that they play important roles in maintenance of homeostasis in adipose tissues (Moro et al., 2010). Moreover, IL-33 and ST-2 expression in adipose tissue has been reported to play a protective role in obesity-driven sterile inflammation through promotion of a type 2 environment. These events were found to occur in association with switching of the macrophage response from a M1 to M2 phenotype raising the possibility that NH cells could also contribute to the regulation of the sterile inflammatory response to obesity by acting as an important orchestrator of the type 2 response (Miller, 2011).

Nuocytes and NH cells are two phenotypically similar cell subsets which respond to IL-33 and appear to play pathogenic roles in models of allergic and parasitic inflammation. However with the emerging importance of IL-33 as an endogenous danger signal further investigation into what role these subsets may play in mediating type 2 immunity under some settings of sterile inflammation could prove beneficial.

CONCLUDING REMARKS

Although the innate immune response to DAMPs is critical to the initiation of sterile inflammation, this often occurs in association with enhanced expression of effector cytokines more commonly associated with adaptive T helper cell responses. The recent identification of several iLC subsets which can respond rapidly to innate stimuli by expressing such cytokines, in the absence of any obvious requirement for antigenic stimulation, indicates that these subsets may be important mediators of inflammation under sterile conditions. Interestingly, the studies highlighted above, demonstrate that these cells can play both proinflammatory and proresolving roles depending on the inflammatory setting and the effector cytokines which are expressed (**Figure 2**). Further investigation will likely reveal whether, as well as being critical regulators at the interface between innate and adaptive immunity under settings of infection and autoimmunity, these cells are also important mediators of sterile inflammation.

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REFERENCES

Ali, S., Mohs, A., Thomas, M., Klare, J., Ross, R., Schmitz, M. L., and Martin, M. U. (2011). The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *J. Immunol.* 187, 1609–1616.

Andersson, U., and Tracey, K. J. (2011). HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu. Rev. Immunol.* 29, 139–162.

Arslan, F., De Kleijn, D. P., and Pasterkamp, G. (2011). Innate immune signaling in cardiac ischemia. *Nat. Rev. Cardiol.* 8, 292–300.

Awong, G., Lamotte-Mohs, R., and Zuniga-Pflucker, J. C. (2012). Key players for T-cell regeneration. *Curr. Opin. Hematol.* 17, 327–332.

Barlow, J. L., Bellosi, A., Hardman, C. S., Drynan, L. F., Wong, S. H., Cruickshank, J. P., and Mckenzie, A. N. (2012). Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J. Allergy Clin. Immunol.* 129, 191–198.e1–4.

Bianchi, M. E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* 81, 1–5.

Caccamo, N., La Mendola, C., Orlando, V., Meraviglia, S., Todaro, M., Stassi, G., Sireci, G., Fournie, J. J., and Dieli, F. (2011). Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood* 118, 129–138.

Cai, Y., Shen, X., Ding, C., Qi, C., Li, K., Li, X., Jala, V. R., Zhang, H. G., Wang, T., Zheng, J., and Yan, J. (2011). Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity* 35, 596–610.

Carriere, V., Roussel, L., Ortega, N., Lacorre, D. A., Americh, L., Aguilar, L., Bouche, G., and Girard, J. P. (2007). IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 104, 282–287.

Cayrol, C., and Girard, J. P. (2009). The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc. Natl. Acad. Sci. U.S.A.* 106, 9021–9026.

Chen, C. J., Kono, H., Golenbock, D., Reed, G., Akira, S., and Rock, K. L. (2007). Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat. Med.* 13, 851–856.

Chen, G. Y., and Nunez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* 10, 826–837.

Cua, D. J., and Tato, C. M. (2010). Innate IL-17-producing cells: the sentinels of the immune system. *Nat. Rev. Immunol.* 10, 479–489.

Dinarello, C. A. (2009). Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 27, 519–550.

Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T., and Tschopp, J. (2008). Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320, 674–677.

Dudakov, J. A., Hanash, A. M., Jenq, R. R., Young, L. F., Ghosh, A., Singer, N. V., West, M. L., Smith, O. M., Holland, A. M., Tsai, J. J., Boyd, R. L., and Van Den Brink, M. R. (2012). Interleukin-22 drives endogenous thymic regeneration in mice. *Science* 336, 91–95.

Duewell, P., Kono, H., Rayner, K. J., Sirois, C. M., Vladimer, G., Bauernfeind, F. G., Abela, G. S., Franchi, L., Nunez, G., Schnurr, M., Espevik, T., Lien, E., Fitzgerald, K. A., Rock, K. L., Moore, K. J., Wright, S. D., Hornung, V., and Latz, E. (2010). NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464, 1357–1361.

Dumitriu, I. E., Baruah, P., Valentinis, B., Voll, R. E., Herrmann, M., Nawroth, P. P., Arnold, B., Bianchi, M. E., Manfredi, A. A., and Rovere-Querini, P. (2005). Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *J. Immunol.* 174, 7506–7515.

Halim, T. Y., Krauss, R. H., Sun, A. C., and Takei, F. (2012). Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 36, 451–463.

Ivanov, I. I., Diehl, G. E., and Littman, D. R. (2006). Lymphoid tissue inducer cells in intestinal immunity. *Curr. Top. Microbiol. Immunol.* 308, 59–82.

Kim, H. Y., Chang, Y. J., Subramanian, S., Lee, H. H., Albacker, L. A., Matangkasombut, P., Savage, P. B., Mckenzie, A. N., Smith, D. E., Rottman, J. B., Dekruyff, R. H., and Umetsu, D. T. (2012). Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J. Allergy Clin. Immunol.* 129, 216–227.e1–6.

Kolivras, A., Theunis, A., Ferster, A., Lipsker, D., Sass, U., Dussart, A., and Andre, J. (2011). Cryopyrin-associated periodic syndrome: an autoinflammatory disease manifested as neutrophilic urticarial

dermatosis with additional perieccrine involvement. *J. Cutan. Pathol.* 38, 202–208.

Koyasu, S., and Moro, K. (2011). Type 2 innate immune responses and the natural helper cell. *Immunology* 132, 475–481.

Krysko, D. V., Agostinis, P., Krysko, O., Garg, A. D., Bachert, C., Lambrecht, B. N., and Vandenabeele, P. (2011). Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. *Trends Immunol.* 32, 157–164.

Laggner, U., Di Meglio, P., Perera, G. K., Hundhausen, C., Lacy, K. E., Ali, N., Smith, C. H., Hayday, A. C., Nickoloff, B. J., and Nestle, F. O. (2011). Identification of a novel proinflammatory human skin-homing Vgamma9Vdelta2 T cell subset with a potential role in psoriasis. *J. Immunol.* 187, 2783–2793.

Lalor, S. J., Dungan, L. S., Sutton, C. E., Basdeo, S. A., Fletcher, J. M., and Mills, K. H. (2011). Caspase-1-processed cytokines IL-1beta and IL-18 promote IL-17 production by gammadelta and CD4 T cells that mediate autoimmunity. *J. Immunol.* 186, 5738–5748.

Lamkanfi, M., and Dixit, V. M. (2009). The inflammasomes. *PLoS Pathog.* 5, e1000510. doi: 10.1371/journal.ppat.1000510

LaRosa, D. F., Gelman, A. E., Rahman, A. H., Zhang, J., Turka, L. A., and Walsh, P. T. (2007). CpG DNA inhibits $CD4^+CD25^+$ Treg suppression through direct MyD88-dependent costimulation of effector $CD4^+$ T cells. *Immunol. Lett.* 108, 183–188.

Lasiglie, D., Traggiai, E., Federici, S., Alessio, M., Buoncompagni, A., Accogli, A., Chiesa, S., Penco, F., Martini, A., and Gattorno, M. (2011). Role of IL-1 beta in the development of human T_H17 cells: lesson from NLPR3 mutated patients. *PLoS ONE* 6, e20014. doi: 10.1371/journal.pone.0020014

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., Towne, E., Tracey, D., Wardwell, S., Wei, F. Y., Wong, W., Kamen, R., and Seshadri, T. (1995). Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell* 80, 401–411.

Liew, F. Y., Pitman, N. I., and Mcinnes, I. B. (2010). Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat. Rev. Immunol.* 10, 103–110.

Martinon, F., Petrilli, V., Mayor, A., Tardivel, A., and Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440, 237–241.

Masters, S. L., Dunne, A., Subramanian, S. L., Hull, R. L., Tannahill, G. M., Sharp, F. A., Becker, C., Franchi, L., Yoshihara, E., Chen, Z., Mullooly, N., Mielke, L. A., Harris, J., Coll, R. C., Mills, K. H., Mok, K. H., Newsholme, P., Nunez, G., Yodoi, J., Kahn, S. E., Lavelle, E. C., and O'Neill, L. A. (2010). Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat. Immunol.* 11, 897–904.

Meng, G., Zhang, F., Fuss, I., Kitani, A., and Strober, W. (2009). A mutation in the Nlrp3 gene causing inflammasome hyperactivation potentiates Th17 cell-dominant immune responses. *Immunity* 30, 860–874.

Miller, A. M. (2011). Role of IL-33 in inflammation and disease. *J. Inflamm. (Lond.)* 8, 22.

Miller, A. M., Asquith, D. L., Hueber, A. J., Anderson, L. A., Holmes, W. M., Mckenzie, A. N., Xu, D., Sattar, N., Mcinnes, I. B., and Liew, F. Y. (2010). Interleukin-33 induces protective effects in adipose tissue inflammation during obesity in mice. *Circ. Res.* 107, 650–658.

Miller, A. M., Xu, D., Asquith, D. L., Denby, L., Li, Y., Sattar, N., Baker, A. H., Mcinnes, I. B., and Liew, F. Y. (2008). IL-33 reduces the development of atherosclerosis. *J. Exp. Med.* 205, 339–346.

Mirchandani, A. S., Salmond, R. J., and Liew, F. Y. (2012). Interleukin-33 and the function of innate lymphoid cells. *Trends Immunol.* doi: 10.1016/j.it.2012.04.005 [Epub ahead of print].

Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J., Ohtani, M., Fujii, H., and Koyasu, S. (2010). Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463, 540–544.

Moussion, C., Ortega, N., and Girard, J. P. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS ONE* 3, e3331. doi: 10.1371/journal.pone.0003331

Nace, G., Evankovich, J., Eid, R., and Tsung, A. (2012). Dendritic cells and damage-associated molecular patterns: endogenous danger signals linking innate and adaptive immunity. *J. Innate Immun.* 4, 6–15.

Neill, D. R., Wong, S. H., Bellosi, A., Flynn, R. J., Daly, M., Langford, T. K., Bucks, C., Kane, C. M., Fallon, P. G., Pannell, R., Jolin, H. E., and Mckenzie, A. N. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464, 1367–1370.

Ness-Schwickerath, K. J., Jin, C., and Morita, C. T. (2010). Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. *J. Immunol.* 184, 7268–7280.

Pantelyushin, S., Haak, S., Ingold, B., Kulig, P., Heppner, F. L., Navarini, A. A., and Becher, B. (2012). Rorγ+ innate lymphocytes and $\gamma\delta$ T cells initiate psoriasiform plaque formation in mice. *J. Clin. Invest.* 122, 2252–2256.

Rock, K. L., Latz, E., Ontiveros, F., and Kono, H. (2010). The sterile inflammatory response. *Annu. Rev. Immunol.* 28, 321–342.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., Gorman, D. M., Bazan, J. F., and Kastelein, R. A. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23, 479–490.

Sutton, C. E., Lalor, S. J., Sweeney, C. M., Brereton, C. F., Lavelle, E. C., and Mills, K. H. (2009). Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31, 331–341.

Takatori, H., Kanno, Y., Watford, W. T., Tato, C. M., Weiss, G., Ivanov, I. I., Littman, D. R., and O'Shea, J. J. (2009). Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J. Exp. Med.* 206, 35–41.

Yang, D., De La Rosa, G., Tewary, P., and Oppenheim, J. J. (2009). Alarmins link neutrophils and dendritic cells. *Trends Immunol.* 30, 531–537.

Yang, D., and Oppenheim, J. J. (2009). Alarmins and antimicrobial immunity. *Med. Mycol.* 47(Suppl. 1), S146–S153.

Zanin-Zhorov, A., Cahalon, L., Tal, G., Margalit, R., Lider, O., and Cohen, I. R. (2006). Heat shock protein 60 enhances $CD4^+$ $CD25^+$ regulatory T cell function via innate TLR2 signaling. *J. Clin. Invest.* 116, 2022–2032.

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Shane Russell 09125159

Novel mediators regulating innate and adaptive type 17 immune responses.

The discovery of a third subset of T helper cells and more recently innate T cell subsets which preferentially express IL-17A has led to intensive investigation into the roles these cells play in both host defence and autoimmune settings As such, understanding the mechanisms related to the of the generation, function and regulation of these cells are crucial to the development of therapeutic interventions to counteract associated inflammation and pathology. In these studies we have focused on two of the more recently described pathways of regulation of IL-17A expressing T cells, namely the IL-2/IL-2 receptor signalling axis and TIR8 a negative regulator of IL-1R1 signalling.

IL-2 has been implicated as a negative regulator of Th17 responses *in-vitro* and *in-vivo.* Mutations at *CD25*, the α chain of the IL-2 receptor, confer susceptibility to a variety of autoimmune diseases with a number of these susceptibility alleles correlating with elevated levels of the soluble form of the α receptor chain (sCD25). We have demonstrated that elevated levels of sCD25 *in-vivo* resulted in enhanced antigen-specific Th17 responses in the periphery and exacerbated experimental autoimmune encephalomyelitis (EAE). We report that sCD25 is capable of enhancing Th17 responses *in-vitro* with no impact on Th1 responses or Treg generation. This enhancement of the Th17 response is characterised by decreased STAT5 signalling downstream of the IL-2 receptor, which facilitates an enhancement of IL-6 receptor driven STAT 3 phosphorylation. sCD25 exerted its effects early during the Th17 developmental programme, leading to increased expression of the signature transcription factor RORγT, as well as IL-17A expression after just 48 hours. sCD25 did not directly impact cellular expression of IL-2 or act at the cell surface, but was found to greatly inhibit the detection of secreted levels of IL-2 by ELISA through direct interaction with IL-2 itself, demonstrating its ability to act as an IL-2 'sink' in the T cell microenvironment. These data identify the ability of sCD25 to promote autoimmune disease pathogenesis and enhance Th17 responses through its ability to sequester local IL-2.

Th17 cells have received considerable interest since their discovery; however, recently it has become clear that a large proportion of the IL-17A expression in both host defence and autoimmune settings is accounted for by innate T cell subsets. One such subset is γδ T cells, which have been shown to express IL-17A directly in response to IL-1β and IL-23 stimulation. TIR8, an orphan receptor of the Toll/IL-1 receptor superfamily has been established as a negative regulator of IL-1R1 signalling and in this role has also been implicated as a key intrinsic regulator of Th17 responses and autoimmunity. However, its role in the modulation of IL-1R1 driven IL-17A expression by innate lymphocyte populations or its potential to modulate inflammation in disease associated with these cells has yet to be established. Expression of TIR8/SIGIRR has been reported to be reduced in the peripheral blood of psoriatic arthritis patients. However, whether TIR8/SIGIRR activity plays a specific role in regulating psoriatic inflammation is unknown. We report that *Tir8/Sigirr* deficient mice develop more severe psoriatic inflammation in both the chemical (Aldara™) and cytokine (recombinant IL-23) induced models of psoriasis. Increased disease severity was associated with enhanced infiltration of $V\gamma4^+$ γδ T cells which express significantly elevated levels of IL-17A. Critically, we also demonstrate that TIR8/SIGIRR activity directly suppressed innate IL-17A expression by γδ T cells *in vitro* and *in vivo.* Importantly, treatment of *Tir8/Sigirr-/-* mice with an IL-17A neutralisation antibody reversed the enhanced disease severity observed in these mice. This study identifies TIR8/SIGIRR as a novel intrinsic negative regulator of innate IL-17A expression and characterises a novel mechanism involved in the regulation of psoriatic inflammation.

Taken together these data further highlights the central role IL-17A plays in inflammatory states mediated by both innate and adaptive T cell and identifies both sCD25 and TIR8/SIGIRR as signalling mediators acting to directly modulate these events i*n-vivo.*