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Characterizing the Role of the T Cell Receptor Repertoire in T Cell Development and Function

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List of Abbreviations

APC	Antigen presenting cell
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CNS	Central nervous system
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DN	CD4- CD8- double negative
DP	CD4+ CD8+ double positive
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
FACS	Fluorescence activated cell sorting
FDR	False discovery rate
Foxp3	Forkhead box P3
GF	Germ free
IBD	Inflammatory bowel disease
IL	Interleukin
IRES	Internal ribosome entry site
IRF4	Interferon regulatory factor
LN	Lymph node
LP	Lamina propria
MHC	Major histocompatibility complex
mLN	Mesenteric lymph node
MOG	Myelin oligodendrocyte
MS	Multiple sclerosis
NFAT	Nuclear factor of activated T-cells
NOD	Non-obese diabetic (mice)
pLN	Peripheral lymph node
RAG	Recombination-activating gene

RORγt	RAR-related orphan receptor γ t
RV	Retroviral
RVBMC	Retroviral bone marrow chimera
SFB	Segmented filamentous bacteria
SPF	Specific pathogen feature
TCR	T cell receptor
T_{Eff}	Effector T cell
T_H	Helper T cell
Treg	Regulatory T cell
TRV	T cell receptor variable segment
WNV	West Nile virus
WT	Wild type

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Abstract

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Expansion and memory of immune cells in response to stimulation of diversified antigen receptors is the hallmark of adaptive immunity. Here, we use antigen receptor sequencing and *in vivo* analysis of monoclonal cell populations to elucidate the development and function of two T cell populations: Foxp3+RORγt+ CD4+ T cells and γδ T cells. Foxp3+RORγt+ T cells have recently been characterized as an immunoregulatory population highly enriched in the colon lamina propria. However, their developmental origin and relation to RORγt- Treg and RORγt+ T_H17 cells remains unclear. Here, we show that despite sharing a subset of TCR specificities with T_H17 cells, Foxp3+RORγt+ T cells first acquire a Foxp3+RORγt- phenotype before co-expressing RORγt, suggesting that Foxp3+RORγt+ cell development can occur via an RORγt- Treg intermediate.

While γδ T cells are considerably well studied relative to Foxp3+RORγt+ T cells, the importance antigen receptor diversification to γδ T cell function is still poorly understood. In order to comprehensively assess the paired-chain γδ T cell repertoire during inflammation, we developed a fixed-TCRδ system. We show that experimental autoimmune encephalomyelitis (EAE) results in dramatic clonal expansion of γδ T cells and that a single expanded TCR clone is sufficient to exacerbate immune pathology. Together, this suggests that γδ T cells can exhibit the clonal

expansion characteristic of an adaptive immune response and that this response is physiologically significant to the outcome of EAE.

Chapter 1: Introduction

Expansion and retention of immune cells base on interactions between antigens and diversified antigen receptors is the hallmark of adaptive immunity. Not only does this phenomenon underlie the principal effector mechanisms of adaptive immune cells, but it is also crucial for understanding their development and physiology. In this thesis, we apply antigen receptor sequencing and *in vivo* analysis of monoclonal T cell populations to two relatively poorly understood T cells subsets, Foxp3+RORγt+ regulatory T cells and γδ T cells, in order to elucidate their development and contribution to adaptive immunity.

Regulatory T cells in mucosal tolerance

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is one of the most common chronic gastrointestinal diseases, affecting as many as 1.4 million individuals in the United States (Loftus, 2004). A multitude of evidence currently suggests that an inappropriate immune response directed against normal, commensal microbes is a major component of IBD pathogenesis (Abraham and Cho, 2009; Bonen and Cho, 2003; Hardenberg et al., 2011; Khor et al., 2011). In order to elucidate how this abnormal immune response arises, it is important to understand how tolerance to commensal, though foreign, microorganisms is maintained in healthy individuals. Originally identified for their role in maintaining tolerance to self-antigens (Hsieh et al., 2012), Foxp3+ regulatory T (Treg) cells are also necessary for maintaining mucosal tolerance (Barnes and Powrie, 2009). However, Treg cells appear susceptible to adopting the inflammatory phenotype of the related RORγt+ T helper (T_H)17 T cell subset (Koenen et al., 2008; Komatsu et al., 2009; Korn et al., 2009; Lee et al., 2009). This potential plasticity between Treg and T_H17 cells may represent a component of IBD pathogenesis.

Interactions between the immune system and commensal microbiota

During steady-state, the majority of CD4+ T cells are found in the mucosal lamina propria (Maynard and Weaver, 2009). Treg and T_H17 CD4+ T cells are particularly enriched in intestinal tissue, with Treg cells primarily localizing to the colonic lamina propria, while T_H17 cells are mostly restricted to the small intestine lamina propria (Ivanov et al., 2008). The enrichment of Treg and T_H17 cell in mucosal tissues suggests that these cells play a role in regulating the immune response to the microbiota. Indeed, in germ free (GF) mice, which lack commensal organisms, the generation of mucosal Treg and T_H17 cells is impaired (Ivanov et al., 2009; Lathrop et al., 2011).

In addition, several bacterial species appear capable of promoting the differentiation of mucosal Treg cells. For example, both *Bacteroides fragilis,* though TLR2-mediated detection of bacterial PSA (Mazmanian et al., 2005, 2008; Round and Mazmanian, 2010; Round et al., 2011), as well as several *Clostridium* species (Atarashi et al., 2011) generate protective Treg cell populations. Similarly, several gut bacteria promote the differentiation of T_H17 cells including segmented filamentous bacteria (SFB) (Ivanov et al., 2008, 2009) and an enterotoxigenic strain of *B. fragilis* (ETBF) (Wu et al., 2009), which stands in stark contrast to the Treg cell-promoting non-enterotoxigenic strain (NTBF) described above.

An unresolved question in our understanding of mucosal tolerance is the role of antigen specificity in the induction of mucosal T cell subsets. The dependence on TLR2 for PSA-mediated colonic Treg cell induction suggests that this interaction is antigen independent. However, our lab has demonstrated that the T cell receptor (TCR) repertoire of colonic Treg cells is unique compared to the repertoire's of Treg cells in other tissues, as well as other mucosal T cell subsets and that many of these TCRs appear to be specific to bacterial components (Lathrop et al., 2011). Similarly,

interactions between specific TCR sequences and SFB-derived antigens have recently been shown to contribute towards SFB-mediated T_H17 induction (Yang et al., 2014) .

Treg and T_H17 cellular plasticity

In the conventional view of CD4+ T cell differentiation, these multiple cell lineages are considered mutually exclusive and terminally differentiated. This is supported by *in vitro* observations that the characteristic cytokines produced by each lineage are capable of inhibiting the development of alternative subsets. However, it is now clear that CD4+ T cell subsets are far more plastic than previously thought. For example, Treg cells downregulate Foxp3 upon transfer to lymphopenic hosts and begin to express inflammatory cytokines, including IL-17 instead (Komatsu et al., 2009). Additional studies using lineage tracking mice capable of identifying "ex-Treg cells" have generated conflicting results regarding the true extent of Treg cell plasticity (Rubtsov et al., 2010; Zhou et al., 2009). However, additional evidence suggests that ex-Treg cells may arise from the outgrowth of a small sub-population of unstable cells within the larger Foxp3+ population (Miyao et al., 2012). The identity and functional role of these unstable cells remains unclear.

Lineage tracing mice have also been used to track the fate of "ex-T_H17 cells" that have lost the expression of IL-17. In the context of experimental autoimmune encephalomyelitis (EAE), a significant proportion of T_H17 cells lose their expression of IL-17 as the disease progresses. In addition, at peak disease severity, approximately 50% of all IFNγ producers are ex-T_H17 cells (Hirota et al., 2011). Ex-T_H17 cells have also been shown to contribute to the population of T_H1 cells in *H. hepaticus* infection (Ding and Morrison, 2013) as well as the pool of follicular helper T (T_{FH}) cells in the Peyer's patches during normal gut homeostasis (Hirota et al., 2013). However, while certain conditions seem to promote a non-inflammatory variant of T_H17 cells (Lee et al., 2012b), it remains to be seen if T_H17 cell are capable of adopting a Treg cell phenotype *in vivo*.

Foxp3 and RORγt co-expression

CD4+ T cell subsets are typically defined by the expression lineage specific transcription factors. For Treg and T_H17 cells, these transcription factors are Foxp3 and RORγt, respectively. Interestingly, despite the fact that Foxp3 and RORγt can directly inhibit one another (Zhou et al., 2008), a large proportion of CD4+ T cells co-express these two transcription factors in mucosal tissues (Ohnmacht et al., 2015). Furthermore, despite the expression of RORγt in these cells, they are believed to largely immunoregulatory due to their lack of IL-17 expression and ability to produce increased IL-10 (Lochner et al., 2008). In addition, Foxp3+RORγt+ cells appear protective in several models of inflammation including immune-mediated diabetes (Tartar et al., 2010) and colitis (Ohnmacht et al., 2015; Sefik et al., 2015; Yang et al., 2015)

However, the significance of RORγt expression in this cellular population remains unclear. Foxp3+RORγt+ T cells have been implicated in the control of T_H17 inflammation, suggesting that RORγt expression is needed to target RORγt-expressing T_H17 cells (Sefik et al., 2015; Yang et al., 2015). However, an additional report demonstrated that Foxp3+RORγt+ cells are important for control of T_H2-mediated inflammation, with little effect on the T_H17 population (Ohnmacht et al., 2015). In addition, it has also been shown that RORγt+ T_H17 cells can come to acquire Foxp3 expression when cultured with dendritic cells (DC)s stimulated with agonists for dectin-1, a pattern recognition receptor for fungal cell wall constituents (Osorio et al., 2008). Thus it is unclear if RORγt expression represents the basis for a suppressive mechanism acquired by Treg cells or instead reflects an origin in plastic T_H17 cells.

In Chapter 2 of this thesis, we seek to address this question through characterization of the Foxp3+RORγt+ TCR repertoire, as well as developmental analysis of a monoclonal population of these cells. We will show that (1) Foxp3+RORγt+ develop via a RORγt- Treg intermediate and (2)

that they share a limited set of high frequency antigen specificities with the T_H17 population. Together, these observations suggest that RORγt expression in Foxp3+RORγt+ cells does not represent T_H17 plasticity and, instead, reflects their convergence with T_H17 cells on a shared set of antigen specificities.

γδ T cells at the intersection of innate and adaptive immunity

αβ vs γδ T lymphocytes

Similar to the more numerous and well-studied αβ T cell, γδ T cells comprise a population of thymically derived lymphocytes that express a characteristic TCR, generated through the actions of recombination activating gene (RAG). As reflected in their names, the precise difference between αβ and γδ T cells is based on the usage of distinct genetic loci in generating rearranged TCR transcripts. For instance, the γδ TCRγ chain utilizes gene segments exclusively contained in the *TRG* locus. However, a multitude of developmental and functional differences underlie this simple genetic distinction.

Perhaps most illustrative of the difference between these two T cell lineages is that, compared to αβ T cells, a generalized model of γδ T cell development and function has remained elusive. While αβ T cells go through a concerted sequence of developmental events, numerous modes of thymic γδ T cell development have been described (Bandeira and Itohara, 1991; Kreslavsky et al., 2008). Nearly all αβ T cells recognize peptide antigens in the context of MHC presentation, whereas γδ T cells can recognize nearly any class of molecule, with or without the aid of presenting molecules (Born et al., 2012). Finally, while αβ T cells follow a general life cycle of antigen-dependent clonal expansion, γδ T cells can perform a range of functions spanning adaptive

and innate immunity (Vantourout and Hayday, 2013). As a result of these barriers to generalization, the biological advantage of maintaining this third antigen receptor-expressing lineage has remained a puzzle.

γδ T cells as adaptive or innate immune cells

Given their expression of a recombined antigen receptor, γδ T cells were initially assumed to represent an additional lineage of adaptive immune cell. However, subsequent research soon showed that γδ T cells defy simple classification, exhibiting characteristic features of both innate and adaptive immune cells. This dual nature is perhaps best exhibited in the diversity of the γδ TCR repertoire. Interestingly, due to the unique orientation of RSS sequences in the *TCRD* locus, recombined TCRδ chains can include two D-gene segments, exponentially increasing theoretical γδ TCR diversity (Elliott et al., 1988). In fact, despite having approximately 10-fold fewer V-gene segments to choose from compared to αβ T cells, the γδ TCR has the highest theoretical diversity of any antigen receptor, immunoglobulins included. Such potential diversity would clearly be advantageous for an adaptive immune response.

However, while many γδ T cells do indeed express diversified receptors, actual γδ TCR repertoire diversity is markedly limited relative to its theoretical potential, even when compared to other *in vivo* antigen receptor repertoires. As an extreme example, Vγ5+ dendritic epidermal γδ T cells (DETC) are nearly monoclonal, expressing a single germline configuration TCR (Asarnow et al., 1988). Despite representing a rearranged antigen receptor, such molecular homogeneity is more reminiscent of the pattern recognition receptors used primarily by innate immune cells.

In addition, γδ T cells are capable of behaving in ways similar to both adaptive and innate immune cells. For example, many γδ T cells exit the thymus expressing a CD44-hi CD25+ phenotype typically associated with activated αβ T cells. As a result, these cells can rapidly respond

to inflammatory stimuli. In fact, such "pre-activated" cells are often capable of responding to inflammatory cytokines alone, bypassing the requirements of antigen stimulation and APC co-stimulation normally required for αβ T cell responses (Jensen et al., 2008). The speed of this response, often within hours, is typically associated with innate immune function. Yet, other γδ T cells leave the thymus as CD44-lo CD25+ cells and, like other adaptive immune cells, require TCR stimulation for activation (Zeng et al., 2012).

γδ T cell function

As mentioned previously, γδ T cells fulfill many biological roles. Most reminiscent of other immune cells is their ability to contribute to the inflammatory response generated against pathogens. γδ T cells can participate in the immune response against all classes of infectious organisms including bacteria such as *Listeria* (Hiromatsu and Yoshikai, 1992) and mycobacteria (Shen et al., 2002), viral infections including West Nile virus (Wang et al., 2006) and vaccinia virus (Selin et al., 2001), as well as eukaryotic parasites such as *Plasmodium* (Hviid et al., 1996). However, while γδ T cells expand and contribute to immune memory in some of these infections (Sheridan et al., 2013), in others, their role is more similar to that of NK cells and other components of innate immunity (Mokuno et al., 2000).

As a corollary to their role during infection, γδ T cells also contribute to the pathogenesis of autoimmune reactions. γδ T cells clearly participate in the development of collagen induced arthritis, as TCRδ-deficient mice show protection against diseases pathology (Roark et al., 2007). Similarly, mice lacking γδ T cells are also protected against the development of EAE (Spahn et al., 1999). However, while the majority of published research implicates γδ T cells in the exacerbation of autoimmunity, there is some evidence that they can also contribute to immune regulation. For example, in EAE, while the Vγ4+ population of γδ T cells does indeed contribute to disease

pathology, the Vγ1+ population appears to limit autoimmune reactivity (Blink and Miller, 2009), suggesting that the role of γδ T cells in autoimmunity is multifaceted.

As reflected in the prominence of DETCs and other γδ T cells at barrier surfaces, γδ T cells can also play a role in wound healing. In γδ T cell deficient mice, closure of epidermal wounds is significantly delayed as a result of reduced keratinocyte proliferation. This delay is likely due to decreased availability of keratinocyte stimulation by KGF-1 and -2, which are highly produced by DETCs (Jameson et al., 2002). The participation of γδ T cells in wound healing also reflects their probable contribution to tissue stress surveillance. For instance, one of the best characterized γδ T cell ligands in mice, T-10, is specifically upregulated during cellular stress (Chien and Konigshofer, 2007). Indeed, the position of the DETC TCR is relocated in the direction of tissue wounds, suggesting that the role of these epidermal resident cells is to survey for local tissue damage (Chodaczek et al., 2012).

In addition, γδ T cells also contribute to several unconventional lymphocyte processes. For example, in humans, γδ T cells can serve as excellent antigen presenting cells, as activated γδ T cells efficiently take up antigen, traffic to lymph nodes, and express both MHC1 and MHC2 molecules (Brandes et al., 2005). γδ T cells may also participate in the induction of central immune tolerance, as interactions between thymic DETC cells and medullary thymic epithelial cells (mTECs) induces the latter's expression of autoimmune regulator (AIRE), which is responsible for the promiscuous expression of non-thymic self-antigens during negative selection (Roberts et al., 2012). Altogether, the functions of γδ T cells are highly diverse.

γδ T cell antigens

As stated above, γδ T cells recognize a diverse range of biomolecules. Perhaps the best characterized γδ TCR ligands are phosphoantigens. Phosphoantigens are intermediates in the

isoprenoid synthetic pathways used by both eukaryotic hosts and prokaryotic pathogens like mycobacteria. Recognition of these soluble molecules by the TCR requires the aid of cell surface accessory molecules including butyrophilin 3A1 and the F1-ATPase (Scotet et al., 2005; Vavassori et al., 2013). However, not all γδ T cell antigens require association with additional proteins, or even a cell surface, as γδ T cells from NOD mice are reportedly able to recognize soluble insulin peptide (Zhang et al., 2010).

In mice, the most well characterized γδ T cell ligands are the MHC-1 like molecules T-10 and T-22. These cell surface proteins are recognized by the TCR clones KN6 and G8, as well as a large percentage of the circulating γδ T cell population, as identified by T-10/22 tetramers (Crowley et al., 2000). Despite their relation to MHC-1, T-10 and T-22 present no peptide and are themselves the ligand of corresponding γδ TCRs (Wingren et al., 2000). Moreover, in addition to the small molecule and protein antigens described so far, γδ TCRs have also been shown to recognize lipids such cardiolipin and α-Galactosylceramide, both in the context of the cell surface molecule CD1d (Dieudé et al., 2011; Uldrich et al., 2013).

Yet, how antigen specificity contributes to the role of γδ T cells during an immune response remains unclear. In Chapter 3, we will show that (1) inflammation associated with EAE results in a shift in the γδ TCR repertoire and (2) that clones that expand during EAE can specifically exacerbate EAE pathogenesis. Together, these observations suggest that γδ T cells are capable of adaptive immune-like clonal expansion and that this expansion directly contributes to the course of an inflammatory response in a TCR-dependent manner.

Chapter 2: Development of Foxp3+RORγt+ T cells from Treg cells

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2.1 Abstract

Foxp3+RORγt+ T cells have recently been characterized as an immunoregulatory population highly enriched in the colon lamina propria. However, their developmental origin and relation to RORγt- Treg and T_H17 cells remains unclear. Here, we use a fixed TCRβ system to show that the TCR repertoire of the Foxp3+RORγt+ population is mostly distinct compared to other colonic T cell subsets. However, a fraction of these TCRs are also found in the T_H17 subset, suggesting that TCR repertoire overlap may contribute to the reported ability of Foxp3+RORγt+ cells to regulate T_H17 immunity. Naïve transgenic T cells expressing a Foxp3+RORγt+ restricted TCR first acquire a Foxp3+RORγt- phenotype before co-expressing *RORγt*, suggesting that Foxp3+RORγt+ cell development can occur via an RORγt- Treg intermediate.

2.2 Introduction

The maintenance of tolerance towards commensal bacteria represents a unique challenge for the host immune system. In order to protect against infection from pathogenic microbes, the host must be capable of clearing harmful bacteria, while simultaneously limiting responses that target commensal, though no less foreign, species. This balance is achieved in part by the induction of effector and tolerogenic CD4+ T cell responses by specific commensal species. For example, segmented filamentous bacteria is capable of promoting the differentiation of retinoid orphan receptor *(ROR)γt*-expressing T helper (T_H)17 cells, which are able to promote anti-bacterial responses (Ivanov et al., 2009). Conversely, Clostridial species (Atarashi et al., 2013) are capable of promoting the differentiation of forkhead box P3 *(Foxp3)*-expressing regulatory T (Treg) cells, which can limit inflammation (Josefowicz et al., 2012). Dysregulation between these subsets and subsequent loss of immune homeostasis is thought to represent an important component of inflammatory bowel disease (IBD) pathogenesis (Frank et al., 2007).

The classical model of CD4+ T cell differentiation involves the generation of several unambiguous subsets, distinguished by expression of lineage-defining transcription factors. However, this paradigm has been challenged in the case of Treg cells as several subsets of Foxp3+ cells have been identified that co-express transcription factors normally associated with effector lineages. This has led to the hypothesis that effector transcription factor co-expression allows Treg cells to specifically target corresponding effector subsets for suppression (Zheng et al., 2009). For example, T-bet+ Treg cells are involved in restraining T_H1-mediated gut inflammation (Koch et al., 2009). However, the precise developmental origin of such transcription factor co-expressing Treg cells is incompletely understood.

Recently, a subset of intestinal CD4+ T cells co-expressing both *Foxp3* and *RORγt* was identified (Ohnmacht et al., 2015; Sefik et al., 2015; Yang et al., 2015). While small numbers of these cells have been found in other peripheral lymphoid tissues (Lochner et al., 2008, 2011), the lamina propria appears uniquely enriched for this population. Foxp3+RORγt+ cells are dependent on the presence of commensal bacteria and are capable of producing IL-10. Moreover, the absence of these cells exacerbates pathogenesis of several models of mucosal autoimmunity, suggesting that these Foxp3+RORγt+ T cells represent another Treg cell subset.

Yet, the unique shared developmental requirements of Treg and T_H17 cells suggest an alternative interpretation for the role of Foxp3+RORγt+ T cells. TGFβ can promote the peripheral development of both Treg and T_H17 cells from naïve CD4+ T cells (Zhou et al., 2008). This raises the possibility that, instead of a stable Treg subset, Foxp3+RORγt+ T cells could represent a common precursor of both Treg and T_H17 cells, as previously proposed (Zhou et al., 2008). Thus, the relationship between Treg, T_H17, and Foxp3+RORγt+ T cells remains poorly defined.

Here, we use TCR sequencing and a TCR transgenic (TCRtg) system to elucidate the relationships between these Foxp3+RORγt+ cells and conventional Treg and T_H17 cells. We found that the TCR repertoire of Foxp3+RORγt+ cells is largely unique compared to other colonic T cell subsets suggesting that TCR specificity is sufficient to mediate Foxp3+RORγt+ cell development. Yet, we also observe a subset of Foxp3+RORγt+ TCRs that are shared with T_H17 cells and may contribute to the ability of this population to suppress T_H17 inflammation. Naïve T cells expressing a TCR restricted to the Foxp3+RORγt+ subset likely develop via an RORγt- Treg intermediate without significant T_H17 differentiation. In addition, we show that, similar to T_H17 cells, Foxp3+ cells are partially dependent on CX3CR1+ antigen presenting cells (APCs) for subsequent *RORγt* expression. Together, our data suggest that the dominant portion of Foxp3+RORγt+ T cells develop as a subset of Treg cells.

2.3 Materials and Methods

Mice

TCliβ TCRtg *Tcra*$^{+/-}$ mice, used as previously described (Hsieh et al., 2006), were bred to *Foxp3*$^{IRES\text{-}Thy1.1}$ (Liston et al., 2008), a gift from A. Rudensky (MSK); and *RORγt*^{gfp}, obtained from Jackson Laboratory (stock# 007572). CT2 transgenic mice were generated as described (Bautista et al., 2009) with microinjection into B6 × 129 fertilized eggs and backcrossed 5+ generations to B6 background. *CD11c*^{Cre} *Notch2*$^{fl/fl}$ (McCright et al., 2006) were a gift from Ken Murphy (WashU). *CX3CR1*^{DTR} (Diehl et al., 2013) were purchased from Jackson Laboratory (stock# 025629) and bred to *CD11c*^{Cre}. CD45.1 mice are housed together and interbred to maintain microbial integrity as assessed by CT2 transfer experiments. Animal experiments were performed in a specific pathogen-free facility under the guidelines of the Institutional Animal Care and Use Committee at Washington University.

Cell isolation and flow cytometry

Lamina propria tissue was isolated and digested in RPMI media containing 3% FBS, 20mM HEPES, 1mM DTT, and 50mM EDTA for 20min at 37º C. Additional washes in RPMI + 22.5mM EDTA was done to completely remove IELs. Remaining tissue was minced and digested in digested in 28.3μg/mL liberase TL (Roche) and 200μg/mL RNase 1 (Roche) for 30min at 37º C to release lamina propria lymphocytes. Suspended cells were filtered through a 40μm filter prior to use. Distal mLN was isolated due to its lymphatic drainage of the colon (Mowat and Agace, 2014). Fluorescently conjugated antibodies were purchased from Biolegend and eBioscience. Samples were sorted and analyzed with a FACSAria IIu (BD) and data was processed in FlowJo (Treestar).

TCR sequencing

CD4+Vα2+ cells were sorted from TCliβ TCRtg *Tcra*$^{+/-}$ *Foxp3*$^{\text{IRES-Thy1.1}}$*RORγt*$^{gfp/+}$ mice at ≥8wks old (mean age 18 weeks). TCRα cDNA was prepared using a Cα-specific primer for reverse transcription. cDNA libraries were generated using a nested PCR protocol with specific to TRAV14. Paired end reads were generated from 250 cycle sequencing using Illumina MiSeq at the Washington University Center for Genome Sciences. V, J, and CDR3 regions were then determined with a custom BLAST application using sequence data from IMGT (Lefranc, 2003).

Adoptive transfer experiments

Naïve (CD4+ CD44-lo CD62L-hi CXCR3- Foxp3- RORγt-) T cells were FACS purified from peripheral LNs and spleen of *Foxp3*$^{\text{IRES-Thy1.1}}$*RORγt*$^{gfp/+}$*Rag1*$^{-/-}$ TCRtg mice. CD45.2 TCRtg mice were used for transfer experiments into wild type CD45.1 hosts. CD45.1 TCRtg mice were used in experiments with CD45.2 DC-deficient hosts. $5x10^4$ cells were injected retro-orbitally into 3 week old hosts and analyzed 3 weeks post-transfer, unless otherwise indicated.

Statistical analysis

Diversity profiles were generated using Renyi entropy values with alpha/order values ranging from 0 (natural logarithm of species richness) through 2 (natural logarithm of the inverse Simpson index) (Jost, 2006; Pacholczyk et al., 2007). This includes alpha = 1, which represents the commonly used Shannon entropy. Evenness was calculated from the ratio of each point on the diversity profile to Renyi entropy at alpha = 0, resulting in an evenness profile of relative evenness indices ($RLE_{0,alpha}$) (Jost, 2010). Pielou's evenness represents the special case of $RLE_{0,alpha=1}$. Euclidean distance was used to generate hierarchical clusters from these diversity profiles and Pearson correlation was used for clustering of Evenness profiles (Greiff et al., 2015). Coverage was calculated as described (Chao and Jost, 2012). For multiple comparisons of individual TCR enrichment between samples, Benjamini-

Hochberg false discovery rate adjusted p-values were used. To generate TCR perturbation scores, CDR3 sequences within a sample were represented by their amino acid length to generate a spectratype distribution (Bergot et al., 2015). These spectratype distributions were then compared using the Morisita-Horn index and hierarchically clustered. All statistical analysis was performed in R (v3.3.0) with the use of the vegan (v2.3-5, diversity and similarity analysis), DESeq2 (v1.12.0, differential TCR usage), and pvclust (v2.0-0, bootstrapped dendrograms) packages. Mann-Whitney U or Kruskal-Wallis with post-hoc Dunn's tests were used for between group analysis. Our analytical code can be found at **https://github.com/BenSolomon/Solomon-Hsieh-JI-2016**.

2.4 Results

Sequencing of the Foxp3+RORγt+ TCR repertoire

Similar to data reported in recent publications, we found enrichment of Foxp3+RORγt+ T cells in mucosal tissue, particularly the colon lamina propria (**Fig. 2.1**). To address how this population relates to other colonic T cell subsets, we asked whether Foxp3+RORγt+ T cells utilized the same TCR repertoire as other Foxp3+ or RORγt+ cells. In order to circumvent analytical problem posed by the diversity of the fully polyclonal TCR repertoire, we analyzed TRAV14 TCRα transcripts from a TCRβ-tg system in which TCR diversity is restricted to the TCRα chain (Hsieh et al., 2006). From mucosal tissues we sorted (**Fig. 2.2**) and sequenced (**Fig. 2.3**) mature CD4+ T cells (CD44-hi CD62L-lo) into RORγt- Treg (Foxp3+RORγt-) and Foxp3+RORγt+ populations, as well as T_H17 (Foxp3- RORγt+) and remaining RORγt- T effector (T_{Eff}) cell (Foxp3- RORγt-) populations. In order to limit overlap with T_H1 cells, all populations except the RORγt- T_{Eff} population were further gated to exclude CXCR3+ cells (Yamamoto et al., 2000) (**Fig. 2.4**).

We first quantified the diversity of the Foxp3+RORγt+ TCR repertoire using Renyi entropies, which depict population diversity across a spectrum of orders that increasingly weight unique TCR sequences according to their abundance (Jost, 2010). Higher Renyi entropy values indicate greater sequence diversity and a steeper decrease in entropy with increasing order reflects a TCR repertoire more dominated by high frequency clones. Compared to the RORγt- T_{Eff} and RORγt- Treg populations, the Foxp3+RORγt+ and T_H17 T cell populations appear to have lower overall diversity (**Fig. 2.5**), with a larger proportion of the repertoire composed of high frequency clones (**Fig 2.6**).

The relationship between the diversity patterns of Foxp3+RORγt+ and T_H17 cell repertoires can be further quantified using hierarchical clustering. A distance metric can be calculated between

two samples' Renyi diversity profiles (Greiff et al., 2015), as defined by each sample's series of entropy values across a range of orders. Similarly, a correlation metric can be applied to evenness, a quantitative measure of clonal dominance reflected in the steepness of a curve of Renyi entropies (Jost, 2010). An evenness profile can be calculated from a series of Renyi entropies relativized to their order 0 entropy. Correlation and clustering of population diversity and evenness profiles across all tissues groups samples into two broad sets, one composed mainly of T_H17 and Foxp3+RORγt+ cell samples and the other composed mostly of RORγt- T_{Eff} and RORγt- Treg cell samples (**Fig. 2.7**). Thus, these data suggest that the TCR repertoire diversity of Foxp3+RORγt+ cells is closer to that of the T_H17 cell population and that these cells may be driven by a more restricted set of antigens than other Foxp3+ cells.

Foxp3+RORγt+ cells share limited sequence similarity with T_H17 cells

To assess similarity of the Foxp3+RORγt+ and T_H17 cell repertories at the level of antigen specificity, we compared the usage of individual TCR sequences across each of the sequenced populations. For most of the sequenced subsets, the majority of top clones for each population are largely unshared by any of the other populations (**Fig. 2.8**). However, the Foxp3+RORγt+ T cell population shares several of its more common TCR specificities with the T_H17 population.

We further quantified this repertoire overlap using the Morisita-Horn (MH) index. This index represents the probability that the sequence drawn from each of the two populations being compared will represent the same TCR, relative to the probability that two sequences drawn from all possible sequences, regardless of population, will represent the same TCR. This metric results in a continuum of values from 0, indicating complete dissimilarity, to 1, indicating complete similarity. Similar to previous observations of effector T cells in our lab, the repertoires of each of the four sorted populations were largely unique. However, the Foxp3+RORγt+ and T_H17 repertoires

demonstrated some degree of similarity that was significantly greater than that between the Foxp3+ RORγt+ population and any other sequenced subset (**Fig. 2.9**). We also utilized TCR repertoire perturbation scores, which applies the MH-similarity index to TCR's according to their amino acid lengths (i.e. CDR3 spectratype), rather than the TCR CDR3 amino acid sequences (Bergot et al., 2015), to compare the general structure of TCRs between samples. This also showed that the Foxp3+RORγt+ repertoire frequently cluster with the T_H17 (**Fig. 2.10**).

However, analysis of the repertoire at the level of individual TCRs revealed that only a limited number of shared high frequency clones accounts for the repertoire similarity seen between these two populations (**Fig 2.11**). This is further supported by the difference in Pearson and Spearman correlation values for comparisons of TCR frequencies between populations. Pearson correlation is more affected by outliers, like the high frequency TCRs shared by the Foxp3+RORγt+ and T_H17 populations, while Spearman correlation is less affected. We observe that, while a modest correlation between the Foxp3+RORγt+ and T_H17 populations is suggested by Pearson correlation, this relationship is reduced or absent when considering Spearman correlation (**Fig 2.12**). These data suggest that the Foxp3+RORγt+ population possesses a largely unique TCR repertoire, yet shares a limited subset of high frequency antigen receptor sequences with the T_H17 population.

TCR specificity can mediate acquisition of the Foxp3+RORγt+ phenotype

Though limited to a subset of clones, the similarity seen between the Foxp3+RORγt+ TCR repertoire and T_H17 TCR repertoire raises the possibility that Foxp3+RORγt+ cells could represent a developmental subset of the T_H17 population. However, as previous work has characterized Foxp3+RORγt+ cells as immunoregulatory, this population could represent a developmental subset of Treg cells instead. In order to distinguish between these alternatives, we utilized a monoclonal T cell-based approach. From our sequencing data, we identified several TCRs highly enriched in the

Foxp3+RORγt+ T cell population. Moreover, as the majority of Foxp3+RORγt+ TCRs are restricted to this population, we narrowed our search to TCRs with high relative enrichment compared to all other sorted populations (**Fig. 2.13**, **2.14**). Notably, the TCR CT2, previously identified by our lab as a colonic Treg TCR specific to gut bacteria (Lathrop et al., 2011), was the single most highly represented TCR in the Foxp3+RORγt+ T cell population.

We first sought to test whether this TCR could specifically mediate Foxp3+RORγt+ T cell development using TCR transgenic (TCRtg) mice expressing CT2. We adoptively transferred sorted naive Foxp3-RORγt- TCRtg cells into wild type host mice. By 3 weeks post-transfer, these cells showed significant co-expression of Foxp3 and RORγt, with greater than 50% of all colonic Treg cells expressing *RORγt* (**Fig. 2.15**). While the proportion of Foxp3+ cells expressing RORγt is similar for transferred polyclonal cells, this conversion is much less efficient than CT2 TCRtg cells. With polyclonal cells, only ~1% of all cells transferred become Foxp3+ RORγt+, compare to ~13% of all CT2 TCRtg cells at 3 weeks in the distal mesenteric LN (**Fig 2.16**). This suggests that different TCRs have varying ability to mediate Foxp3+RORγt+ T cell development and, thus, that antigen specificity is an important determinant for differentiation into this phenotype. This is further supported by the observation that an alternative mucosal Treg TCR, CT6, also mediates Foxp3+RORγt+ conversion, but to a lesser degree than CT2 (**Fig. 2.16**).

CT2 TCRtg T cells develop into Foxp3+RORγt+ T cells via a Treg cell intermediate

To more precisely delineate the developmental relationships between Foxp3+RORγt+, Treg, and T_H17 cells, we next performed kinetic experiments using this monoclonal TCR system. After transfer of naïve CT2 cells into wild type hosts, we analyzed *Foxp3* and *RORγt* expression in the transfer population at multiple time points. *Foxp3* expression was first observed as early as day 4 post-transfer. *RORγt* expression followed by day 7 post-transfer and was mostly restricted to the

Foxp3+ population (**Fig 2.17**). By 3 weeks post-transfer, *RORγt* expression is substantial and entirely restricted to the Foxp3+ population in both the colon and distal mLN.

We also validated these results in a polyclonal setting. We obtained similar results as seen with CT2 TCRtg cells, although the kinetics and extent of differentiation were somewhat diminished in the polyclonal population (**Fig 2.18**). In addition, the T_H17 population was more prevalent in the polyclonal setting. Together, these data suggest that the majority of Foxp3+RORγt+ T cells begin as naïve CD4+ T cells in the periphery and pass through a RORγt- Treg intermediate before co-expressing *RORγt*.

CX3CR1+ APCs can provide signals for Foxp3+RORγt+ differentiation

There are several phenotypically distinct APC subsets found in the gut, several of which support the differentiation of particular CD4+ T cells subsets. For example, monocyte-derived MHC2-hi APCs (Panea et al., 2015) contribute to the development of mucosal T_H17 cells, while Treg cells in the gut broadly require CD103+ cDCs (Coombes et al., 2007). Recent work from our lab suggests that mucosal Treg development is also partially dependent on the CD11b+ fraction of CD103+ DCs in a monoclonal setting (Nutsch et al.). Previous work has demonstrated that CD11c+ DCs are necessary for Foxp3+RORγt+ T cell development (Ohnmacht et al., 2015), but the role of specific APC subsets remains unclear.

To test whether there are similar APC requirements for *Foxp3* and *RORγt* expression in RORγt+ Treg cells, we adoptively transferred naïve CT2 TCRtg T cells into hosts that lack specific subsets of APCs. Since we have previously demonstrated a role for *Notch2*-dependent CD103+CD11b+ DC in CT2 Treg development, we first tested if RORγt+ Treg cells are similarly dependent on this DC. While we confirmed a general defect in Foxp3+ cells development, DC-

specific *Notch2* deficiency in host mice (Satpathy et al., 2013) did not specifically affect RORγt+ Treg cells (**Fig 2.19**).

Because of their reported contribution to SFB-mediated T_H17 induction, we also tested the role of CX3CR1+ APCs in Foxp3+RORγt+ T cell development using CX3CR1-DTR mice. These mice express DTR upon CD11c-Cre mediated excision of a Loxp-Stop cassette, resulting in loss of the of the CX3CR1+ APC population after DT treatment. Indeed, we observed a specific defect in the generation of *RORγt*-expressing Treg cells (**Fig 2.20**), similar to the impaired development of RORγt+T_H17 cells reporter previously. Interestingly, we also observed a deficit in overall Foxp3+ cell frequencies in absence of CX3CR1+ APCs. This shows that the CX3CR1+ APC may be involved in the differentiation of multiple T cell subsets and are important for the specific development of RORγt+ Treg cells.

2.5 Discussion

The expression of *Foxp3* or *RORγt* in T cells leads to the acquisition of generally antagonistic cellular phenotypes. Yet, a significant proportion of gut lamina propria T cells co-express these two transcription factors. Despite evidence suggesting that these Foxp3+RORγt+ T cells play a regulatory, rather than inflammatory role, it is still unclear how these cells develop in relation to classical Treg and T_H17 cells. Here, we present data which shows that Foxp3+RORγt+ T cells are a developmental subset of regulatory T cells.

Among CD4+ T cell subsets, Treg and T_H17 cells possess a unique relationship. Peripheral development of both subsets can be induced by TGFβ raising the possibility that Treg and T_H17 cells share a developmental intermediate (Zhou et al., 2008). The identification of Foxp3+RORγt+ T cells represents an appealing candidate for such an intermediate. However, using our TCRtg system, we demonstrate that the first cells to arise from naïve precursors are RORγt- Treg cells, preceding the appearance of Foxp3+RORγt+ T cells by several days. These TCRtg cells infrequently develop into T_H17 cells, despite reaching a Foxp3+RORγt+ state, suggesting that this is a mature rather than intermediate phenotype. This conclusion is supported by data showing the stability of the Foxp3+RORγt+ T cell gene expression profile (Yang et al., 2015).

If Foxp3+RORγt+ T cells do represent a subset of Treg cells, one might expect to see more similarity between the TCR repertoires of these two populations. However, as previously reported, >70% of all Foxp3+RORγt+ T cell development occurs by 9 weeks of age (Ohnmacht et al., 2015). As the mice in our experiments were sequenced at a mean 17 weeks of age, the majority of cells in our data set capable of differentiating into Foxp3+RORγt+ cells likely had already done so. Therefore, even if the Foxp3+RORγt+ population is a subset of Treg cells, these two groups could still appear distinct by TCR sequencing.

In fact, the repertoire of Foxp3+RORγt+ cells appears largely distinct from all sequenced populations. The simplest explanation for this observation is that Foxp3+RORγt+ cells recognize a distinct set of antigens. However, it remains possible that they may recognize the same antigens, but that different affinities to these or other antigens results the differential expression of Foxp3 or RORγt, akin to that seen with Treg cell selecting ligands in the thymus (Lee et al., 2012a; Shafiani et al., 2013). Future studies will be required to determine the precise features of antigen specificity that dictate Foxp3+RORγt+ development.

Yet, the Foxp3+RORγt+ population does share some TCR sequences with the T_H17 population and, though they represent a limited subset of the overall repertoire, this raises the question of how these clones develop. One possibility is that cells with these shared TCRs sequentially develop from T_H17 to Foxp3+RORγt+ cells, in contrast to the CT2 TCR studied. Alternatively, the Foxp3+RORγt+ and T_H17 phenotypes may develop via parallel pathways due to stochastic expression of cytokines or other signals associated with the cognate antigen. Further studies with additional TCRs are required to determine the extent that CT2 represents Foxp3+RORγt+ development, as well as establish the progression of transcription factor expression in cells expressing TCR shared by Foxp3+RORγt+ and T_H17 repertoires.

Notably, our sequencing data differs from that of a previous study which found no repertoire similarity between Foxp3+RORγt+ T cells and any other subset (Yang et al., 2015). However, these experiments used samples pooled from the spleen and peripheral lymph nodes, two tissues in which the population of RORγt+ Treg cells is dramatically smaller than that seen in the lamina propria. Given the relationship between Foxp3+RORγt+ T cells and the gut microbiota, we believe our analysis, which includes the lamina propria, more accurately reflects the physiology of these cells. Moreover, the use of fully polyclonal mice in the former study can make identification of shared clones difficult due the unknown TCRαβ pairing, as well as the greater diversity in the TCR

repertoire. Our use of a fixed TCRβ system restricts the TCR repertoire to a size capable of revealing clonal relationships, such as that seen between the Foxp3+RORγt+ and T_H17 cell populations.

The developmental path of Foxp3+RORγt+ cells is reflected in their requirements for specific APC subsets. As we have shown previously (Nutsch et al.) and in this report, Foxp3+ T cell development is influenced by *Notch2*-dependent, CD103+CD11b+ DCs. Moreover, we demonstrate that development of the RORγt+ fraction of Treg cells is specifically affected by the loss of CX3CR1+ APCs. Although future studies are required to determine the specific role of these APC subsets, one hypothetical model consistent with the current data is that naïve T cells first upregulate Foxp3 in response to signals from CD103+CD11b+ DCs and then express RORγt in response to CX3CR1+ APC-derived factors.

Several groups have demonstrated that co-expression of effector T cell transcription factors by a Treg cell confers upon it the ability to specifically regulate the corresponding effector T cell subset. In the case of T-bet+ Treg cells, this is achieved, in part, by the T-bet-mediated expression of the chemokine receptor CXCR3, which allows these cells to traffic to the same locations as CXCR3-expressing T_H1 cells (Koch et al., 2009). However, it remains to be shown whether these Treg subsets recognize antigens similar to those of their corresponding effector subsets and if such an overlap in TCR specificity contributes to the function of these Treg cells.

Here, we show that a noticeable fraction of high frequency Foxp3+RORγt+ TCR clones are shared with the T_H17 population. As previous work demonstrated that T_H17 inflammation is enhanced in the absence of RORγt+ Treg cells (Sefik et al., 2015; Yang et al., 2015), our observation suggests effector-specific Treg populations can share antigen specificities with their target effector populations. TCR-dependent activation of these cells would be expected to occur at the same sites of corresponding T_H17 activation, thus providing T_H17-specific regulation. Loss of these clones in

Foxp3^{Cre}RORγt^{flx} could account for the reported T_H17-regulatory phenotype. One caveat to this model comes from an additional study, which instead reported that RORγt+ Treg cells are involved in regulating type-2 immunity (Ohnmacht et al., 2015). However, as we did not specifically sequence the Th2 subset, we cannot rule out the possibility of a similar set of Foxp3+RORγt+ TCRs that overlap with this population. Future work is needed to determine the role of overlap in antigen specificity for regulatory T cell control of specific effector T cell subsets.

2.6 Figures

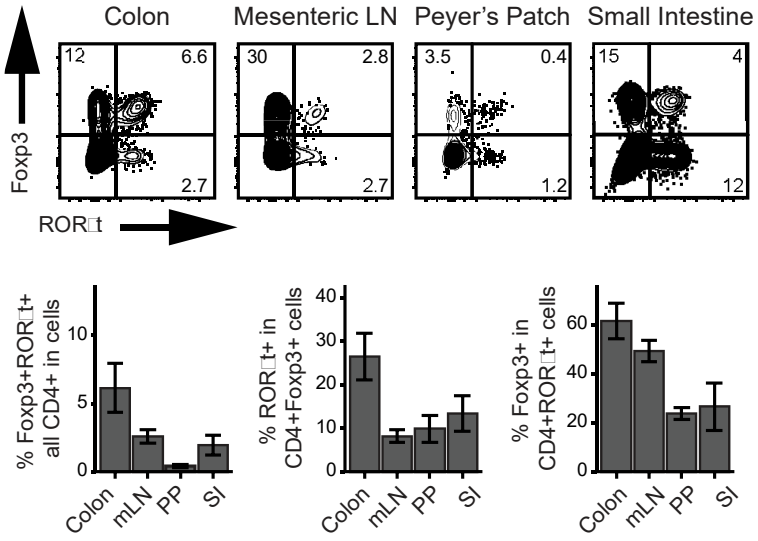


Figure 2.1 – Frequency of mucosal Foxp3+RORγt+ T cells

Representative FACS plots of CD4+ CD44-hi CD62L-lo CXCR3- cells in indicated tissues and associated quantification. n=6, each of 2 pooled mice. Error bars = ± 1 s.e.m.

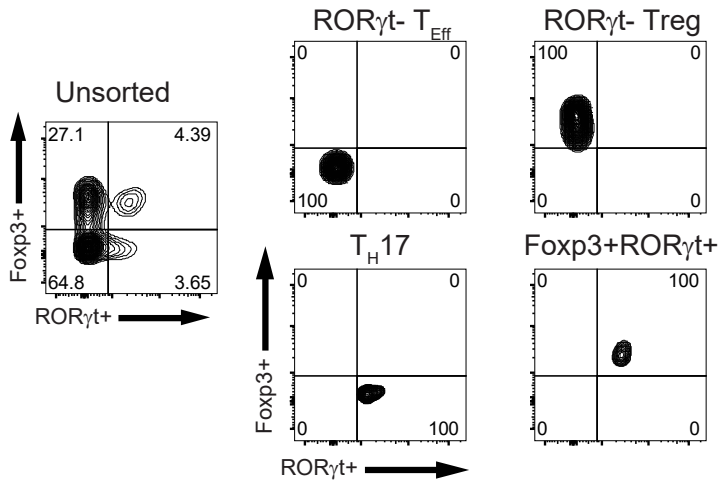


Figure 2.2 – Sorting purity

Representative post-sort purity of sequencing populations. Gated on CD4+ CD44-hi CD62L-lo CXCR3- cells.

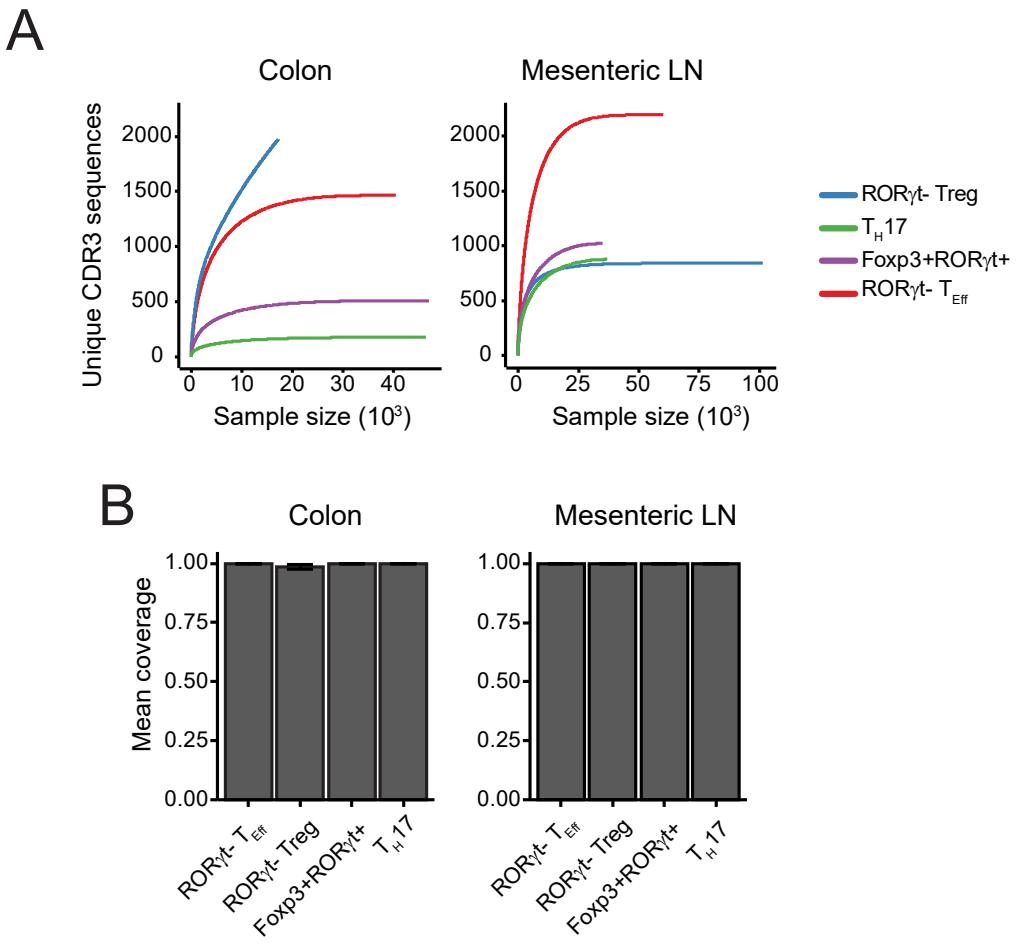


Figure 2.3 – Sequencing coverage of TCR repertoire

(A) Representative rarefaction curves for indicated sequencing populations. **(B)** Mean of calculated population coverage across all sequencing experiments for indicated populations and tissues. n=6, each of 2 pooled mice. Error bars = ± 1 s.e.m.

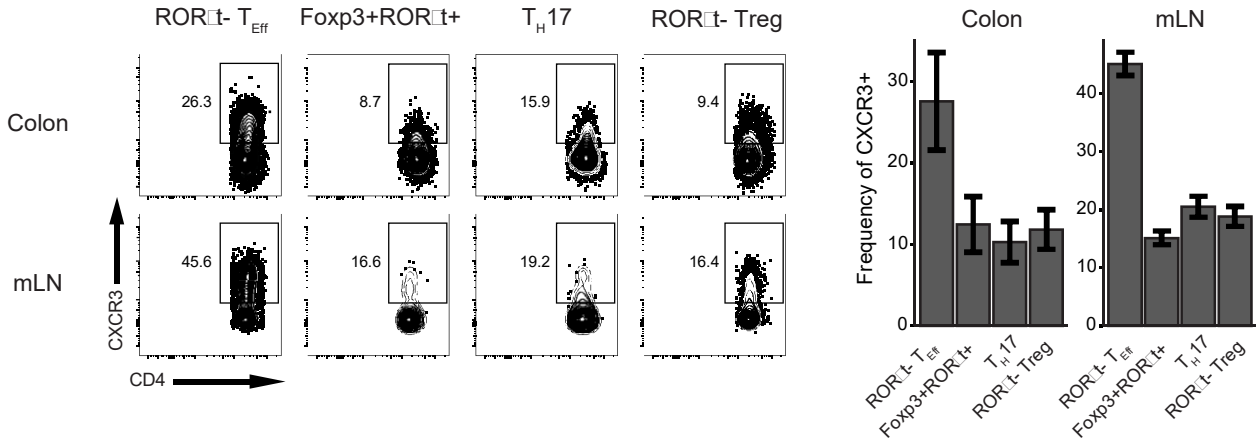


Figure 2.4 – CXCR3 expression in mucosal T cell populations

Frequency of CXCR3+ cells within indicated populations of CD4+ CD44-hi CD62L-lo cells. n=6, each of 2 pooled mice. Error bars = ± 1 s.e.m.

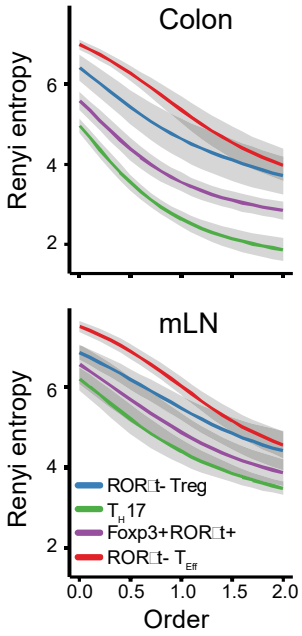


Figure 2.5 – Reduced TCR diversity in Foxp3+RORγt+ T cells

Mean Renyi entropy of indicated populations. Increasing entropy indicates higher population diversity. Increasing order indicates increasing weight on high frequency clones. n=6, each of 2 pooled mice. Error ribbon = ± 1 s.e.m.

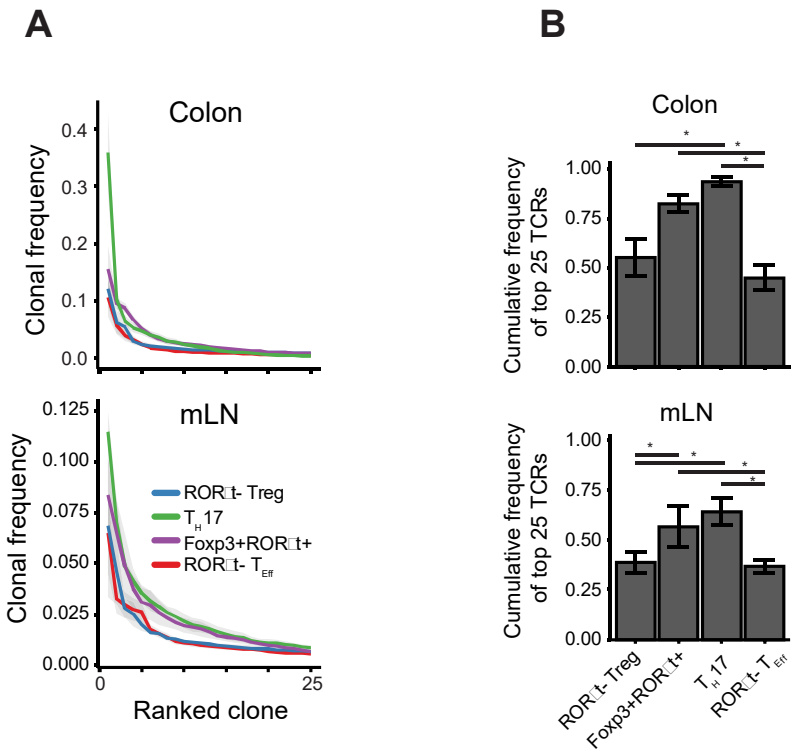


Figure 2.6 – Dominance of most common TCR clones in Foxp3+RORγt+ T cells

(A) Mean clonal frequency of top 25 clones in each population. **(B)** Oligoclonality indicated by average cumulative frequency of top 25 clones. n=6, each of 2 pooled mice. Error bars/ribbon = ± 1 s.e.m. (*) adjusted p-value < 0.05. P-values reflect FDR corrected Wilcox rank sum test.

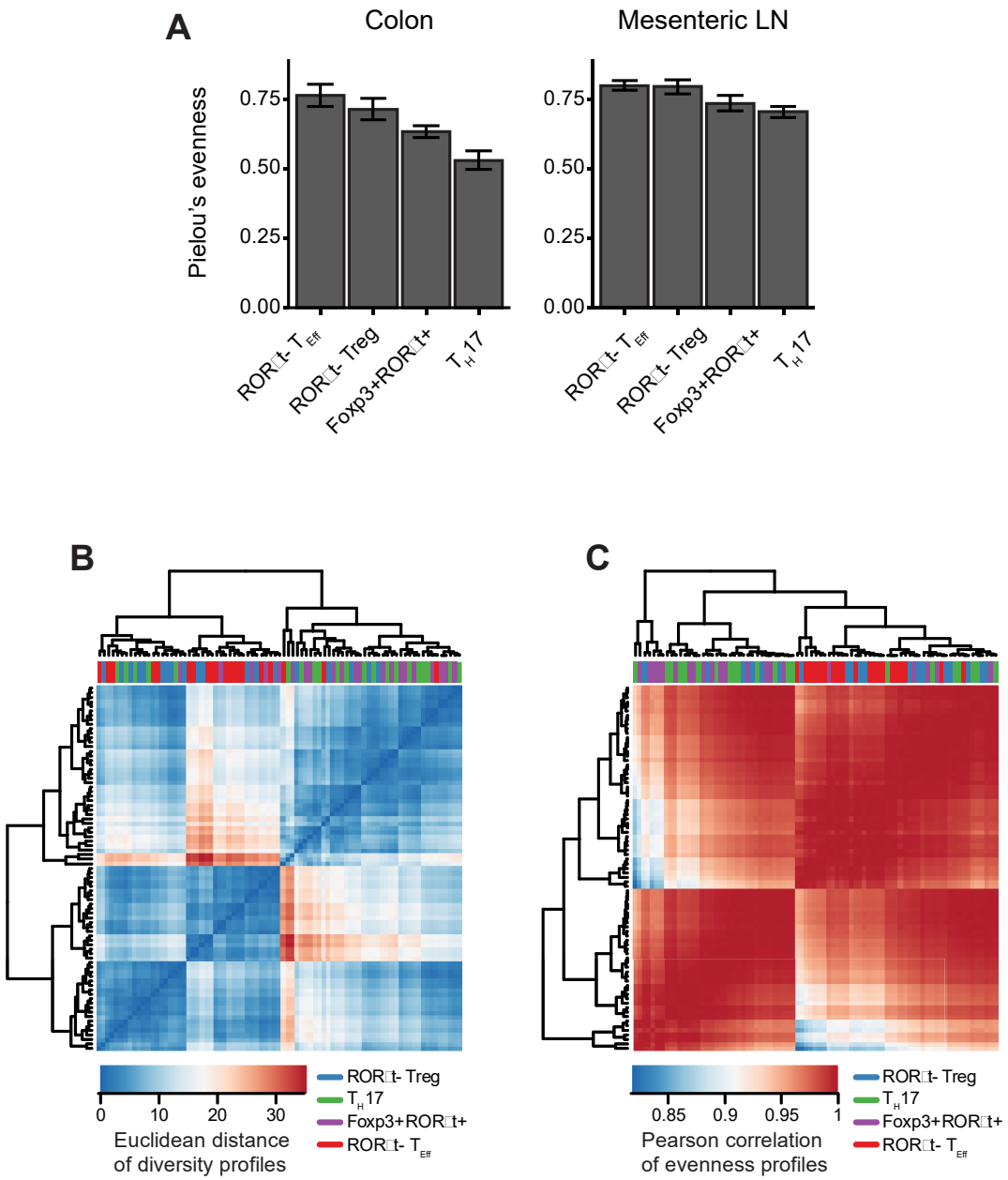


Figure 2.7 – Clustering of Foxp3+RORγt+ and T_H17 T cell populations by TCR repertoire diversity and evenness

(A) Polyclonality indicated by average Pielou's evenness (evenness at order = 1) for each population in indicated tissues. Hierarchical clustering of **(B)** diversity and **(C)** evenness profiles calculated from TCR sequence counts. n=6, each of 2 pooled mice. Error bars = ± 1 s.e.m.

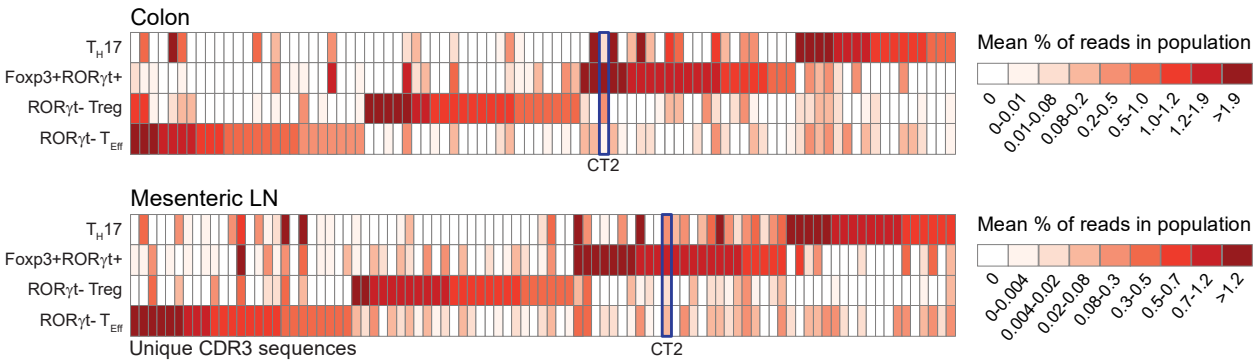


Figure 2.8 – Relative proportions of top 25 TCRs from mucosal T cell populations

Top 25 TCR sequences from each population plotted by mean relative frequency in each population. The TCR CT2 is indicated. n=6, each of 2 pooled mice.

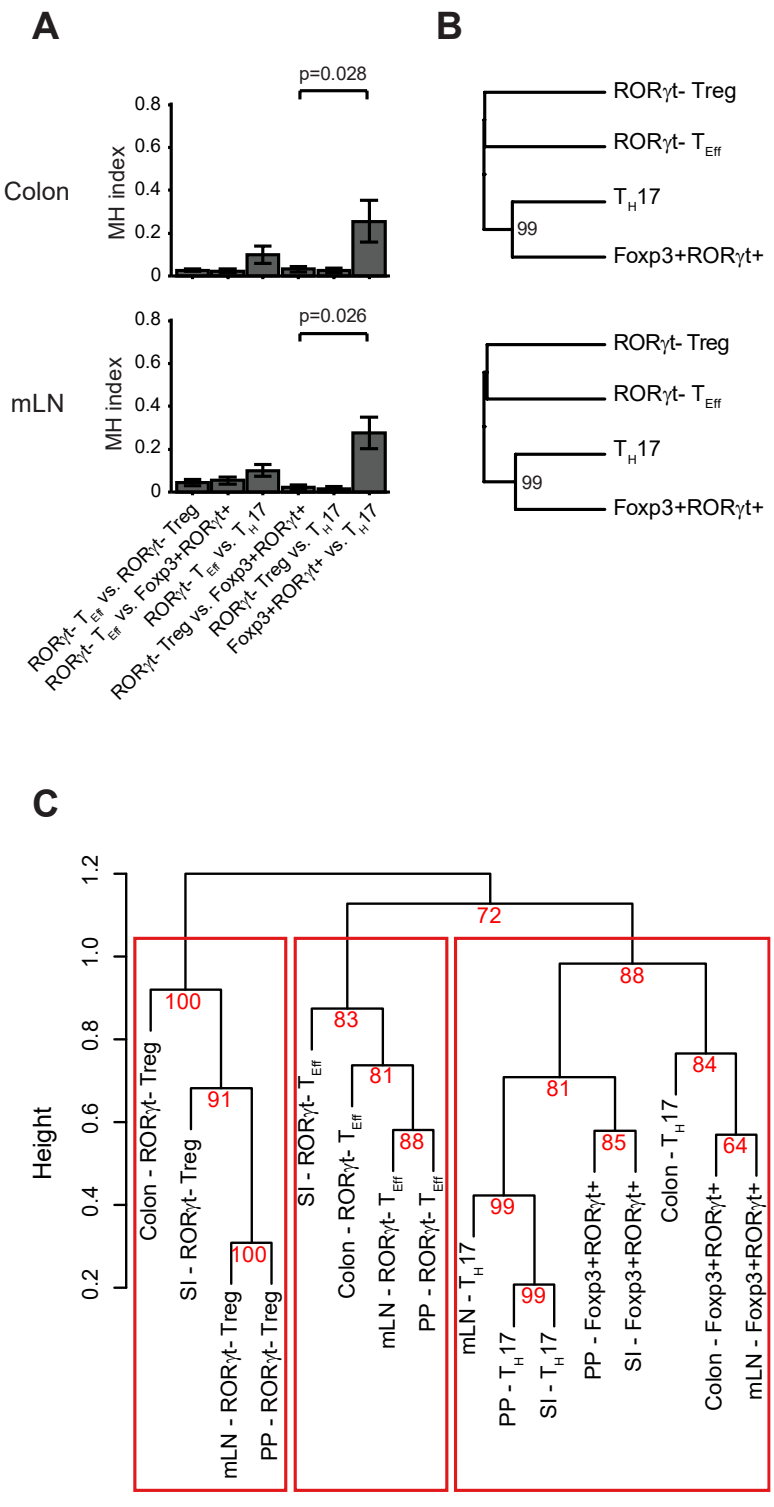

A
B
C
Colon
mLN
MH index
p=0.028
p=0.026
RORγt- Treg
RORγt- T_Eff
T_H17
Foxp3+RORγt+
99
Height
72
100
91
83
81
88
84
85
64
Colon - RORγt- Treg
SI - RORγt- Treg
mLN - RORγt- Treg
PP - RORγt- Treg
SI - RORγt- T_Eff
Colon - RORγt- T_Eff
mLN - RORγt- T_Eff
PP - RORγt- T_Eff
mLN - T_H17
PP - T_H17
SI - T_H17
PP - Foxp3+RORγt+
SI - Foxp3+RORγt+
Colon - T_H17
Colon - Foxp3+RORγt+
mLN - Foxp3+RORγt+

Figure 2.9 – TCR repertoire similarity of mucosal T cell populations

(A) Mean Morisita-Horn (MH) similarity index (0=complete dissimilarity, 1=identical) between indicated populations. **(B)** Dendrogram based on similarity values in A. **(C)** Hierarchical clustering of all sequenced populations and tissues based on Euclidean distance of combined set of Morisita-Horn values from each mouse for a given population and tissue. Dendrogram node values indicates bootstrap reproducibility of branch point. Error bars = ± 1 s.e.m. P-values reflect FDR corrected Wilcox rank sum test.

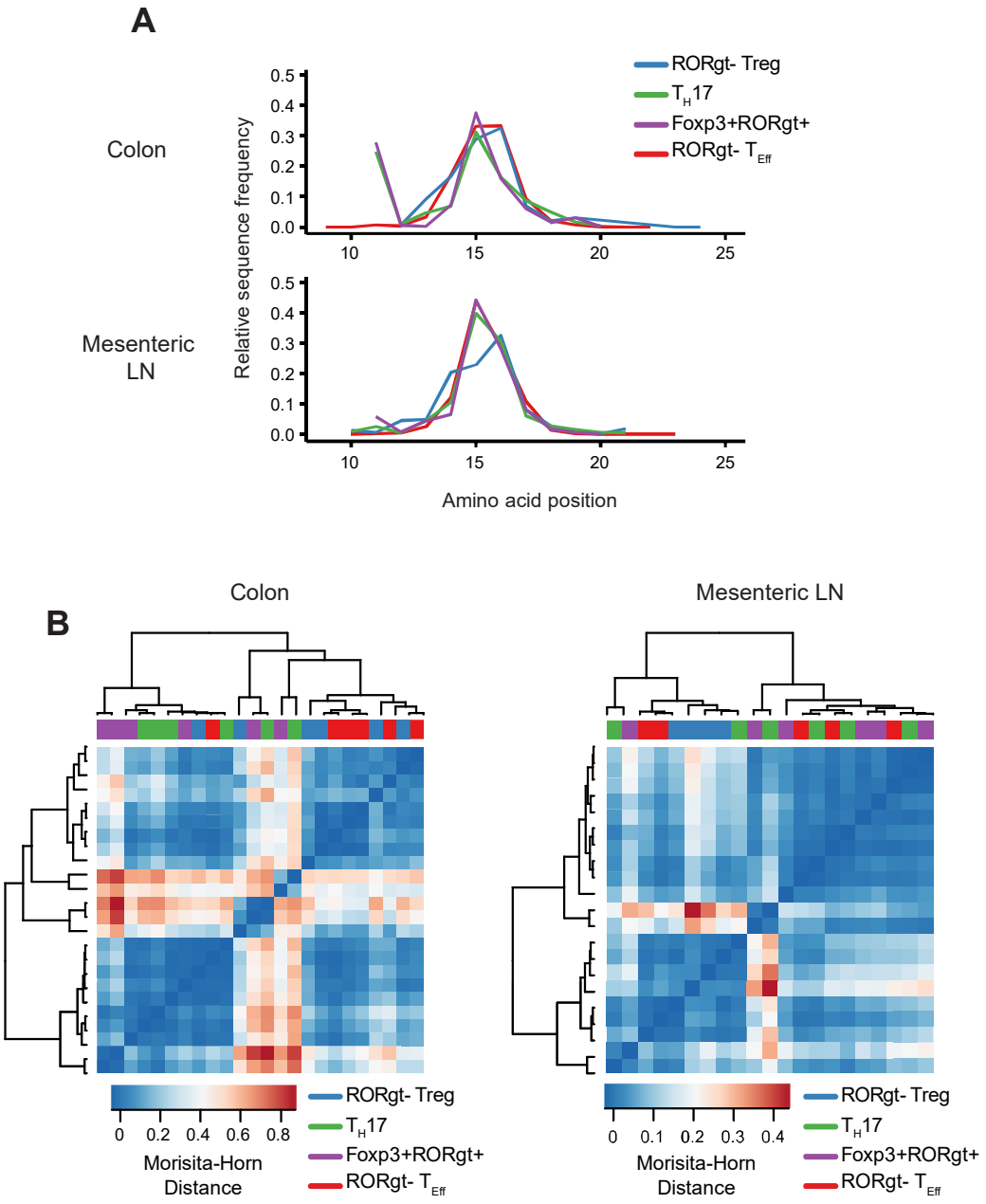


Figure 2.10 - Clustering of Foxp3+RORγt+ and T_H17 T cell populations by CDR3 length

(A) Spectratype analysis of TCRs from sorted populations. Lines represent mean frequency of indicated sequence length for each population. **(B)** Hierarchical clustering of TCR perturbation calculated from MH distance (inverse of MH similarity with 0=identical, 1=complete dissimilarity) of spectratype profiles (set of relative frequency for each sequence length) between individual sequencing samples of indicated populations and tissues. n=6, each of 2 pooled mice.

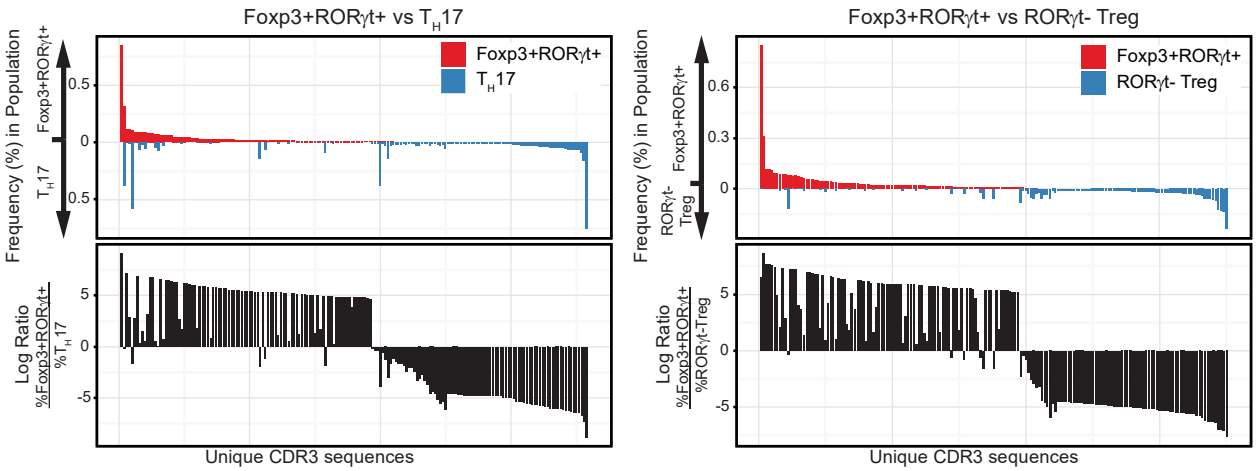


Figure 2.11 – Few, high-frequency clones contribute to Foxp3+RORγt+ and T_H17 repertoire similarity

Mean clonal frequencies (top) or ratio (bottom) of all TCRs with ≥0.01% clonal frequency in either of indicated populations in the colon. n=6, each of 2 pooled mice.

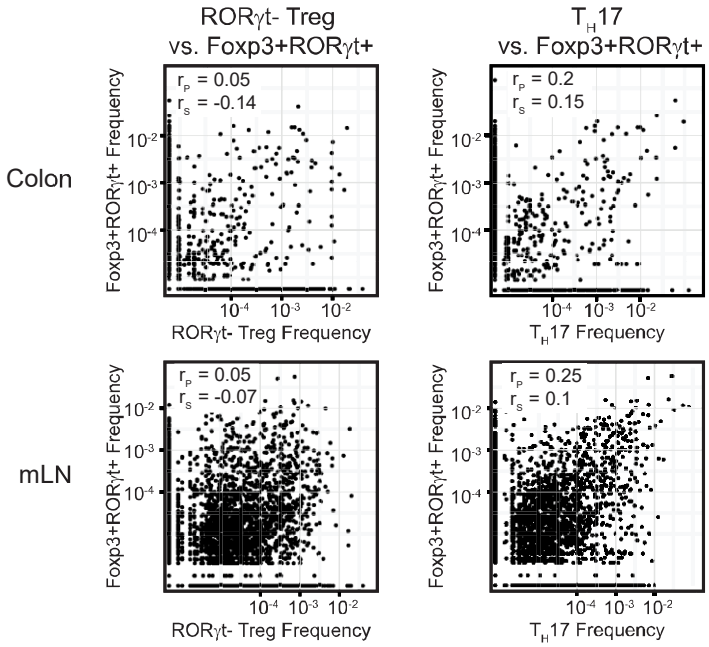


Figure 2.12 – Correlation between TCR frequencies in mucosal T cell populations

Pearson (r_P) and Spearman (r_S) correlation of TCR frequencies between indicated populations. n=6, each of 2 pooled mice.

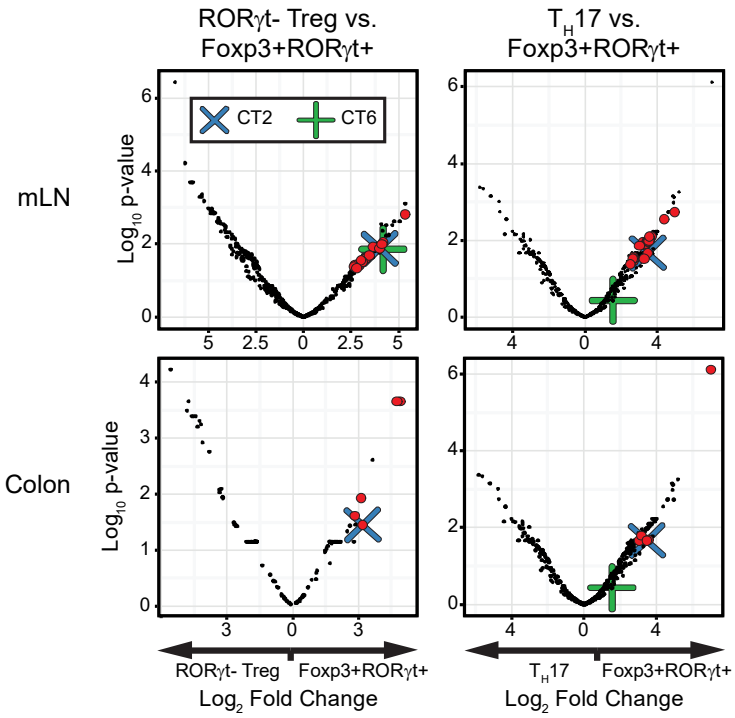


Figure 2.13 – Identification of top TCRs in Foxp3+RORγt+ T cell population

Enrichment of TCRs in Foxp3+RORγt+ population relative to indicated population vs. FDR adjusted p-value. Red dots are TCRs > 2-fold enriched in the Foxp3+RORγt+ population over both Treg and T_H17 populations with an adjusted p-value < 0.05. Blue X indicates TCR CT2. Green cross indicates TCR CT6. n=6, each of 2 pooled mice.

	Average TCR frequency				Foxp3+RORγt+ vs. RORγt- Treg		Foxp3+RORγt+ vs. T_H17	
TCR	RORγt- T_{Eff}	RORγt- Treg	Foxp3+RORγt+	T_H17	log2fold	P-adj	log2fold	P-adj
Colon								
TRAV14-3*01_ FCAASAIWNTGYQNFYFG	1.2E-04	2.2E-03	3.9E-02	5.3E-04	3.186	6.5E-02	3.47	4.1E-02
TRAV14-2*02_ FCAAMGYQNFYFG	3.3E-05	0	4.6E-03	0.0E+00	4.960	4.4E-04	2.58	9.9E-02
TRAV14-2*02_ FCAASWASGYNKLTFG	1.9E-03	2.9E-04	4.5E-03	1.5E-04	2.798	8.8E-02	2.48	9.9E-02
TRAV14-2*02_ FCAASVTGNTGKLIFG	4.0E-04	1.4E-05	3.3E-03	1.0E-04	4.897	4.0E-04	3.55	4.0E-02
TRAV14D-3/DV8*01_ FCAASEYSALGRLHFG	6.6E-05	0	1.8E-03	0	4.755	4.0E-04	7.00	1.4E-06
TRAV14D-3/DV8*01_ FCAASDGNYAQGLTFG	2.5E-04	1.7E-05	2.3E-04	0	1.775	8.8E-02	2.93	4.9E-02
TRAV14-2*02_ FCAASWASGDNKLTFG	4.9E-05	9.5E-06	1.2E-04	0	1.928	8.8E-02	2.64	5.7E-02
TRAV14-2*02_ FCAAMGDQNFYFG	0	0	1.1E-04	0	2.198	8.8E-02	2.04	9.9E-02
TRAV14-3*01_ FCAASGYNQGKLIFG	0	0	1.0E-04	0	2.060	8.8E-02	3.66	1.5E-02
TRAV14-1*01_ FCAASYNNAGAKLTFG	0	0	7.8E-05	0	1.874	8.8E-02	2.02	9.9E-02
Mesenteric LN								
TRAV14-3*01_ FCAASAIWNTGYQNFYFG	4.9E-04	1.9E-04	9.7E-03	1.1E-03	4.00	2.3E-02	3.47	4.1E-02
TRAV14D-3/DV8*02_ FCAASYNNAGAKLTFG	0	0	6.9E-03	1.2E-04	5.34	2.7E-03	4.95	3.5E-03
TRAV14-2*02_ FCAAMGYQNFYFG	1.1E-04	0	1.3E-03	6.0E-04	4.09	1.8E-02	2.58	9.9E-02
TRAV14D-3/DV8*02_ FCAAAGANTGKLTFG	1.3E-04	0	8.4E-04	2.1E-04	4.19	1.5E-02	3.26	5.8E-02
TRAV14D-3/DV8*03_ FCAASASSGSWQLIFG	2.7E-04	0	7.4E-04	1.2E-04	3.75	2.7E-02	2.17	9.9E-02
TRAV14-1*01_ FCAASWASGYNKLTFG	6.4E-05	3.8E-05	5.4E-04	1.0E-04	2.88	7.2E-02	2.01	9.9E-02
TRAV14D-3/DV8*03_ FCAASEYSALGRLHFG	0	4.4E-05	5.3E-04	0.0E+00	2.66	9.7E-02	5.18	1.0E-03
TRAV14D-3/DV8*02_ FCAASRGGSAKLIFG	0	0	4.6E-04	0.0E+00	4.11	1.8E-02	4.36	5.3E-03
TRAV14-2*02_ FCAASDGSNYQLIWG	2.7E-05	0	3.2E-04	0.0E+00	3.21	4.6E-02	3.33	2.4E-02

Figure 2.14 – Table of top Foxp3+RORγt+ TCRα sequences

Top 10 Foxp3+RORγt+ TCRα sequences from indicated tissues, sorted by frequency, meeting criteria of ≥1 log2fold enrichment and ≥0.1 false discovery rate adjusted p-value for comparison with both the RORγt- Treg and T_H17 populations, calculated by DESeq2. Bold indicates CT2 sequence

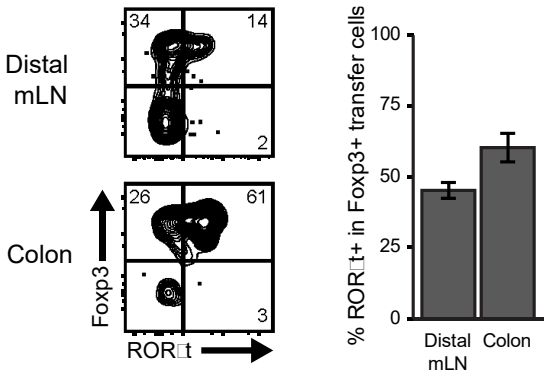


Figure 2.15 – CT2 efficiently adopts a Foxp3+RORγt+ phenotype

Phenotype of transferred CT2-TCRtg CD4+ cells in indicated tissues 3 weeks after transfer of $5x10^4$ naïve cells into WT hosts. n≥5 for each group. Data representative of ≥2 independent experiments. Error bars = ±1 s.e.m.

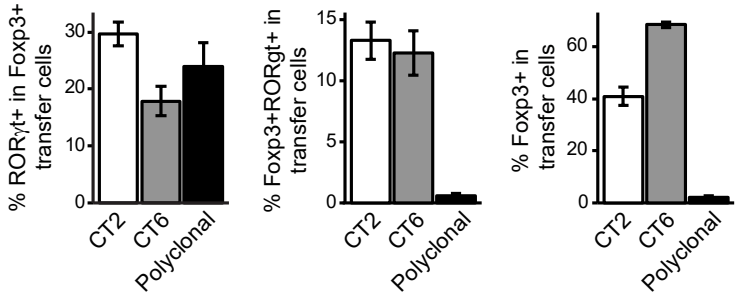


Figure 2.16 – CT2 Foxp3+RORγt+ T cell differentiation is TCR dependent

Frequencies of indicated populations from distal mLN of WT host mice 3 weeks after transfer of $5x10^4$ naïve cells. n≥5 for each group. Data representative of ≥2 independent experiments. Error bars = ±1 s.e.m

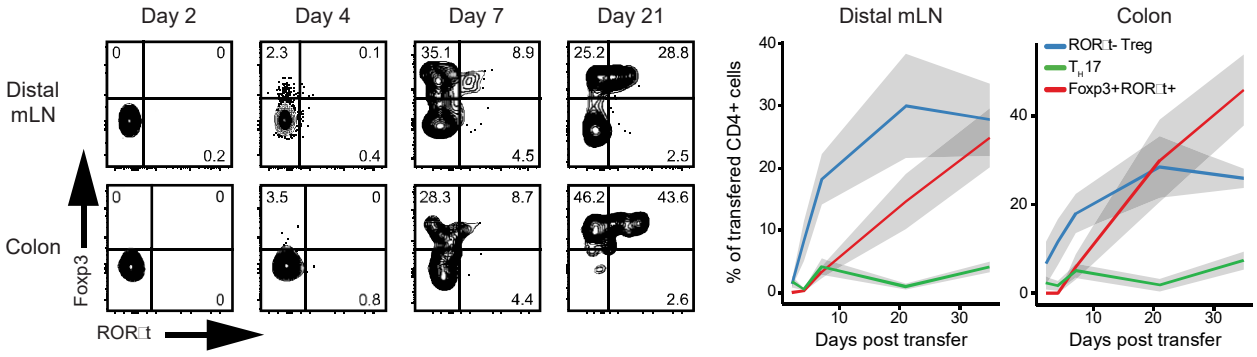


Figure 2.17 – Naïve CT2 T cells adopt a Foxp3+RORγt+ phenotype via a RORγt- Treg cell intermediate

Phenotype of transferred CD4+ cells at indicated time points after transfer of $5x10^4$ naïve CT2 TCRtg cells into WT hosts. n≥5 for each group/time point. Data representative of ≥2 independent experiments. Error ribbon = ±1 s.e.m.

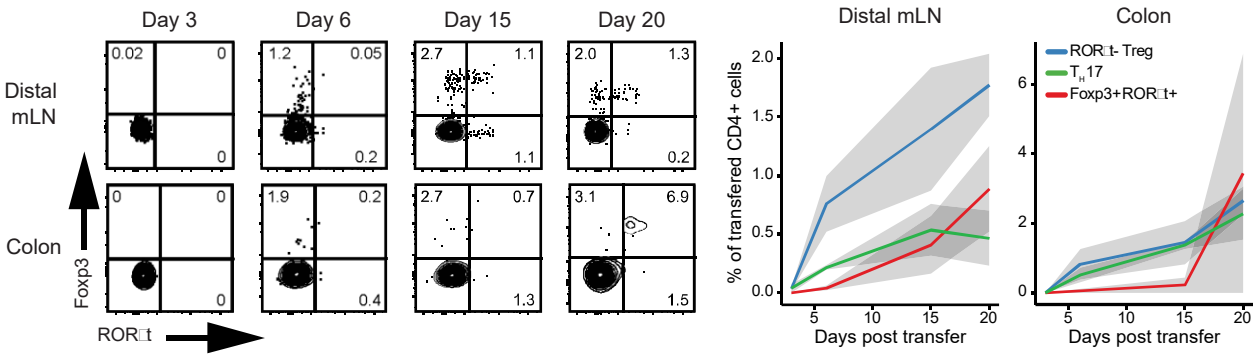


Figure 2.18 – Naïve polyclonal T cells adopt a Foxp3+RORγt+ phenotype with limited prior T_H17 differentiation

Phenotype of transferred CD4+ cells at indicated time points after transfer of $5x10^4$ naïve polyclonal cells into WT hosts. n≥5 for each group/time point. Data representative of ≥2 independent experiments. Error ribbon = ±1 s.e.m.

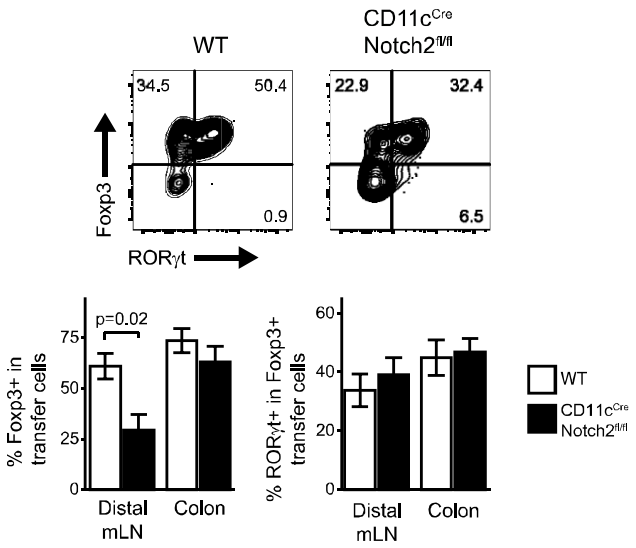


Figure 2.19 – RORγt+ Treg cell development is not significantly affected in the absence of Notch2-expressing APCs

Phenotype of CT2 TCRtg CD4+ cells in Notch2+ APC-deficient host mice 3 weeks after transfer of $5x10^4$ naïve cells. n≥7 for each group. Data representative of ≥2 independent experiments. Error bars = ±1 s.e.m.

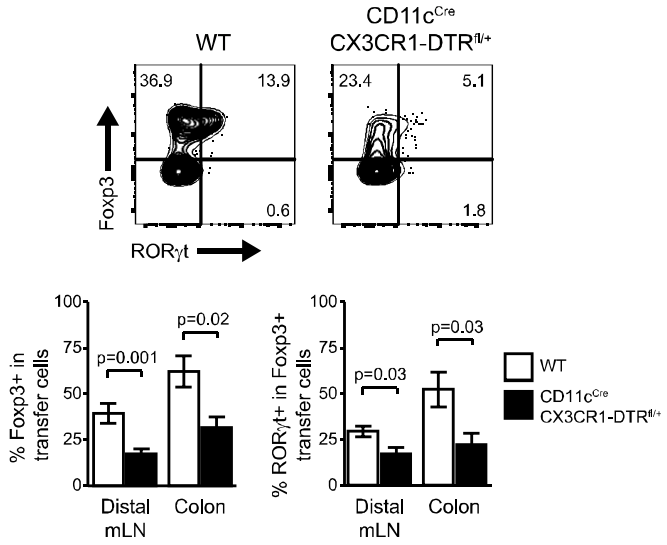


Figure 2.20 - RORγt+ Treg cell development is significantly impaired in the absence of CX3CR1-expressing APCs

Phenotype of CT2 TCRtg CD4+ cells in CXC3R1-APC deficient host mice 10 days after transfer of $5x10^4$ naïve cells. For deletion of CX3CR1+ APCs, DT was administered (20ng/g) for two days prior to transfer and (5ng/g) every other day after transfer until analysis. n≥7 for each group. Data representative of ≥2 independent experiments. Error bars = ±1 s.e.m.

Chapter 3: Antigen-specific exacerbation of CNS inflammation by clonally expanded γδ T cells

3.1 Abstract

γδ T cells represent a unique intersection of adaptive and innate immunity. While capable of responding rapidly to inflammatory stimuli in a manner reminiscent of many innate immune cells, γδ T cells express a somatically rearranged antigen receptor similar to B and T lymphocytes. Yet, despite its evolutionary conservation, the importance of a diversified γδ TCR is unclear. Here, we describe a fixed-TCRδ system that allows high-throughput analysis of the paired chain γδ TCR repertoire *in vivo*. Using this system, we show that γδ T cells with diversified T cell receptors clonally expand during experimental autoimmune encephalomyelitis (EAE). Moreover, these expanded clones directly contribute to disease pathogenesis, as a single γδ T cell clone enriched during EAE is capable of exacerbating disease severity in a TCR-specific manner.

3.2 Introduction

Importance of the γδ TCR

The importance of the TCR for the development and function of αβ T cells is well understood. αβ T cells require stimulation through their TCR during thymic maturation in order to proceed through several developmental checkpoints. Moreover, all conventional αβ T cells exit the thymus displaying a naïve phenotype and require antigen-mediated TCR stimulation to clonally expand, activate, and exert their various effector functions throughout the course of an immune response.

However, for γδ T cells the importance of TCR is much less clear. While TCR signaling does play a role in thymic development (Kreslavsky et al., 2008), it remains to be shown if this process depends on stimulation via idiotype-specific ligands as is required for αβ T cell positive and negative section. Moreover, in the thymus, γδ T cells are capable of acquiring a pre-activated phenotype normally associated with antigen-experienced peripheral cells. This allows cells to respond directly to inflammatory cytokines in the absence of antigen. For example, "natural" IL-17 producing γδ T (nTγδ17) cells leave the thymus with a CD44-hi CD62-lo IL-1R+ IL-23R+ phenotype (Jensen et al., 2008) and can produce IL-17 within several hours of inflammatory cytokine stimulation (Shibata et al., 2007), compared to the several days needed for IL-17 production by T_H17 αβ cells or γδ T cells that exit the thymus with a CD44-lo CD62-hi phenotype (Zeng et al., 2012, 2014). In addition, the γδ TCR repertoire contains a high frequency of TCR sequences arranged in germline configuration, suggesting limited pressure for extensive receptor diversification. Together, this has led many to suggest that the γδ TCR functions merely as a pattern recognition receptor or may even be irrelevant to the function of mature γδ T cells (Kapsenberg, 2009).

However, several lines of evidence argue against a marginalized view of the γδ TCR. Notably, though γδ T cells expressing a pre-activated phenotype can produce effector molecules in response to inflammatory cytokines alone, this response is significantly blunted by inhibitors of TCR signaling (Zeng et al., 2012). Similarly, genetic defects in components of TCR signaling can impair the function of pre-activated γδ T cells, while defects in the signaling pathways of several pattern recognition receptors do not (Duan et al., 2010; Wencker et al., 2014). Finally, unlike what would be expected of an irrelevant or vestigial gene, the primate *TRG* locus shows a high rate of evolutionary change and appears to be undergoing selection for diversifying mutations and against inactivating mutations (Kazen and Adams, 2011).

Diversity of the γδ TCR repertoire

The γδ TCR repertoire has an extremely high theoretical diversity (Davis and Bjorkman, 1988), yet is notably restricted *in vivo*, even compared to other lymphocyte lineages. However, rather than a general property of the entire γδ TCR repertoire, this reduced diversity appears to be due to an averaging of γδ T cells subsets with high TCR diversity and populations with low or even monoclonal diversity. On one extreme of this spectrum are epidermal dendritic T cells (DETCs), a subset of Vγ5+ γδ T cells that bear a single germline configuration TCR sequence (Asarnow et al., 1988). Yet despite its highly restricted repertoire, the DETC TCR appears to have functional importance, as it is required for keratinocyte-mediated activation of DETCs (Havran et al., 2016). Moreover, DETCs exhibit a sustained level of TCR signaling at steady state and the sub-cellular localization of this signaling is redirected in response to nearby epidermal wounds (Chodaczek et al., 2012).

In contrast to DETCs, many populations of γδ T cells possess diverse TCR repertoires, particularly those that reside in peripheral lymphoid tissue, such as the spleen and lymph nodes. For

example, the TCR repertoire of Vγ4+Vδ4+ γδ T cells is moderately diverse in naïve mice, with the highest frequency TCR clones representing approximately 5-20% of the entire repertoire (Roark et al., 2007). Moreover, upon immune challenge in collagen induced arthritis (CIA), the TCR repertoire of this population of cells becomes substantially more oligoclonal, with the top frequency clones representing 20-50% of the entire repertoire. Thus, the γδ TCR repertoire appears to span a range of diversity, with the TCR able to directly contribute to function at both ends of this spectrum.

γδ T cell memory

How then does a diverse antigen receptor repertoire contribute to the function of T cells? For αβ T cells, it is believed that the purpose of a diverse TCR repertoire is to provide a sufficiently broad range of antigen-specific lymphocytes so as to be capable of recognizing novel immune challenges and establish subsequent immune memory. Initially, γδ T cells appeared to lack the ability to generate memory. For example, though γδ T cells contribute to the protective immune response against West Nile virus (WNV), there is no difference between the response of γδ T cells during primary and secondary challenge (Wang et al., 2006). Interestingly, while having no memory response of their own in WNV infection, γδ T cells do play a role in the ability of CD8+ αβ T cells to form WNV-specific immune memory. As such, γδ T cell-deficient mice do exhibit decreased resistance to secondary WNV infection, though this is a direct consequence of impaired CD8+ T cell memory (Wang et al., 2006).

However, recent evidence has led investigators to reexamine the ability of γδ T cells to form memory responses. In the imiquimod-induced model of psoriasis, Vγ4Vδ4 γδ T cells expand in draining lymph nodes and eventually traffic back to dermal tissue. Secondary challenge results in enhanced proliferation of these dermal-resident cells. This memory response is intrinsic to γδ T cells, as γδ T cells transferred from imiquimod-sensitized mice into healthy hosts still demonstrate

an enhanced response when the hosts are subsequently challenged (Ramírez-Valle et al., 2015). Similarly, *S. aureus* infection results in an expansion of Vγ6+ γδ T cells, which further expand upon secondary challenge. Moreover, transfer of expanded γδ T cells from *S. aureus* challenged mice to naïve hosts provides a small protective advantage when the hosts are similarly immunized (Murphy et al., 2014). Finally, γδ T cells are also capable of contributing to the memory response to oral *Listeria* infection and this response correlates with the specific expansion of Vγ6+ γδ T cells (Sheridan et al., 2013).

γδ TCR specificity in clonal expansion

However, whether this memory response can be attributed directly to antigen recognition by the γδ TCR remains to be shown. Certain γδ T cell phenotypes, such as the nTγδ17 cell subset, are imprinted in the thymus and correlate with specific Vγ and Vδ containing TCRs, particularly those in germ line conformation (Shibata et al., 2014). Thus, it is possible that the TCR only contributes to the thymic development of such γδ T cell clones and their expansion during an immune response is dictated by the imprinted phenotype, rather than the TCR itself.

By comparison, the involvement of the TCR in clonal expansion and memory of αβ T cells has been well described. The αβ TCR 2D2, identified based on its expansion during experimental autoimmune encephalomyelitis (EAE), is an illustrative example. T cells that transgenically express 2D2 can directly recognize myelin oligodendrocyte (MOG) peptide and contribute towards the pathogenesis of both spontaneous and transferred EAE (Bettelli et al., 2003).

Though limited, there is some evidence that suggests expanded γδ TCR clones are capable of directly contributing to an immune response. In the imiquimod model of psoriasis discussed above, Vγ4+Vδ4+ γδ T cells specifically involved in the memory response upregulate *Nur77*, while Vγ4+Vδ4- γδ T cells, which are not involved in the memory response, do not. Nur77 is a

transcription factor whose expression has previously been described to correlate with stimulation of the TCR (Moran et al., 2011), suggesting that only the cells involved in imiquimod induced expansion and memory are receiving TCR stimulation. Notably, the Vγ4+Vδ4+ γδ T cell fraction is the same population to clonally expand in collagen induced arthritis (CIA) (Roark et al., 2007). Moreover, in the γδ T cell memory response to oral listeria, internalization of the Vγ6 TCR results in loss of γδ T cell memory, while internalize of other γδ TCRs has no effect (Sheridan et al., 2013). However, evidence of a specific T cell clone like 2D2 that expands in response to cognate antigen and actively contributes to the associated immune response is still lacking for γδ T cells.

γδ T cells in EAE

In looking for TCR mediated expansion and function, there are many possible immune models with reported γδ T cell involvement to investigate. However, one of the clearest examples of γδ T cell contribution to an immune phenotype can be found in EAE, a mouse model of multiple sclerosis (MS). In multiple variations of the EAE model, TCRδ-deficient mice, which lack all γδ T cells, exhibit decreased disease severity (Blink and Miller, 2009; Petermann et al., 2010; Spahn et al., 1999). Indeed, in the hands of some investigators, TCRδ-deficient mice are completely resistant to developing EAE. Moreover, enriched numbers of γδ T cells are readily found in the CNS of WT mice with active EAE. These observations are mirrored in human MS patients, where γδ T cells can be detected in CNS plaques (Freedman et al., 1991). Moreover, γδ T cells isolated from these lesions are capable of lysing oligodendrocytes *in vitro*. Together, these observations suggest that γδ T cells can actively contribute to demyelinating pathology.

γδ T cell may contribute to EAE in several ways. IL-17 is an important component of EAE pathogenesis (Komiyama et al., 2006; Yang et al., 2008) and γδ T cells are a significant, if not the principal source of IL-17 during EAE (Price et al., 2012). In addition, γδ T cells contribute several

other pro-inflammatory cytokines during EAE, as the levels of IL-1, IL-6, TNF, and IFNγ, are diminished in γδ T cell deficient mice (Rajan et al., 1998). γδ T cells may also contribute to the pathogenicity of αβ T cells, as γδ T cell deficient mice have reduced αβ T cell CNS infiltration during EAE (Spahn et al., 1999). Even upon transfer with autoreactive αβ T cells, TCRδ-deficient mice still exhibit decreased EAE disease severity (Odyniec et al., 2004), although conflicting data has also been reported (Clark and Lingenheld, 1998).

However, not all evidence is in agreement regarding the pathological role for γδ T cells in EAE and MS. For instance, at least one group has reported that antibody-mediated depletion of γδ T cells results in enhanced EAE disease severity. However, in a more recent study of antibody-based depletion of γδ T cells using *TCRD-GFP* mice, Blink *et al.* demonstrated that several anti-γδ antibody clones (UC7, UC3, and 2.11) actually result in cellular activation and downregulation of cell surface TCR molecules (Blink et al., 2014). Since the γδ TCR is the only reliable way to detect γδ T cells by cell surface staining, this would give the erroneous appearance of a loss of γδ T cells, when in truth, only the means to detect them by flow cytometry has been lost. Moreover, these investigators showed that γδ T cells can be categorized into at least two subsets in relation to their role in EAE pathogenesis. As indicated by anti-Vγ4 antibody-mediated activation, Blink *et al.* showed that Vγ4+ γδ T cells enhance disease pathology. Conversely, using anti-Vγ1 antibodies, they showed that Vγ1+ γδ T cell activation can constrain EAE severity. These opposing functions within the γδ population could explain why different investigators could observe different outcomes when manipulating the bulk γδ T cell population in EAE.

Despite a clear role for γδ T cells in EAE and MS, the role of antigen specificity in disease pathology remains uncertain. The limited studies of the γδ TCR repertoire during EAE and MS have consistently reported significant TCR oligoclonality, whether it be in CNS tissue from EAE-immunized mice (Olive, 1995) or the plaques and CSF of MS patients (Shimonkevitz et al., 1993;

Wucherpfennig, 1992). However, of these oligoclonal TCR sequences, many are found in a germ-line VDJ conformation, suggesting little diversification of the TCRs involved. Indicative of this trend, one study noted that all CNS sequences detected were composed of a monoclonal Vγ6-Jg1 germline TCR rearrangement, although only Vγ6 TCRs were sequenced and no TCRδ sequencing was performed (Olive, 1995). This raises the possibility that the clonal expansion seen in EAE and MS might not represent antigen specificity, but instead antigen-independent proliferation of an already monoclonal population. However, as the sequencing methodologies used in EAE and MS repertoire studies have substantial limitations, the role of the γδ TCR in these immune processes remains an open question.

Limitations of current γδ TCR data

One of the major limitations of currently available γδ TCR repertoire data comes from the technology used to obtain it. The majority of our understanding of the γδ TCR repertoire comes from early studies relying on Sanger sequencing, where the number of DNA reads obtained is limited to the number of individual sequencing reactions carried out. Typical repertoire sequencing studies of this kind analyze less than 100 DNA reads (Elliott et al., 1988; Olive, 1995, 1996; Takagaki et al., 1989) and, as such, even if taken from only a single sample, are unlikely to detect TCR sequences that represent less than 1% of the total repertoire. Although the γδ T cell repertoire is more restricted, the average αβ TCR frequency is approximately 0.001% (Casrouge et al., 2000), suggesting that previous studies may be drastically undersampling the γδ TCR repertoire. By comparison, deep-sequencing methodologies can yield millions of DNA reads, offering a much more comprehensive picture of the γδ T cell repertoire. However, compared to the study of the αβ TCR repertoire, the application of deep-sequencing to the γδ TCR repertoire has been extremely limited (Kashani et al., 2015; Schumacher et al., 2014; Sherwood et al., 2011; Wei et al., 2015).

Another major challenge in studying the γδ TCR repertoire comes from the fact that the two receptor chains that jointly contribute to TCR antigen specificity are encoded by separate genes, *TRG* and *TRD*. As a result, the *in vivo* pairing of TCRγ and TCRδ sequences cannot be determined from transcripts obtained from a bulk population of γδ T cells. To date, repertoire studies have either had to acknowledge this as a limitation to the data obtained from bulk samples or sequence individual cells. Early approaches of single cell sequencing relied on the generation of γδ T cell hybridomas, from which individual clones could be cultured *in vitro* and then sequenced (Shin et al., 2005). Current methods now incorporate automated single cell sorting, which increases the number of total cells that can be analyzed in a single cell sequencing approach (Wei et al., 2015). However, both strategies are still limited to the number of cells that can be independently processed, usually on the order of tens to hundreds.

In order to address this same problem in the context of the αβ TCR repertoire, our lab and others have previously utilized transgenic mice in which one chain of the TCR remains fixed, while the second chain is left free to rearrange (Correia-Neves et al., 2001; Hsieh et al., 2006; Lathrop et al., 2011; Merkenschlager et al., 1994). Sequences from the freely rearranging TCR chain can then be obtained from a bulk sample and pairing to the sequence of the fixed TCR chain can be inferred. As such, fixed TCR systems represent a way to obtain paired-chain sequence data without the need for single cell analysis. When combined with a deep-sequencing platform, this has provided a significantly more comprehensive view of the αβ TCR repertoire. Yet, such methods have yet to be applied to the study of the γδ TCR repertoire.

Here we utilize a fixed TCRδ-retroviral bone marrow chimera system to investigate the response of the γδ TCR repertoire to inflammatory stimuli and the role this response plays in the resulting immune response. Using EAE, we show that the γδ TCR repertoire can shift significantly

in response to inflammation. Moreover, using a TCR clone isolated during this response, we show that γδ T cells can contribute to immune pathology in a TCR dependent manner.

3.3 Materials and Methods

Cell isolation and flow cytometry

IELs were isolated by digesting intestinal tissue in RPMI media containing 3% FBS, 20mM HEPES, 1mM DTT, and 50mM EDTA for 20min at 37º C. Additional washes in RPMI + 22.5mM EDTA was done to completely remove IELs. Suspended cells were filtered through a 40μm filter prior to use. CNS lymphocyte isolation was performed as described (Lin et al., 2016). Briefly, mice were perfused with PBS and the spinal cord and brain were dissected. CNS tissue was then digested with 500μg/mL collagenase I (Sigma-Aldrich) and 10μg/mL DNAse I (Sigma-Aldrich) in the presence of 0.1μg/mL TLCK trypsin inhibitor (Sigma-Aldrich) and 10mM HEPES in HBSS followed by enrichment by Percoll gradient. Fluorescently conjugated antibodies were purchased from Biolegend and eBioscience. TCRγδ (GL3) antibodies were used at 0.8μg/mL and CD3 (145-2C11) antibodies were used at 2μg/mL. Samples were sorted and analyzed with a FACSAria IIu (BD) and data was processed in FlowJo (Treestar).

TCR sequencing

cDNA was prepared from RNA of sorted populations using gene specific, reverse transcription primers. cDNA libraries were then prepared by multiplex PCR containing forward primers for each *TRV* and reverse primers for each TRC. 250-cycle paired-end sequences were then obtained using Illumina MiSeq at the Washington University Genome Sequencing Center. *TRV*, *TRJ*, and *TRC* segments were then identified using a custom BLAST program incorporating sequence data from IMGT (Lefranc, 2003).

TCR retroviral bone marrow chimeras

Retrogenic mice were generated as described previously (Holst et al., 2006; Lee et al., 2012a). Briefly, TCRβ- x TCRδ- (for fixed-TCRδ retrogenic mice) or RAG- (for full TCRγδ retrogenic mice) bone marrow was obtained and retrovirally transduced with the indicated TCR-IRES-Thy1.1 expression vector. Lethally irradiated (950 rads) mice were then injected with transduced bone marrow and congenically distinguished filler bone marrow as indicated by the experiment at a 4:1 ratio. At 6-8 weeks mice were utilized for experiments.

EAE immunization

EAE immunization was performed as described (Lin et al., 2016). Briefly, mice were immunized with 100μg of MOG_{35-55} (CS Bio Co.) in CFA s.c. Mice were additionally immunized with 300ng of pertussis toxin i.p. on days 0 and 2. For sorting or FACS analysis, mice were analyzed at d12 post-immunization.

T cell hybridoma assays

Hybridoma cells expressing GFP under an NFAT promoter were retrovirally transduced with D3G1-IRES-Thy1.1. Hybridomas were then stimulated with plate bound anti-CD3 antibody overnight and assessed for GFP expression by flow cytometry.

Statistical analysis

Diversity profiles were generated using Renyi entropy values with alpha/order values ranging from 0 (natural logarithm of species richness) through 2 (natural logarithm of the inverse Simpson index) (Jost, 2006; Pacholczyk et al., 2007). This includes alpha = 1, which represents the commonly used Shannon entropy. Coverage was calculated as described (Chao and Jost, 2012). For multiple

comparisons of individual TCR enrichment between samples, Benjamini-Hochberg false discovery rate adjusted p-values were used. All statistical analysis was performed in R (v3.3.0) with the use of the vegan (v2.3-5, diversity and similarity analysis), and DESeq2 (v1.12.0, differential TCR usage) packages. Mann-Whitney U or Kruskal-Wallis with post-hoc Dunn's tests were used for between group analysis.

3.4 Results

Deep sequencing of the TCRγ and TCRδ repertoires

In order to comprehensively investigate the γδ TCR repertoire, we designed a multiplex DNA sequencing system capable of detecting all γδ TCR transcripts. TCR transcripts that have successfully undergone VDJ rearrangement and mRNA splicing can utilize one of several V-gene segments and, in the case of TCRγ transcripts, multiple C-gene segments (Lefranc, 2003). In order to account for all possible combinations of these gene segments contributing to the 3' and 5' ends of a TCR transcript, our multiplex system included multiple forward and reverse primers, each specific to a corresponding V- or C-gene segment, respectively. TCR transcripts were PCR amplified using these primers and sequenced using the high-throughput Illumina MiSeq platform.

In order to test the ability of our multiplex amplification to successfully amplify all possible TCRγδ gene families, we first performed sequencing experiments utilizing each V-gene-specific primer individually. Notably, the TCRδ locus is located within the larger TCRα locus and TCRδ transcripts can utilize a subset of TCRα V-gene (*TRAV*) segments in addition to *TRDV* segments (Lefranc, 2003). Therefore, our TCRδ sequencing workflow also included several *TRAV* specific primers. Overall, *TRDV* and *TRAV* containing sequences were highly specific to the corresponding V-gene primer used (**Fig. 3.1**). The minimal cross reactivity observed with some primers was largely restricted to those TCRδ transcripts utilizing *TRAV* V-segments. This is not surprising as, in general, TRAV gene families show closer homology from one to the other than *TRGV* gene families.

Our TCRγ primers we also able to detect sequences utilizing all *TRGV* genes segments (**Fig. 3.1**). However, due to the high sequence homology between *TRGV1*, *TRGV2*, and *TRGV3* gene families, primers specific to these segments exhibited a substantial degree of cross reactivity. Unlike

the TCRδ locus, which only contains one C-gene, the TCRγ gamma locus contains three Cg genes and the use of a particular *TRGC* segment is reported to correspond directly to a subset of TRGV and *TRGJ* genes. Our sequencing system was able to detect transcripts containing each of the three TRGC segments and confirm a unique pattern of upstream VJ-segment usage associated with each C-region. Specifically, *TRGC4* associates with the *TRGJ4* and *TRGV1* gene segments, *TRGC2* associates with the *TRGJ2* and *TRGV2* genes, and *TRGC1* associates with the *TRGJ1* J-segment and the *TRGV4*, *5*, *6*, and *7* V-segments (**Fig. 3.2**).

Across all experiments described in this study, our sequencing approach was able to detect a high number of unique TCRδ and TCRγ CDR3 sequences. As discussed previously, the use of Sanger sequencing has limited the vast majority of γδ TCR repertoire studies to typically fewer than 100 sequence reads, often equating to a considerably smaller number of unique CDR3 amino acid sequences. Yet in our data, we detect approximately 400,000 TCRδ CDR3 sequences and close to 25,000 TCRδ CDR3 sequences (**Fig. 3.3**), demonstrating the degree of improved repertoire coverage we obtain with a deep sequencing approach.

TRV usage and diversity

We next sought to validate our sequence data against several well described aspects of the γδ TCR repertoire. One of the earliest described features of the γδ TCR repertoire is the restricted use of certain Vγ and Vδ chains in different tissues (Vantourout and Hayday, 2013). Indeed, we observed similar biases in TRV gene usage based on the tissue sequenced, with several well characterized patters (**Fig. 3.4**). For instance, the skin is one of the only tissues with notable *TRDV4* or *TRGV5* expression, both associated with the TCR of highly restricted DETC cells.

In addition to *TRV* gene usage, γδ TCR diversity is also reported to vary by tissue and *TRV* gene families. Interestingly, we found that, in general, TCR diversity if relatively uniform across

tissues within a particular *TRV* gene family (**Fig. 3.5**). As suggested by the data described above, it instead appears that it is the relative proportion of γδ T cells bearing these different *TRV* genes that determines the apparent repertoire restriction of the tissue as a whole. We also noted that, overall, TCRδ diversity is greater than TCRγ diversity. This is not unexpected, as TCRδ transcripts can incorporate a second D-gene segment, creating a second opportunity for diversifying N- and P-nucleotides at the joint region (Vantourout and Hayday, 2013).

This observation is also reflected in the overall length of γδ TCR sequences, as the incorporation of a second D-gene segment would be expected to lead to longer TCRδ transcripts. As a result, the CDR3 loop of TCRδ chains often protrudes from the surface of the TCR structure and may contribute to the γδ TCR's ability to bind certain ligands in an immunoglobulin-like fashion (Chien et al., 2014). In support of this, we observed that TCRδ CDR3 sequences are typically longer than TCRγ CDR3 sequences (**Fig. 3.6**). Interestingly, with the exceptions of *TRDV4* and *TRGV5*, the TCRγ and TCRδ chain V-genes could be differentiated based on the uniformity of CDR3 amino acid length distributions, with *TRDV* genes having the most variability in length.

Ontogeny of the γδ TCR repertoire

An additional unique feature of the γδ TCR repertoire is that different Vγ gene are used preferentially at different times during ontogeny (Vantourout and Hayday, 2013). The reported sequence begins with Vγ5+ DETC cells, followed by Vγ6+ and Vγ4+ cells during the embryonic period. Vγ4+ T cell development continues throughout the life of the mouse and is joined by expression of the remaining Vγ families in the neonatal period. However, as these original observations were not made using information at the clonal level we sought to determine if subtler changes in the CDR3 repertoire could be detected throughout ontogeny.

We first confirmed that our data mirrored the *TRV* kinetic patterns previously observed. Using mature, CD24-lo thymocytes, we assessed the γδ TCR repertoire at birth, 1 week of age, and 6 weeks of age (**Fig. 3.7**). At birth, we can still detect transcription of TCRs utilizing *TRV* segments previously attributed to fetal development, such as the *TRDV4* and *TRGV5* genes of the DETC TCR. In fact, we were able to detect significant levels of *TRGV5* up to 1 week of age. However, these early *TRV* segments are eventually replaced by high diversity *TRV* segments like *TRDV4* and *TRGV4*.

We then further characterized this repertoire progression in the context of TCR diversity. Interestingly, regardless of the *TRV* gene used, the γδ TCR repertoire appears to get more diverse with age (**Fig. 3.7**). While it is not surprising that overall diversity would increase with age, it was unexpected that this would hold true for all groups of *TRV* segments, given that certain *TRV*-containing TCRs are mostly constrained to early development. This is surprising given the association of restricted repertoires and preferential *TRV* usage in early life. Due to the characteristic fetal development of γδ T cells, some have argued that the functions of these cells is mostly relevant to this period in a host's life cycle. However, the fact that γδ TCR diversity continues to grow even after birth would suggest that γδ T cells remain relevant in adults.

Polyclonal γδ T cells clonally expand during EAE

As discussed previously, a major limitation of all methods currently used to analyze the γδ TCR repertoire is their inability to obtain paired TCRγ and TCRδ sequence information with the throughput of deep-sequencing. In order to address this, we developed a fixed-TCRδ retroviral bone marrow chimera system to generate TCR "retrogenic" mice. In this approach, a single TCRδ chain is fixed in developing γδ T cells, while the TCRγ locus can freely rearrange. As there is only one TCRδ sequence to which resulting TCRγ transcripts can pair with, we are able to determine the complete

sequence of a γδ TCR from TCRγ sequences alone. Therefore, we can obtain full TCRγδ sequences from bulk populations, rather than limited single cell samples.

To study the dynamics of the γδ TCR repertoire, we chose to utilize EAE as a disease model, due to its well described involvement of γδ T cells (Spahn et al., 1999). In order to obtain a TCRδ sequence likely to be used by γδ T cells involved in EAE inflammation, we first sequenced the TCRδ repertoire of polyclonal mice immunized for EAE. Immunized mice displayed a noticeable shift in their TCRδ repertoire, suggesting an antigen specific γδ T cell response during EAE (**Fig. 3.8**). We then analyzed the use of specific clones in these immunized mice and identified the TCR D3 for its approximately 10-fold enrichment in the draining LN of immunized mice relative to control mice, as well as its representation in inflamed CNS tissue (**Figs. 3.9, 3.10**).

Fixed-TCRδ retrogenic mice provide enhanced coverage

We then proceeded to generate fixed-TCRδ retrogenic mice using this TCR, termed D3G0 (D3 for the TCRδ chain and G0 reflecting the absence of a retrovirally expressed TCRγ chain). We first tested whether γδ T cells in the fixed-TCRδ system develop normally. A thymic CD4 and CD8 double negative (DN) γδ T cell phenotype is often considered indicative of appropriate γδ T cell lineage commitment. Alternatively, CD4 and CD8 double positive (DP) γδ T cells are often abnormal and develop into cells phenotypically resembling αβ T cells (Kreslavsky et al., 2008). We find that D3-expressing γδ T cells develop normally, with almost all cells found in the DN fraction (**Fig. 3.11**). Moreover, the cellular fraction derived from the retrovirally transduced bone marrow efficiently generates γδ T cells, with approximately $2/3^{rd}$ of all γδ T cells in these mice expressing D3G0, while the remaining $1/3^{rd}$ represent polyclonal γδ T cells derived from the WT bone marrow compartment. Finally, we confirmed that these phenotypes were stable, as only mature γδ T cells reach the peripheral lymphoid tissue.

Single cell γδ T cell sorting and sequencing is a common approach currently used to address the challenge of pairing TCRγ and TCRδ sequences. Therefore, we compared the repertoire coverage obtained from sequencing the TCRγ repertoire of our fixed-TCRδ retrogenic mice to that obtained from a recent single cell sorting-based repertoire study (Wei et al., 2015). As expected, our method provided substantially improved repertoire sampling, with essentially complete coverage in the tissues assayed (**Fig. 3.12**). This is compared to the single-cell sorting method which, while achieving high coverage in several tissues shown to have limited overall diversity, appeared to cover less than 50% of the γδ TCR repertoire in high diversity tissues. Together, these data suggest that our methodology will significantly enhanced repertoire resolution.

Clonal expansion of fixed-TCRδ γδ T cells in EAE

Interestingly, we noticed that the TCRγ repertoire observed in cells expressing the D3G0 TCR did not significantly differ from the TCRγ repertoire of the polyclonal fraction of γδ T cells within the same mouse (**Fig. 3.13**). Additionally, we compared the TCRγ repertoires of cells expressing two additional fixed-TCRδ sequences, D1G0 and D2G0, and found that they showed no difference to the TCRγ repertoire of D3G0 γδ T cells. This suggests that that the TCRδ chain used by a γδ T cell does not impart restriction on what TCRγ sequences it can pair with.

To test if the choice of TCRδ chain has no bearing on the TCRγ repertoire during inflammation, we immunized D3G0 retrogenic mice for EAE. Unlike what we observed during homeostasis, the TCRγ repertoires of polyclonal and D3G0-expressing γδ T cells diverged during EAE (**Fig 3.13**). As the TCRδ sequence seems to affect the choice of TCRγ chain during an immune response, but not during homeostasis, this suggests that specific antigen interaction is not important for γδ T cell thymic development, but does contribute to the peripheral function of these cells.

During EAE, D3G0 expressing γδ T cells are capable of trafficking to the CNS (**Fig. 3.14**), suggesting that the repertoire changes observed above are due to active involvement in the immune response. As might be expected given the divergence of polyclonal and D3G0 associated TCRγ repertoires in EAE, the TCRγ repertoire of γδ T cells expressing D3G0 changed substantially between EAE and healthy control mice (**Fig. 3.15**). Moreover, this shift included an expansion of *TRGV4*-utilizing clones, which are associated with a subset of γδ T cells that have been previously implicated in EAE pathology (Blink et al., 2014).

When analyzing specific TCRγ sequences, we identified the TCR G1 for its prominent representation during EAE. G1 is significantly enriched in the draining LNs of EAE immunized mice, with a greater than 6-fold increase over health control samples (**Fig 3.16**). Moreover, G1 represented a dramatically high proportion of all D3G0-associated TCRγ sequences in the CNS, reaching 15% of all sequences (**Fig 3.17**). This enrichment appears to be due to clonal expansion of the entire population of G1-expressing cells rather than a founder event by a single clone, as the distribution of DNA sequences used to encode the G1 CDR3 region are similar between mice and between immunized and control conditions (**Fig. 3.16**). Together, the repertoire changes and specifically enriched TCR clones that we observe strongly suggests antigen-dependent clonal expansion of γδ T cells can occur during EAE.

Generation of full γδ TCR retrogenic mice

Having demonstrated significant expansion of individual γδ TCR clones during EAE, we next sought to determine how these clones contribute to the pathogenesis of EAE. To do this, we utilized γδ T cells bearing both the D3 TCRδ and G1 TCRγ chains, termed D3G1. To observe these cells *in vivo*, we modified our fixed-TCRδ system in order to express both γδ TCR chains in RAG-deficient bone marrow, which would otherwise generate no lymphocytes. These mice also contain

congenically marked TCRδ-deficient bone marrow. Thus, these mice have a normal complement of lymphocytes, with the exception of γδ T cells, which are derived entirely from the D3G1-expressing bone marrow. After generating D3G1 retrogenic mice, we first assessed the thymic development of D3G1-expressing γδ T cells. Notably, the proportion of retrogenic γδ T cells in these mice was significantly smaller than the fraction observed in fixed-TCRδ mice, perhaps due to intraclonal competition of γδ T cells all expressing the same TCR. However, as with our fixed-TCRδ retrogenic mice, the majority of D3G1-expresssing T cells developed normally as DN thymocytes (**Fig. 3.18**). However, we did observe a noticeable fraction of D3G1 γδ T cells with a DP phenotype, suggesting some developmental abnormalities with these retrogenic cells. However, peripheral D3G1 γδ T cells are all DN, suggesting that any aberrantly developing D3G1 thymocytes do not leave the thymus. Indeed, analysis of cellular maturity showed that DP D3G1 γδ T cells have a CD24-hi immature phenotype, whereas the mature CD24-lo phenotype is largely restricted to the DN D3G1 γδ T cells.

D3G1-expressing γδ T cells can contribute to EAE pathology

In order to determine if D3G1-expressing γδ T cells can contribute to EAE, we immunized D3G1 retrogenic mice and TCRδ-deficient bone marrow chimeras. While both groups developed clinical signs of EAE at approximately the same time, D3G1 retrogenic mice developed significantly more severe disease (**Fig. 3.19**). On average, the clinical signs observed in the D3G1 group were one full grade higher than TCRδ- only control bone marrow chimeras. Moreover, D3G1 retrogenic mice exhibited enhanced EAE mortality when compared to TCRδ-deficient bone marrow chimeras. As the latter specifically lacks all γδ T cells, while D3G1 retrogenic mice possess only D3G1 expressing γδ T cells, this suggests that the D3G1 TCR alone is sufficient to mediate this increase in disease severity. In addition, we confirmed that D3G1-expressing γδ T cells are capable of trafficking to the

CNS during EAE (**Fig. 3.20**), further supporting the conclusion that they can directly contribute to disease pathogenesis.

The response of D3G1 in EAE is antigen specific

However, it is possible that simply the presence of any γδ T cell population, and not D3G1-expressing cells specifically, is sufficient to enhance EAE severity. To evaluate this alternative hypothesis, we performed additional TCR retrogenic experiments using the addition of congenically marked WT bone marrow. These mice contain both D3G1-expressiong γδ T cells, as well as polyclonal γδ T cells, which can be distinguished by congenic markers. Upon immunization with EAE, the reaction of these two γδ T cell fractions, which differ only in their use of TCRs, can be compared.

Using these mice, we first looked at the expansion of the two γδ T cell fractions in EAE. In control retrogenic mice the population of D3G1 TCR-expressing cells is relatively small compared to the polyclonal population of γδ T cells. However, Upon EAE immunization, the D3G1-expressing population expands significantly (**Fig. 3.21**). In fact, D3G1 γδ T cells come to represent the majority of γδ T cells in retrogenic mice during EAE, while the proportion of polyclonal cells correspondingly shrinks. Moreover, this response is unique to the brachial and axillary lymph nodes, which drain the sites of the MOG-CFA subcutaneous immunization. In comparison, the distant inguinal lymph nodes show no change in the proportion of D3G1 to polyclonal γδ T cells during EAE. Together, this data suggests that D3G1 TCR-bearing γδ T cells respond in an antigen-specific manner to MOG-CFA immunization during EAE.

D3G1 γδ T cells display a pre-activated phenotype and can produce IL-17

We then sought to determine how D3G1 γδ T cells could be contributing to EAE pathogenesis. As discussed earlier, some γδ T cells leave the thymus already expressing a cell surface phenotype associated with lymphocyte activation. While assaying D3G1 γδ T cells for such markers, we found that these cells are uniformly CD25+ and CD44-hi in healthy control mice (**Fig. 3.22**). γδ T cells can also be categorized based on their ability to naturally produce high levels of the inflammatory cytokine IL-17. Indeed, under homeostatic conditions approximately 20% of all D3G1-expressing γδ T cells produce IL-17, compared to only 2% of the polyclonal γδ T cell population. As the role of IL-17 is well described in the pathogenesis of EAE (Komiyama et al., 2006; Yang et al., 2008), this data suggests that D3G1 γδ T cells can contribute to neuropathology through their production of this cytokine.

Next, we evaluated how several markers of activation change during the course of EAE in D3G1 γδ T cells. Interestingly, while these markers, including CD25, CD44, CD69, and Nur77 all increase within the polyclonal population of γδ T cells, they remain constant or even decrease in D3G1 γδ T cells (**Fig. 3.23**). For CD25 and CD44, this result is not surprising, given that nearly 100% of D3G1 γδ T cells express these markers even in the absence of EAE. However, the lack of CD69 or Nur77 upregulation was puzzling given their relation to TCR signaling and our previous data suggesting that D3G1 γδ T cells contribute to EAE in an antigen-dependent manner.

Downregulation of the γδ TCR upon stimulation

As mentioned above, Nur77 is regarded as a downstream marker of TCR signal transduction (Moran et al., 2011). Therefore, we were particular surprised to see a significant decrease in *Nur77* expression within D3G1-expressing γδ T cells during EAE. Since some examples of inconsistent γδ T cell data have been resolved upon recognition that TCR downregulation can render cells

undetectable by flow cytometry (Blink et al., 2014), we sought to determine if this could also be the reason behind the apparent downregulation of *Nur77*.

To test this, we generated T cell hybridomas expressing the D3G1 TCR *in vitro*. The hybridomas harbored an NFAT-driven GFP construct, allowing us to visualize TCR stimulation via GFP signal. These D3G1 hybridomas readily expressed GFP upon stimulation with anti-CD3 antibodies. However, when stratified based on the level of TCRγδ expression, the cells with highest GFP expression were those with the lowest TCRγδ expression (**Fig 3.24**). Moreover, we noted that the population of TCRγδ-lo cells was increased upon stimulation. These results were not due to non-specific GFP expression, as GFP was entirely absent without CD3 stimulation. Together, this data suggests that strong TCR stimulation can result in downregulation of the γδ TCR, and that the apparent decrease in *Nur77* expression during EAE, could instead be caused by loss of the ability to detect highly activated cells via TCRγδ expression.

3.5 Discussion

γδ T cells play numerous roles within the host immune system. Yet, the contribution of the TCR to the function of γδ T cells during the course of an immune response remains unclear. Here, we present data demonstrating that these cell expansion during EAE leads to the enrichment of clones that can take part in associated immune pathology in a TCR dependent manner.

We believe our study represents a substantial improvement in the analysis of the γδ TCR repertoire. As discussed previously, nearly all available studies of γδ TCR specificity rely on low coverage sequencing that likely undersample the total repertoire. Through the use of deep sequencing, we have been able to comprehensively reevaluate many long standing views regarding γδ TCR diversity. For the most part, our data supports much of the current understanding of the γδ TCR repertoire. For example, we observe a similar progression in the use of Vγ gene families during ontogeny, as well as varying amounts of TCR diversity between anatomical locations. However, our data also expands on several of these features. For instance, thymic development of the Vγ5+ DETC TCR is considered to be restricted to fetal development (Garman et al., 1986), yet we show that this TCR can be detected up to 8 days after birth. Similarly, we demonstrate that the nTγδ17 phenotype, whose development is also thought to be restricted to the fetal period (Shibata et al., 2014), can arise in the adult mouse, as D3G1-expressing γδ T cells acquire this phenotype in bone marrow chimeras.

We also show that TCR diversity increases with age, which is in agreement with previous data describing the limited repertoire diversity of fetal and neonatal γδ T cells (Elliott et al., 1988). However, we additionally observe that this increase in diversity also holds when evaluating the γδ TCR repertoire within each *TRV* gene family. This is surprising, as the increase in γδ TCR diversity with age was presumed to be due to the abundant production of γδ T cells utilizing low diversity

TRV gene segments, such as *TRGV5*, in early life later being supplanted by γδ T cells utilizing high diversity *TRV* segments, such as *TRGV1*. Instead, we show that diversity increases in age across nearly all *TRV* gene families, regardless of the level of expression of the particular *TRV* gene.

Our sequencing methodology further improves on previous approaches in its ability to maintain TCRγ and TCRδ sequence pairing. The majority of available γδ TCR repertoire data is unable to account for this pairing, as sequences are derived from bulk γδ T cell samples. Those studies that do maintain TCRγ and TCRδ sequence pairing do so via single cell methods that further limits possible coverage of the repertoire. Our use of a fixed-TCRδ system addresses this obstacle, as we are able to infer TCRγ and TCRδ pairing from TCRγ sequencing alone, allowing for the use of deep sequencing methodologies.

One potential caveat to this system is the choice of TCRδ sequence in fixed-TCRδ retrogenic mice. However, we show that the use of several different fixed-TCRδ sequences does not significantly alter the appearance of the TCRγ repertoire. Yet, it should be noted that all tested TCRδ sequences in this study belong to the same Vδ family and future experiments should incorporate sequences from other Vδ families to determine the generalizability of these results. However, emerging technologies, such as droplet sequencing (Lan et al., 2016), may soon allow for deep sequencing of paired γδ TCR transcripts without the need for a complex TCR retrogenic system.

Data obtained from our fixed-TCRδ system demonstrate that the γδ TCR repertoire shifts in response to EAE associated inflammation. Interestingly, in the absence of EAE, we observe that the TCRγ repertoire associated with a fixed-TCRδ chain is no different from that of a fully polyclonal population, suggesting that a particular TCRδ chain imparts no restriction on possible TCRγ binding partners during homeostasis. This observation is in agreement with previous data showing that B2M-deficient mice have no defect in the production of γδ T cells that recognize

B2M-containing molecules (Jensen et al., 2008). However, we also note that during EAE, the TCRγ repertoires of fixed-TCRδ and polyclonal γδ T cells populations do diverge. Together, this suggests that while antigen specificity is not significant for thymic development of γδ T cells, it plays an important role in the peripheral functions of these cells.

One such function we demonstrate is the ability of D3G1-expressing γδ T cells to contribute to EAE pathogenesis in a TCR-specific manner. However, one caveat to our data is the lack of additional TCRs with which to compare the results obtained with D3G1. However, in the absence of an additional TCR, we provide several pieces of evidence to support a TCR-dependent view of D3G1 γδ T cell function. First, the development and function of D3G1 expressing γδ T cells is uniform. If the TCR played no role in the character of these γδ T cell, one would expect D3G1 expressing cells to exhibit a range of phenotypes, consistent with the diversity of the polyclonal γδ T cell population. This distinction is made clear in retrogenic experiments by direct comparison to internal controls of polyclonal γδ T cells, which do show phenotypic diversity similar to that the γδ T cell population in a wild type mouse. The results obtained using D3G1 retrogenic cells are similar to those obtained using αβ TCRtg cells, such as CT2, in which the expression of a single TCR specificity drives the development of a largely uniform cellular phenotype.

In addition, the expansion of D3G1 expressing cells relative to the polyclonal population during EAE also suggests the importance of the TCR. While the polyclonal population harbors many different TCRs, if the TCRs themselves had no role in the response to EAE, one would expect this population to have a similar degree of responsiveness during EAE as D3G1-expressing γδ T cells. Instead, the expansion of the polyclonal γδ T cell population during EAE is significantly lower than that of the D3G1 population. As the major difference between these two populations relates to the particular TCR they express, these results are consistent with a TCR-dependent process.

Nonetheless, future study with additional TCRs will be beneficial to determine how much γδ TCR variation is needed to manifest different phenotypes. For example, KN6, one best studied γδ TCRs, shares the same *TRGV4* segment as D3G1. As Vγ4+ γδ T cells are broadly implicated in EAE pathogenesis (Blink and Miller, 2009), KN6 could serve as a control to determine if the contribution of the D3G1 TCR can be attributed to its CDR3 specificity or simply its TCRγ family.

3.6 Figures

A

	TRDV Primer													
TRDV Sequence	**DV1**	**DV2**	**DV2**	**DV4**	**DV5**	**AV12**	**AV13**	**AV14**	**AV15**	**AV16**	**AV18**	**AV21**	**AV4**	**AV9**
TRDV1	**0.90**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.21**
TRDV2-2	0.00	**1.00**	**1.00**	0.00	0.00	0.00	0.00	0.00	0.00	**0.12**	0.00	0.00	0.00	**0.32**
TRDV4	0.00	0.00	0.00	**1.00**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRDV5	0.00	0.00	0.00	0.00	**1.00**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.21**
TRAV4-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.06**	0.00
TRAV4D-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.63**	0.00
TRAV4D-4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.46**	0.00	**0.31**	0.00
TRAV12N-3	0.00	0.00	0.00	0.00	0.00	**0.99**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV13-2	0.00	0.00	0.00	0.00	0.00	0.00	**0.01**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV13-4/DV7	0.00	0.00	0.00	0.00	0.00	0.00	**0.37**	0.00	0.00	**0.03**	0.00	0.00	0.00	0.00
TRAV13-5	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV13D-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV13D-2	0.00	0.00	0.00	0.00	0.00	0.00	**0.57**	0.00	0.00	**0.07**	0.00	0.00	0.00	0.00
TRAV13D-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV13D-4	0.00	0.00	0.00	0.00	0.00	0.00	**0.03**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV13N-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV13N-4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV14-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.25**	0.00	0.00	0.00	0.00	0.00	**0.05**
TRAV14-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.37**	0.00	0.00	0.00	0.00	0.00	**0.21**
TRAV14-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV14D-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.11**	0.00	0.00	0.00	0.00	0.00	0.00
TRAV14D-3/DV8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.27**	0.00	0.00	0.00	0.00	0.00	0.00
TRAV15-1/DV6-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.09**	0.00	0.00	0.00	0.00	0.00
TRAV15-2/DV6-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.18**	0.00	0.00	0.00	0.00	0.00
TRAV15D-1/DV6D-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAV15D-2/DV6D-2	**0.10**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.33**	0.00	0.00	0.00	0.00	0.00
TRAV15N-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.40**	0.00	0.00	0.00	0.00	0.00
TRAV16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00
TRAV16D/DV11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.73	0.00	0.00	0.00	0.00
TRAV16N	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
TRAV18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.54**	0.00	0.00	0.00
TRAV21/DV12	0.00	0.00	0.00	0.00	0.00	**0.01**	0.00	0.00	0.00	0.00	0.00	**1.00**	0.00	0.00

B

	TRGV Primer					
TRGV Sequence	**TRGV1A**	**TRGV2A**	**TRGV4B**	**TRGV5B**	**TRGV6**	**TRbGV7B**
TRGV1	**0.99**	**0.55**	0	0	0	0
TRGV2	**0.01**	**0.45**	0	0	0	0
TRGV4	0	0	**1**	0	**0.52**	0
TRGV5	0	0	0	**1**	0	0
TRGV6	0	0	0	0	**0.19**	0
TRGV7	0	0	0	0	**0.29**	1

Figure 3.1 – Specificity of TCRδ and TCRγ sequencing primers

(A) Frequency of TCR reads using indicated *TRDV/TRAV* V-gene segments for each Vδ sequencing primer. **(B)** Frequency of TCR reads using indicated *TRGV* V-gene segments for each Vγ sequencing primer.

	Cg1			Cg2			Cg4		
	Jg1	**Jg2**	**Jg4**	**Jg1**	**Jg2**	**Jg4**	**Jg1**	**Jg2**	**Jg4**
TRGV1	0	0	0	0	0	0	0	0	**1**
TRGV2	0	0	0	0	**0.75**	0	0	0	**0.25**
TRGV4	1	0	0	0	0	0	0	0	0
TRGV5	**0.98**	0	0	**0.02**	0	0	0	0	0
TRGV6	**1**	0	0	0	0	0	0	0	0

Figure 3.2 – Usage of Cγ gene segments by Vγ gene families

Frequency each Cγ-gene and associated Jγ-gene is utilized with indicated *TRGV* Vγ segment in TCRγδ sequencing reads.

	Colon	IEL	mLN	pLN	Skin	Spleen	Thymus	All
TCRδ	3320	6797	38074	58133	428	130310	119446	400370
TCRγ	1248	2154	3184	4559	486	9655	13356	24834

Figure 3.3 – Table of unique TCRγ and TCRδ sequences obtained from all sequencing data

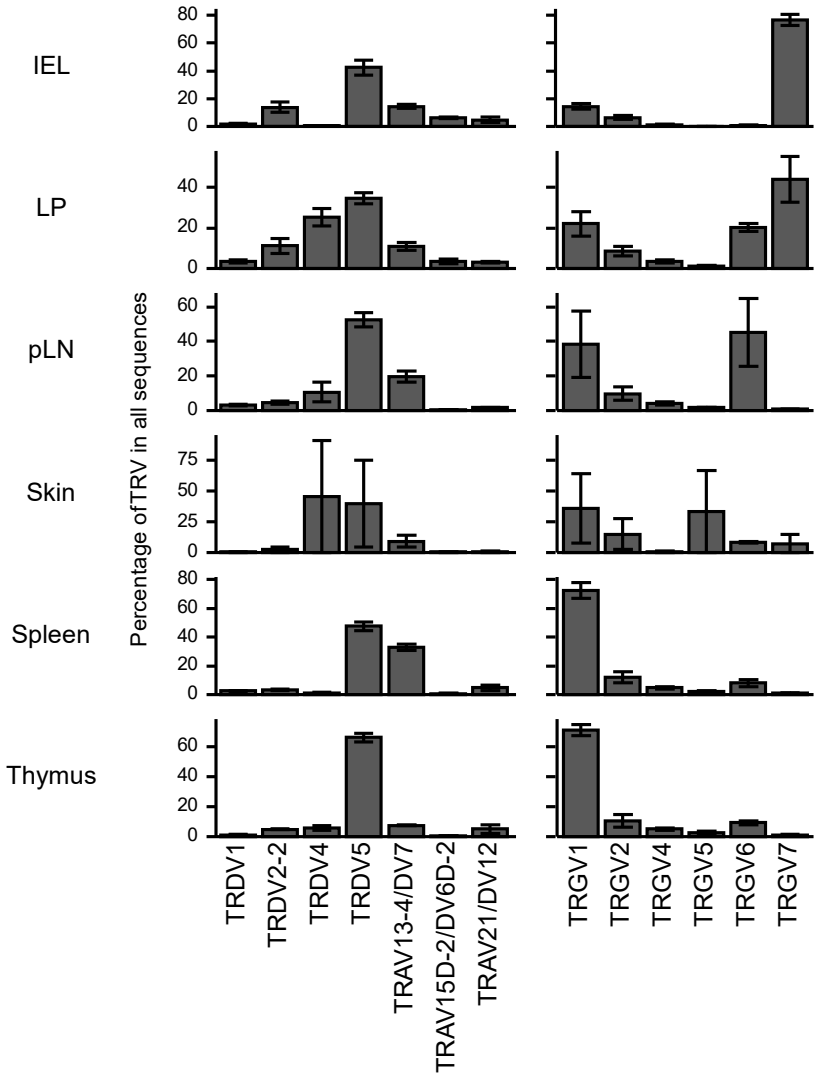


Figure 3.4 – Tissue specific Vγ usage

Relative use of indicated *TRV* gene segments by TCR transcripts from indicated tissues. n≥4 for each tissue. Data representative of ≥2 independent experiments. Error bars = ±1 s.e.m.

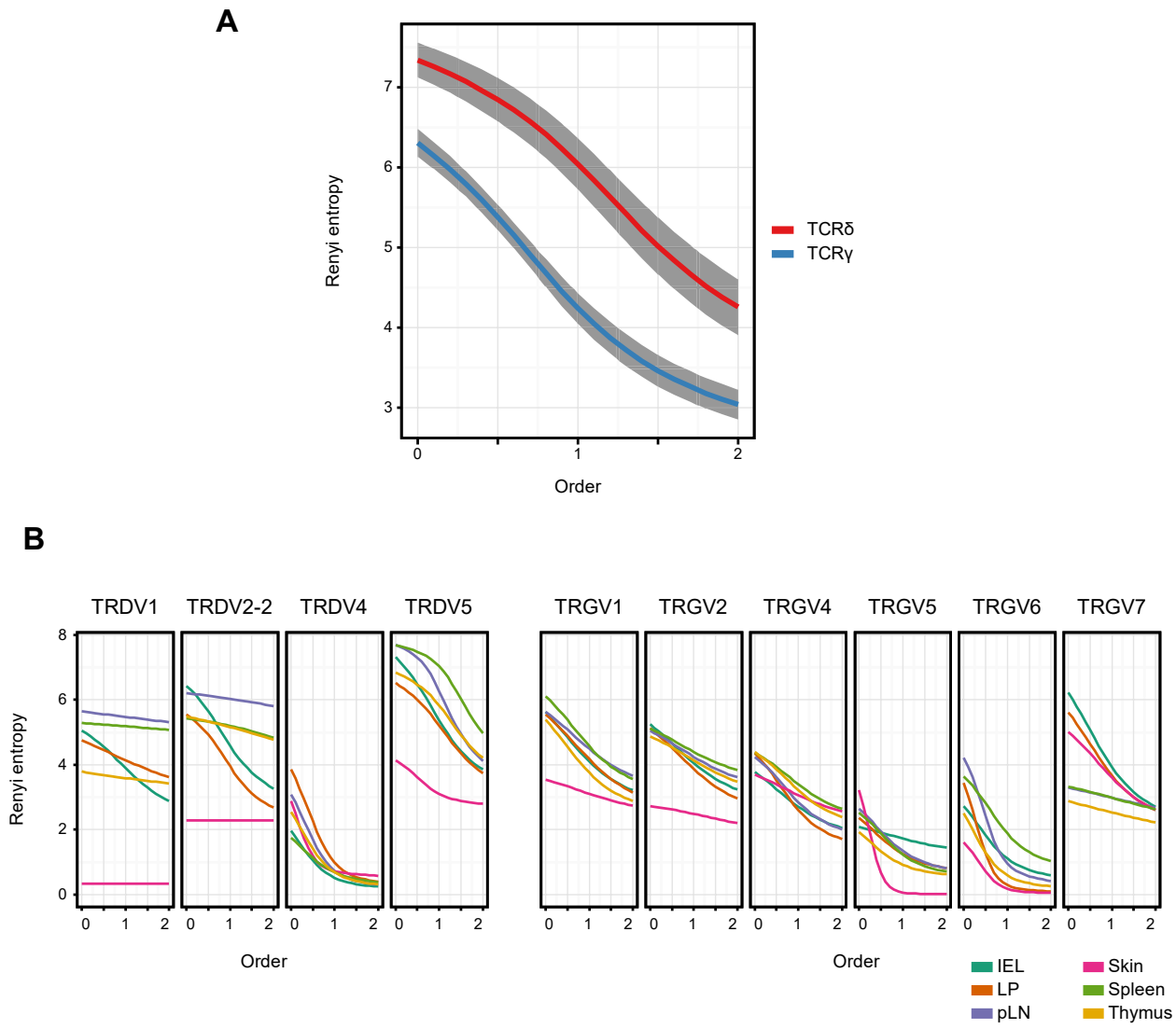


Figure 3.5 – Increased diversity of TCRδ sequences compared to TCRγ sequences

Mean Renyi entropy of **(A)** TCRγ and TCRδ sequences as well as **(B)** stratified by specific *TRV* usage and tissue source. Increasing entropy indicates higher population diversity. Increasing order indicates increasing weight on high frequency clones. n≥4 for each tissue/TCR. Data representative of ≥2 independent experiments. Error ribbon = ±1 s.e.m.

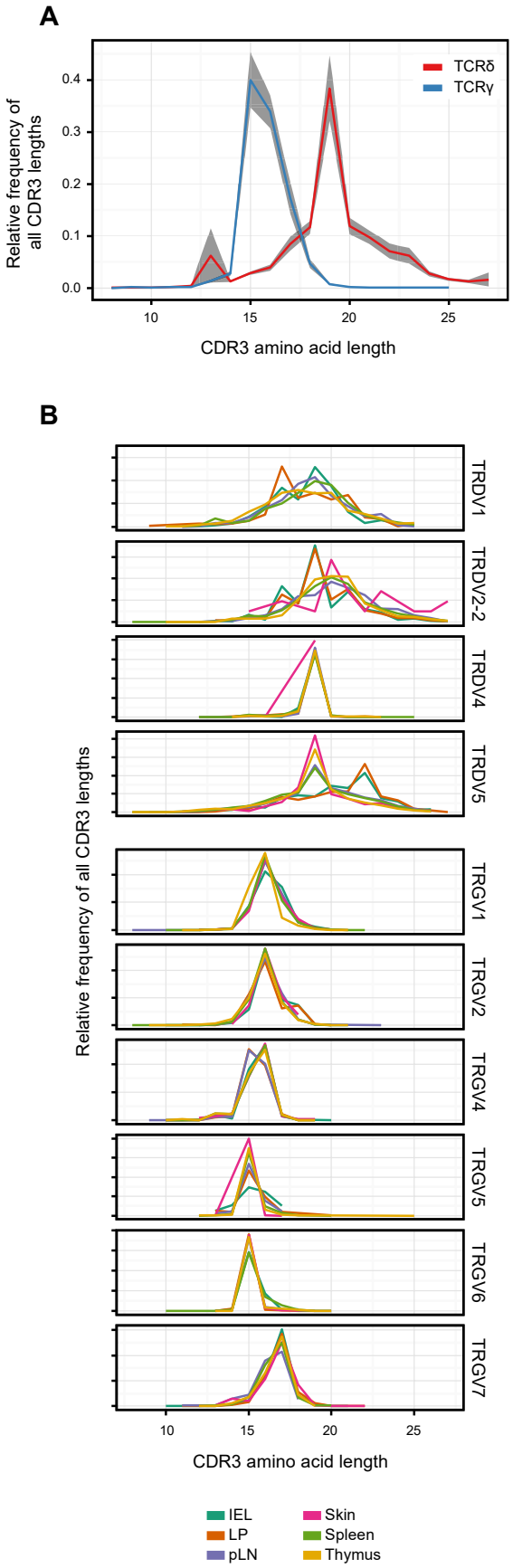


Figure 3.6 – TCRδ sequences have a longer CDR3 region than TCRγ sequences

(A) Mean proportion of TCR sequences with indicated CDR3 amino acid length for the designated TCR chain. **(B)** Mean proportion of TCR sequences with indicated amino acid length stratified by tissue source and *TRV*-gene segment used in sequence. n≥4 for each tissue/TCR. Data representative of ≥2 independent experiments. Error ribbon = ±1 s.e.m.

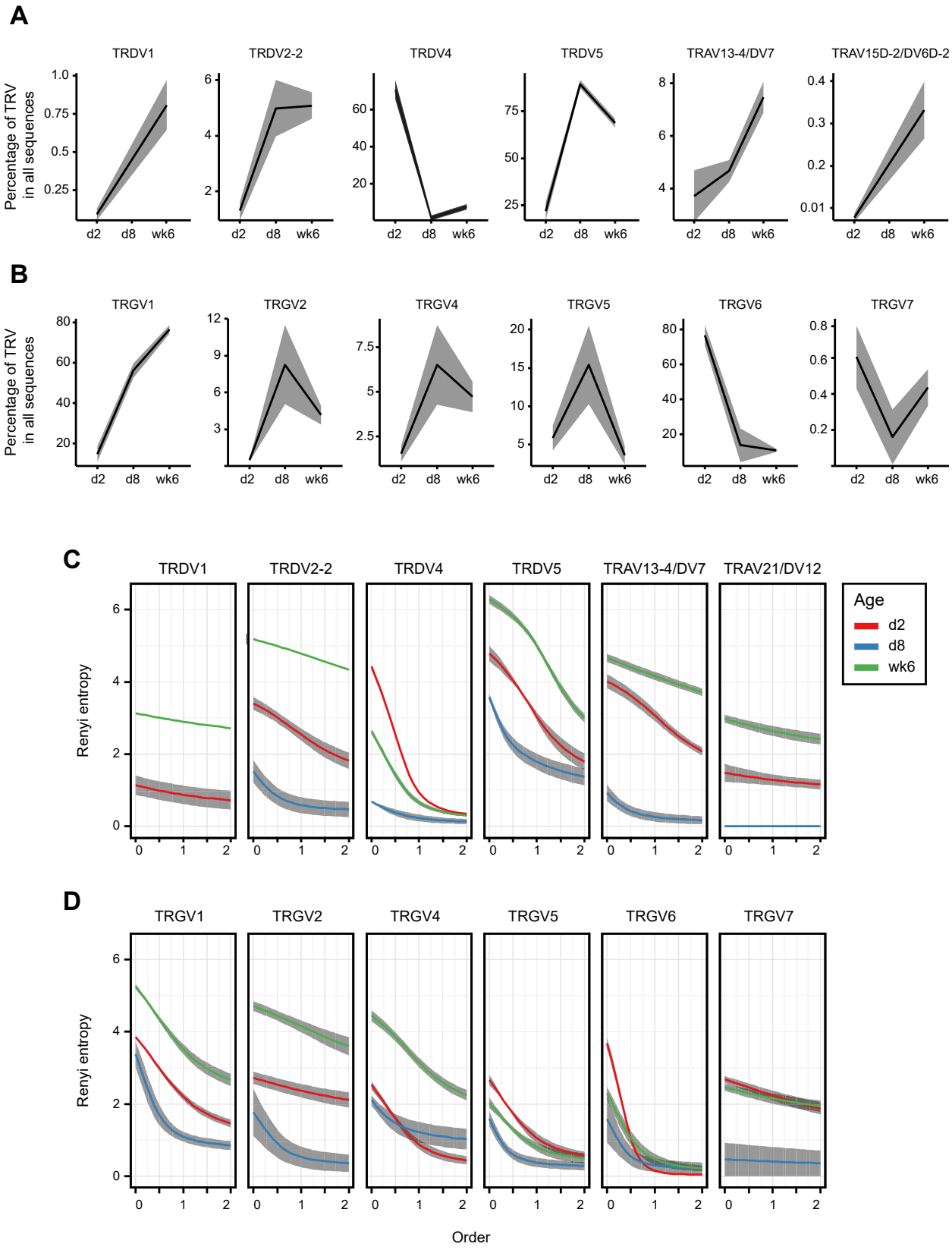

A
TRDV1
TRDV2-2
TRDV4
TRDV5
TRAV13-4/DV7
TRAV15D-2/DV6D-2
Percentage of TRV in all sequences
d2
d8
wk6
B
TRGV1
TRGV2
TRGV4
TRGV5
TRGV6
TRGV7
C
TRDV1
TRDV2-2
TRDV4
TRDV5
TRAV13-4/DV7
TRAV21/DV12
Renyi entropy
Age
d2
d8
wk6
D
TRGV1
TRGV2
TRGV4
TRGV5
TRGV6
TRGV7
Order

Figure 3.7 – Changes in Vγ usage and diversity with age

Relative usage of **(A)** TCRδ and **(B)** TCRγ V-gene segments at indicated time points after birth. Renyi entropy of **(C)** TCRδ and **(D)** TCRγ V-gene segments at indicated time points after birth. Increasing entropy indicates higher population diversity. Increasing order indicates increasing weight on high frequency clones. n≥5 for each time points. Data representative of ≥2 independent experiments. Error ribbon = ±1 s.e.m.

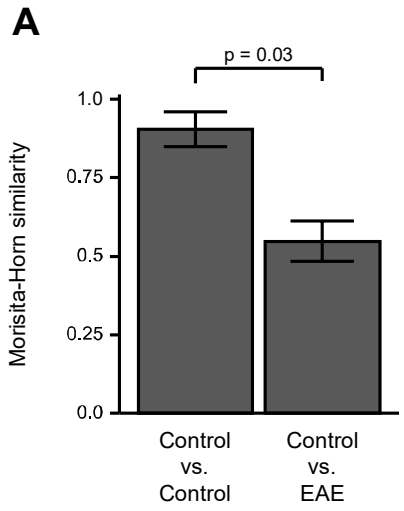


Figure 3.8 – EAE causes a shift in the polyclonal γδ TCR repertoire

Morisita horn index of similarity (0=complete dissimilarity, 1=identical) between indicated experimental groups using TCR sequences obtained from draining lymph nodes. In EAE group, cells isolated 12 days after immunization. n≥9 for each experimental group. Data representative of 2 independent experiments. Error bars = ±1 s.e.m. P-value reflects Wilcox rank sum test.

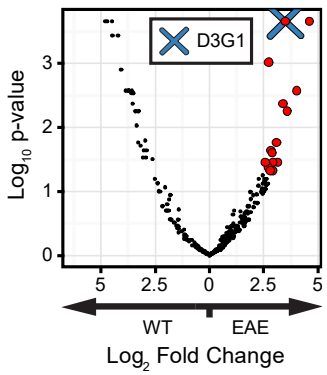


Figure 3.9 – Identification of TCRδ sequences enriched in polyclonal mice during EAE

Enrichment of TCRδ sequences in mice immunized for EAE relative to healthy control mice vs. FDR adjusted p-value. Red dots are TCRs > 2-fold enriched in EAE group with an adjusted p-value < 0.05. Blue X indicates D3 TCRδ sequence utilized in subsequent experiments. n≥9 for each experimental group. Data representative of 2 independent experiments.

CDR3 sequence	TRV	log_2FoldChange	Adjust P-value	CNS frequency
FCALMERHIGGIRATDKLVFG	TRDV2-2	4.582133	0.0002	0.0141
FCALMERADGGIRATDKLVFG	TRDV2-2	4.030265	0.0018	0.0040
FCALMERGVGGIRATDKLVFG	TRDV2-2	3.598694	0.0035	0.0034
FCALMERGGGIRATDKLVFG	**TRDV2-2**	**3.420161**	**0.0006**	**0.1061**
LCAIPATDKLVFG	TRAV13-2	3.154277	0.0210	0.0016
LCAIPATDKLVFG	TRAV13-4/DV7	3.154277	0.0210	0.0016
LCAIPATDKLVFG	TRAV13N-1	3.154277	0.0210	0.0016
LCAIYRRATDKLVFG	TRAV13-4/DV7	2.943935	0.0280	NA
YCASGHIGGIRAPDKLVFG	TRDV5	2.93261	0.0210	0.0005
FCALMERGGGGIRATDKLVFG	TRDV2-2	2.831978	0.0165	0.0173
FCALMERGAGGIRATDKLVFG	TRDV2-2	2.795652	0.0270	0.0098
YCASGYIGGIRGDKLVFG	TRDV5	2.707134	0.0249	0.0016
LCAMPSTDKLVFG	TRAV13-4/DV7	2.703019	0.0458	NA
LCAMPSTDKLVFG	TRAV13D-2	2.703019	0.0458	NA
LCAMPSTDKLVFG	TRAV13N-1	2.703019	0.0458	NA
FCALMERGSGGIRATDKLVFG	TRDV2-2	2.642604	0.0394	0.0030
LCAMEDGGIRATDKLVFG	TRAV13-4/DV7	2.623673	0.0475	NA
LCAMEDGGIRATDKLVFG	TRAV13D-1	2.623673	0.0475	NA
LCAMEDGGIRATDKLVFG	TRAV13D-2	2.623673	0.0475	NA
YCASGYIGGIRPATDKLVFG	TRDV5	2.613362	0.0472	NA

Figure 3.10 – Table of top TCRδ sequences enriched in EAE immunized mice

Enrichment of top 20 TCRδ sequences in mice immunized for EAE relative to healthy control mice with FDR adjusted p-value and CNS frequencies. Data representative of 2 independent experiments. NA indicates sequence was not detected in CNS γδ T cell population. Bold indicates the D3 TCRδ sequence.

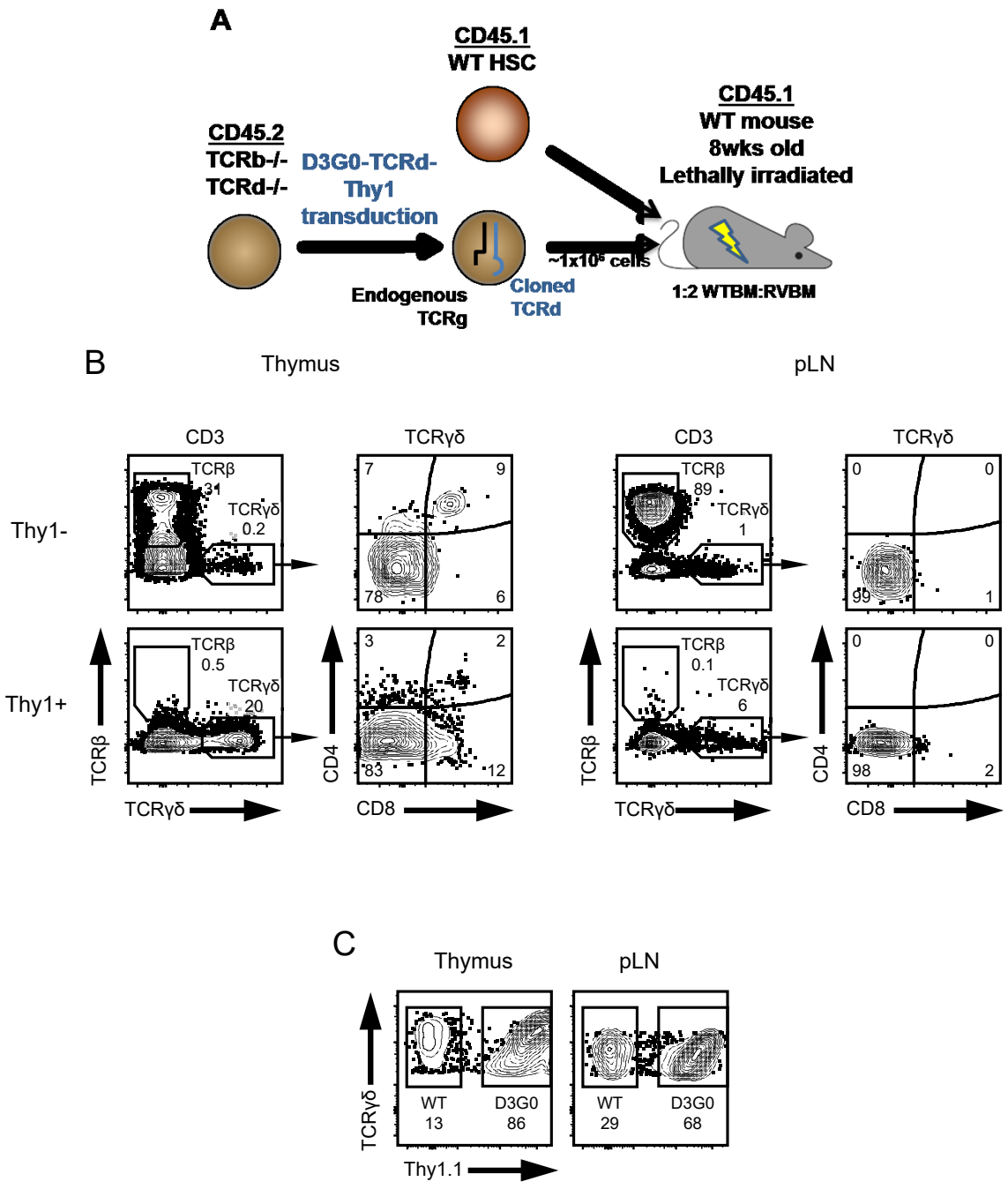


Figure 3.11 – Development of fixed-TCRδ retrogenic mice

(A) Outline of fixed-TCRδ retrogenic mouse setup. **(B)** Representative FACS plots of thymic and pLN lymphocytes of D3G0 fixed-TCRδ retrogenic mice. Thy1.1 represents an expression marker for the D3G0 construct.

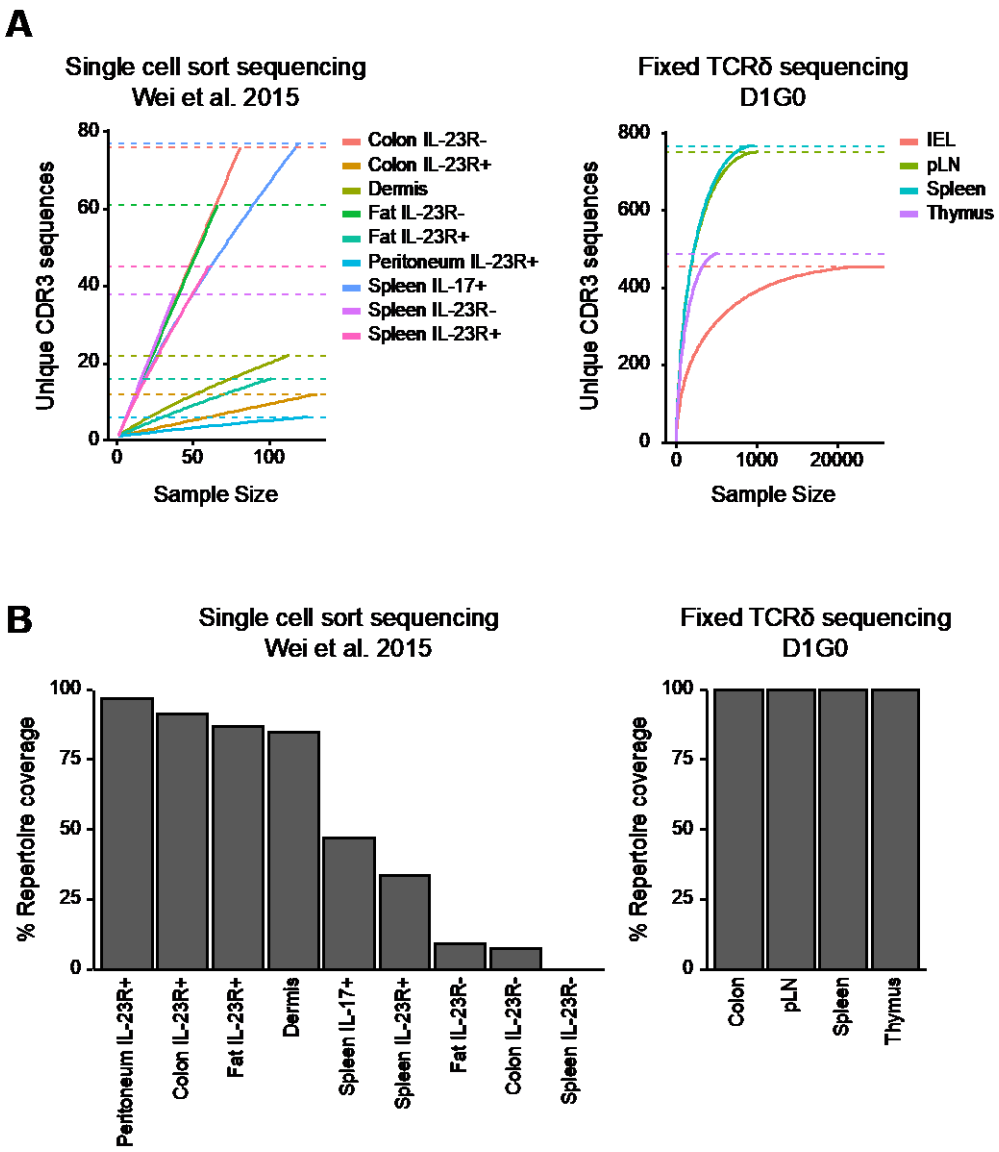


Figure 3.12 – Fixed-TCRδ retrogenic mice allow for greater γδ TCR repertoire coverage than single cell sorting

(A) Rarefaction and **(B)** coverage of TCRγ repertoire in indicated populations. For rarefaction, dashed lines indicated actual population richness. Data from Wei *et al.* based on single cell sorting from indicated population and subsequent TCR sequencing. For fixed-TCRδ sequencing, sampling data derived from representative mouse.

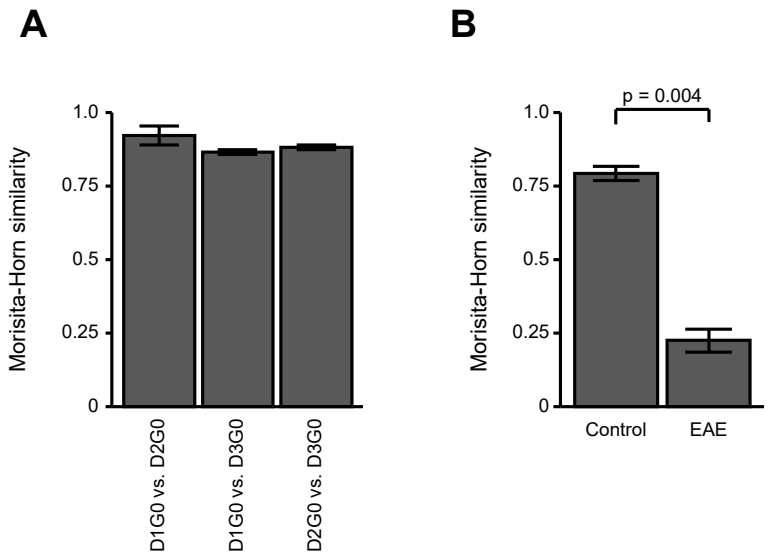


Figure 3.13 – Fixed-TCRδ chain does not constrain TCRγ repertoire during homeostasis

(A) Morisita horn index of TCRγ repertoire similarity (0=complete dissimilarity, 1=identical) between fixed-TCRδ retrogenic mice utilizing three different TCRδ sequences. n≥2 for each fixed-TCRδ sequence. **(B)** Morisita horn similarity between TCRγ repertoire of polyclonal and fixed-TCR fractions of D3G1 mice kept as healthy controls or during EAE. In EAE group, cells isolated 12 days after immunization. n≥9 for each experimental group. Data representative of 2 independent experiments. Error bars = ±1 s.e.m.

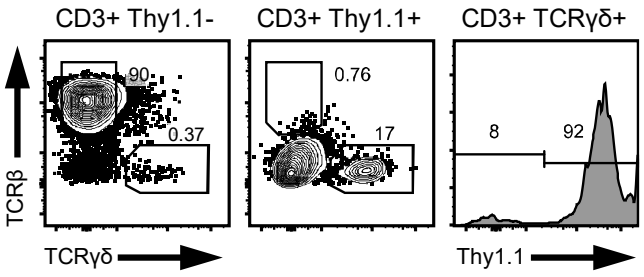


Figure 3.14 – D3G0 γδ T cell can traffic to the CNS during EAE

Representative FACS plots from indicated populations in D3G0 fixed-TCRδ retrogenic mice immunized for EAE. Thy1.1 represents an expression marker for the D3G0 construct, discriminating D3G0 fixed-TCRδ cells (Thy1.1+) and fully polyclonal cells (Thy1.1-).

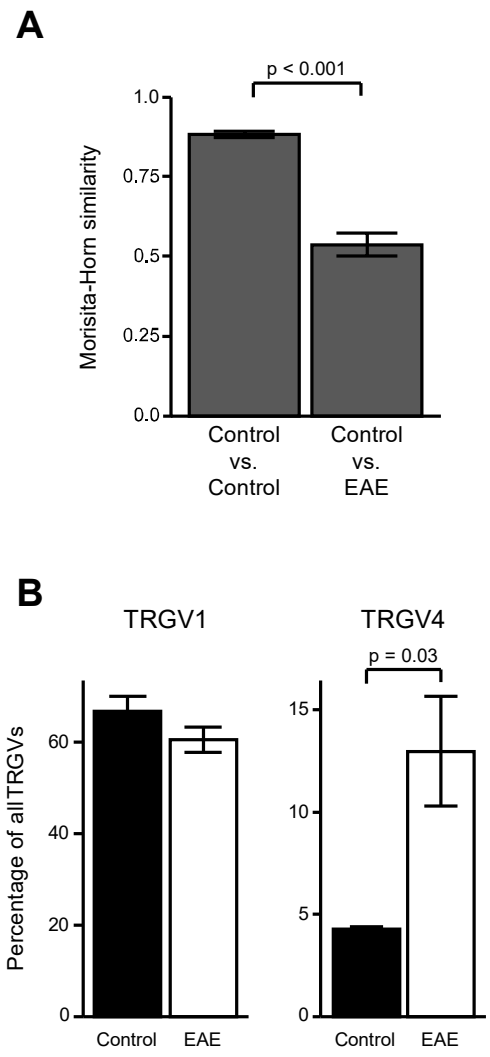
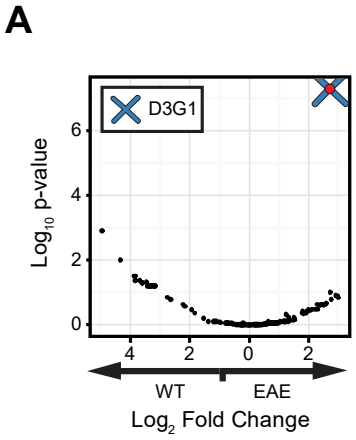


Figure 3.15 – EAE causes a shift in the TCRγ repertoire of D3G0 retrogenic mice

(A) Morisita horn index of similarity (0=complete dissimilarity, 1=identical) between indicated experimental groups of D3G0 retrogenic mice. **(B)** Relative usage of *TRGV1* and *TRGV4* gene segments by TCR sequences from indicated populations. In EAE group, cells isolated 12 days after immunization. n≥9 for each experimental group. Sequences obtained from the draining LN. Data representative of 2 independent experiments. Error bars = ±1 s.e.m. P-value indicates results of Wilcox rank sum test.



C

TRGV	N	P	TRGJ
tgt.....	tcctacggct	tat	atagctcaggttttcacaaggtattt
tgt.....	tcctacggc	ctat	atagctcaggttttcacaaggtattt
tgt.....	tcctacggt	ctat	atagctcaggttttcacaaggtattt

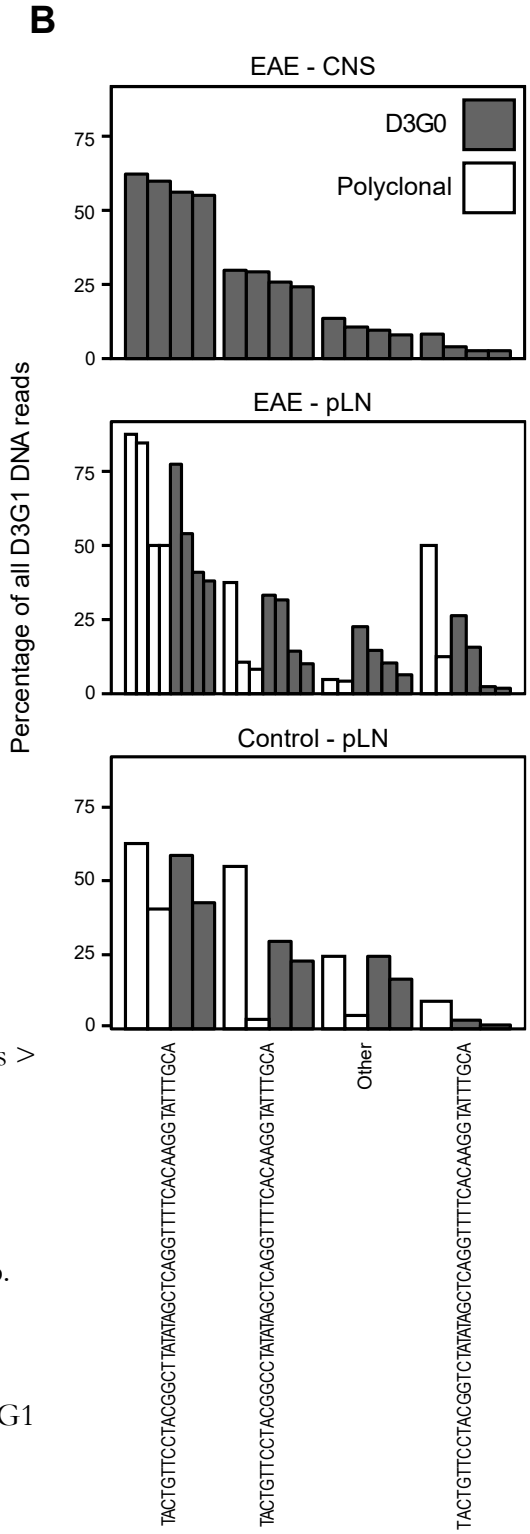


Figure 3.16 – Identification TCRγ sequences enriched in D3G0 retrogenic mice during EAE

(A) Enrichment of TCRγ sequences in D3G0-fixed retrogenic mice immunized for EAE relative to healthy control mice vs. FDR adjusted p-value. Red dots are TCRs > 2-fold enriched in EAE group with an adjusted p-value < 0.05. Blue X indicates G1 TCRγ sequence utilized in subsequent experiments. n≥9 for each experimental group. Data representative of 2 independent experiments **(B)** Relative usage of indicated DNA sequences encoding the G1 TCRγ. Each bar represents 1 mouse. **(C)** Junctional composition of G1-encoding sequence reads.

CDR3 Sequence	TRV	log_2FoldChange	Adjust P-value	CNS Frequency
YCAVWIRVSGTSWVKIFA	TRGV1	3.00	0.14	NA
YCAVWLGGTSWVKIFA	TRGV1	2.98	0.13	NA
YCAVWIRASGTSWVKIFA	TRGV1	2.91	0.12	0.0001
YCAVWIRASGTSWVKIFA	TRGV2	2.91	0.12	0.0001
YCAVWTGPGTSWVKIFA	TRGV1	2.76	0.16	NA
YCSYGFYSSGFHKVFA	TRGV4	2.73	0.10	0.0114
YCSYGLYSSGFHKVFA	**TRGV4**	**2.71**	**5.0E-08**	**0.1466**
YCAVWQRSGTSWVKIFA	TRGV1	2.60	0.21	NA
YCAVWQRSGTSWVKIFA	TRGV2	2.60	0.21	NA
YCAVWTGGTSWVKIFA	TRGV1	2.57	0.23	NA
YCAVWIEGAGTSWVKIFA	TRGV1	2.54	NA	NA
YCAVWMVSGTSWVKIFA	TRGV1	2.53	NA	NA
YCAVWMVSGTSWVKIFA	TRGV2	2.53	NA	NA
YCAVWINIGTSWVKIFA	TRGV1	2.51	NA	NA
YCAVWINIGTSWVKIFA	TRGV2	2.51	NA	NA
YCAVWGPTGTSWVKIFA	TRGV1	2.50	NA	NA
YCAVWKRSGTSWVKIFA	TRGV1	2.50	0.25	NA
YCAVWKRSGTSWVKIFA	TRGV2	2.50	0.25	NA
YCAVWTGSGTSWVKIFA	TRGV1	2.49	0.23	0.0001
YCAVWTGSGTSWVKIFA	TRGV2	2.49	0.23	0.0001

Figure 3.17 - Table of top TCRγ sequences enriched in D3G0 retrogenic mice during EAE

Enrichment of top 20 TCRγ sequences in D3G0 retrogenic mice immunized for EAE relative to healthy control mice with FDR adjusted p-value and CNS frequencies. Data representative of 2 independent experiments. NA indicates sequence was not detected in CNS γδ T cell population. Bold indicates the G1 TCRγ sequence.

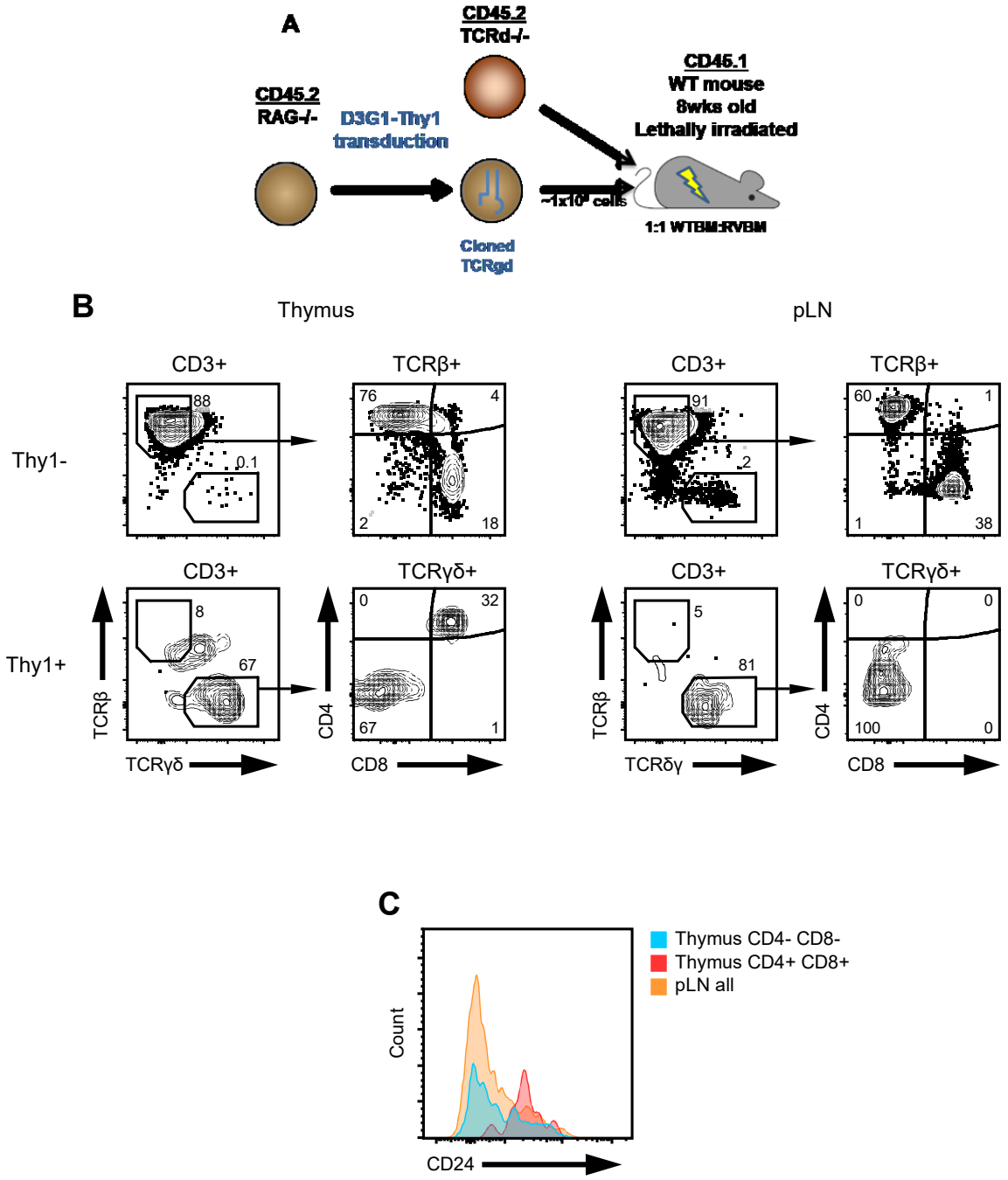


Figure 3.18 – Development of full-TCRγδ retrogenic mice

(A) Outline of full TCRγδ retrogenic mouse setup. **(B)** Representative FACS plots of thymic and pLN lymphocytes from D3G1 retrogenic mice. Thy1.1 represents an expression marker for the D3G1 construct. **(C)** Expression of CD24 as a maturity marker for indicated populations of γδ T cells.

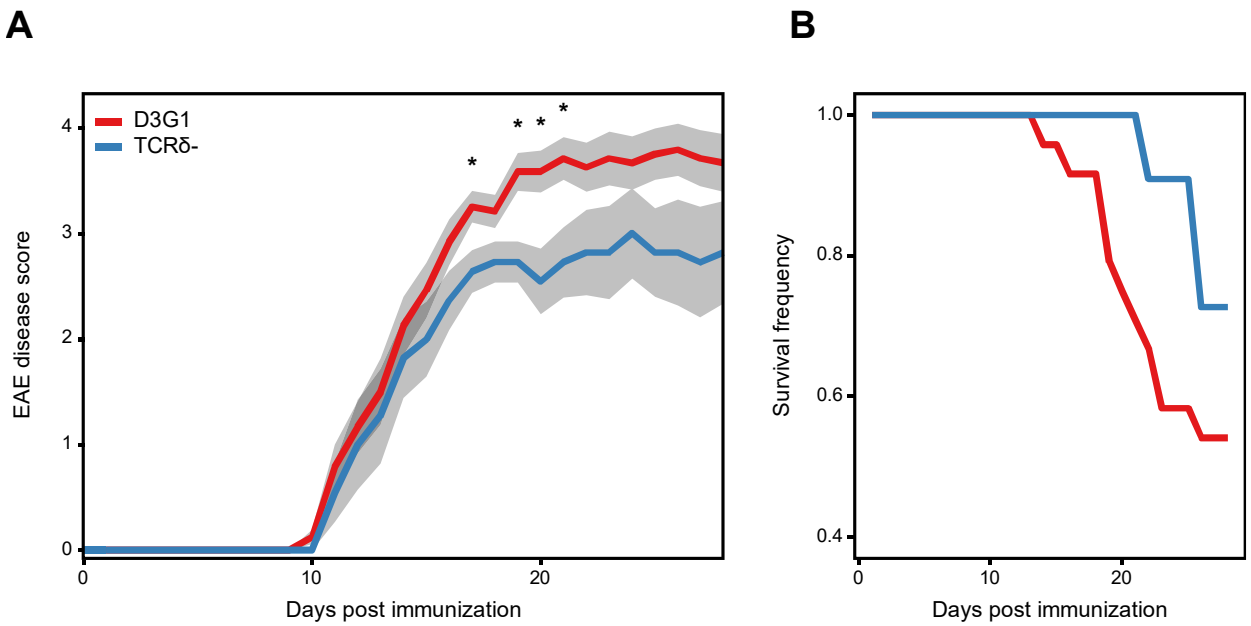


Figure 3.19 – D3G1 γδ T cells are sufficient to exacerbate EAE

(A) Clinical disease scores and **(B)** survival of EAE-immunize mice. Experimental groups include D3G1 retrogenic mice (n = 22) or TCRδ- bone marrow chimeras (n = 11) containing no γδ T cells. (*) indicates $p < 0.05$. Error ribbon = ±1 s.e.m.

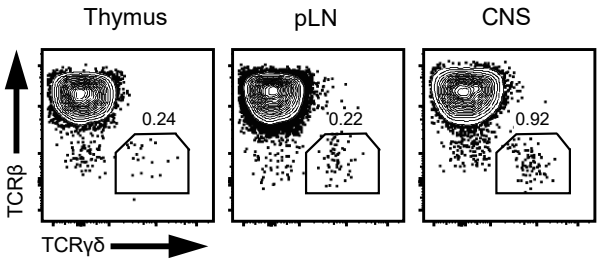


Figure 3.20 – D3G1 γδ T cells can traffic to the CNS

Representative FACS plots from indicated tissues of D3G1 retrogenic mice immunized for EAE. Previously gated on CD3+ lymphocytes with exclusion of Thy1- (residual host cells, post-irradiation) γδ T cells.

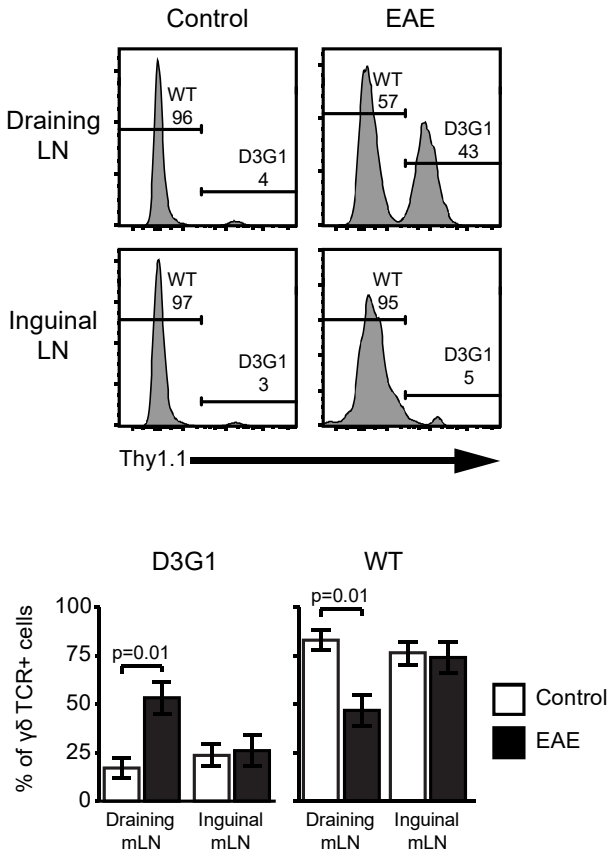


Figure 3.21 – D3G1 γδ T cell expansion in EAE is TCR-specific

D3G1 mice, with additional congenically marked WT compartment, immunized for EAE or kept as controls. Representative plots and quantification of D3G1 and polyclonal γδ T cell proportions. n≥9 for each experimental group. Data representative of 2 independent experiments. Error bars = ±1 s.e.m. P-value indicates results of Wilcox rank sum test.

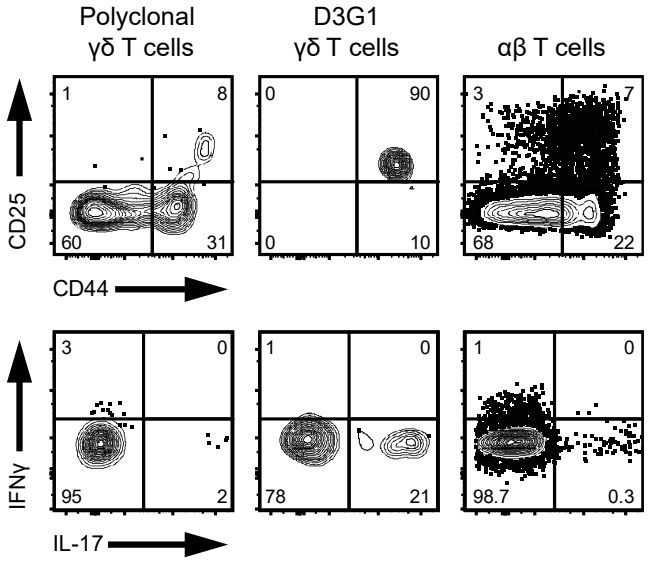


Figure 3.22 – D3G1 γδ T cells exhibit a pre-activated phenotype

Representative FACS plots of indicated populations from D3G1 retrogenic mouse with additional WT bone marrow compartment.

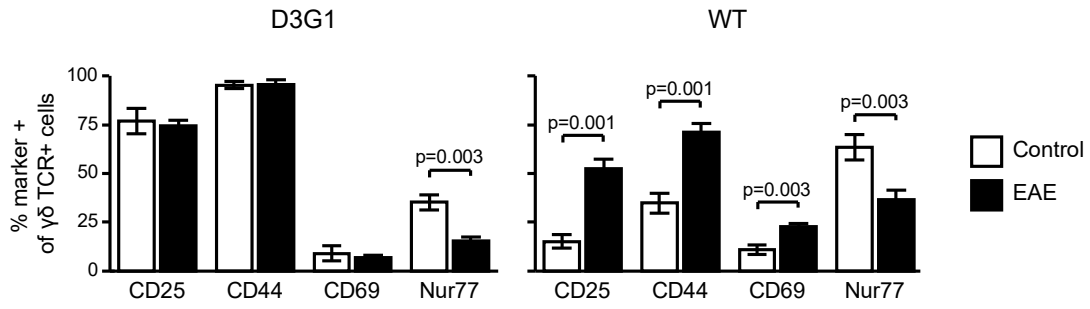


Figure 3.23 – D3G1 γδ T cell activation markers do not increase during EAE

Proportions of indicated γδ T cell fractions expressing corresponding activation markers in EAE-immunized or control mice. n≥9 for each experimental group. Data representative of 2 independent experiments. Error bars = ±1 s.e.m. P-value indicates results of FDR-corrected Wilcox rank sum test.

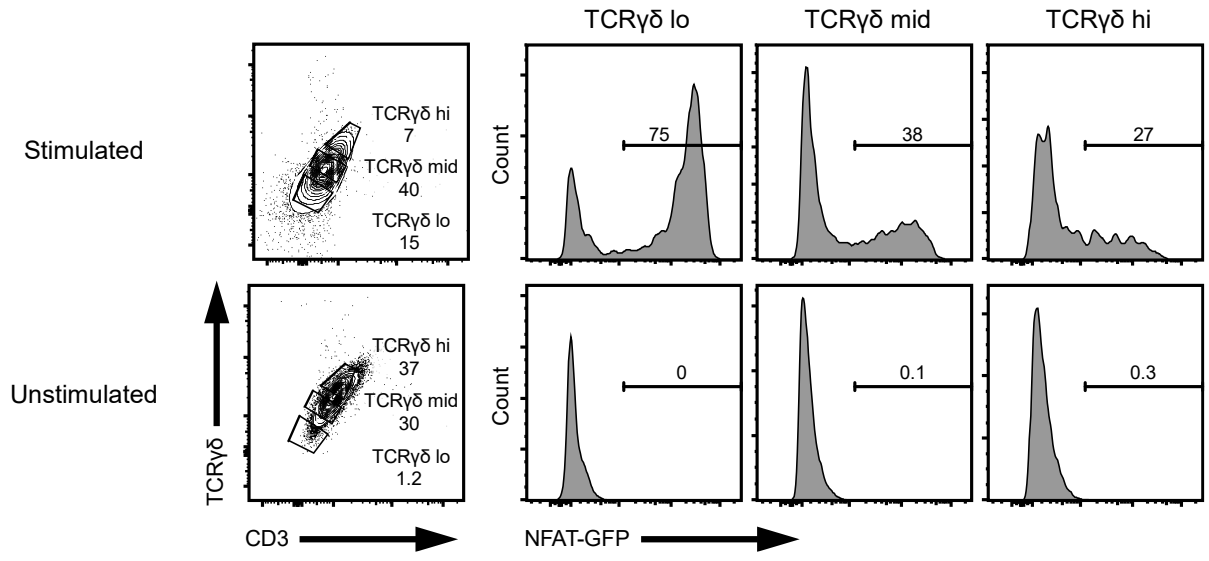


Figure 3.24 – The γδ TCR is downregulated upon activation

Representative FACS plots of D3G1 expressing T cell hybridomas bearing an NFAT-GFP reporter construct. Indicated cells stimulated by culture in plate coated with 25 μg/mL of anti-CD3.

Chapter 4: Conclusions and Future Directions

RORγt+ Treg cells in immune tolerance

Role of transcription factor co-expression in Treg cells

In our study, we show that TCR specificity is sufficient to drive the development of Foxp3+RORγt+ cells and that these cells can develop through an RORγt-Foxp3+ intermediate. These observations, combined with data demonstrating that Foxp3+RORγt+ can play an immune regulatory role, strongly suggest that these cells represent a subset of Treg cells that co-express RORγt. As discussed previously, the co-expression of a generally pro-inflammatory transcription factor with Treg cell-specific Foxp3 is surprising, though not unprecedented. Examples of Treg cell subsets co-expressing T-bet (Koch et al., 2009), GATA-3 (Wang et al., 2011), IRF4 (Zheng et al., 2009), and STAT3 (Chaudhry et al., 2009) have all been previously identified. While all of these Treg populations have been shown to possess immunoregulatory properties, the necessity for transcription factor co-expression is not completely understood.

The clearest explanation for transcription factor co-expression in Treg cells comes from T-bet+ Treg cells. T-bet expression allows these cells to traffic to sites of T_H1 inflammation due to the upregulation of chemokine receptors shared with T-bet+ T_H1 cells (Koch et al., 2009). Similarly, STAT3+ Treg cells express CCR6, which is used by T_H17 cells for trafficking to mucosal tissues (Wang et al., 2009a). However, for both STAT3+ and T-bet+ Treg cells, the role of chemokine receptors in target suppression is still speculatively based on correlation and future study using Foxp3-specific deletion of these receptors is needed.

Interestingly, in the absence of CCR6, mice develop a T_H1 mediated colitis, with a loss of both mucosal T_H17 and Treg cells (Wang et al., 2009b). This suggests that loss of CCR6 affects the

homing of both T_H17 cells and their corresponding STAT3+ Treg cells. However, given that immune pathology occurs in the absence of the effector subset they are proposed to most specifically target, it is clear that the immunoregulatory function of STAT3+ Treg cells extend beyond a simple one-to-one relationship with T_H17 cells. Moreover, this represents a disconnect between the T_H17 suppression of STAT3+ Treg cells and the proposed T_H1 suppression of CCR6+ Treg cells. While these alternatives are not necessarily mutually exclusive, the readily distinguished phenotypes of STAT3 and CCR6 deficient mice suggest that additional mechanisms likely underlie the suppressive capabilities of transcription factor co-expressing Treg cells and additional study is necessary to uncover them.

Antigen specificity of RORγt+ Treg cells

With our TCR sequencing data, we demonstrate that, though largely distinct, the Foxp3+RORγt+ TCR repertoire shares a set of high frequency TCRs with the T_H17 repertoire. This overlap of antigen specificity lends support to the hypothesis that RORγt+ Treg cells can suppress T_H17 mediated inflammation. However, since the majority of RORγt+ Treg TCRs are not shared with T_H17 cells, this raises the question of what particular ligands are recognized by the remainder of this population. One possible hypothesis is that RORγt+ Treg and T_H17 cells do, in general, recognize ligands derived from the same source of antigens, but that the difference in TCR repertoire reflects variation in specific TCR epitopes or TCR affinities to the same epitopes. A similar phenomenon is observed with thymically derived Treg cells, which can recognize antigens shared with conventional CD4+ T cells, despite possessing a distinct TCR repertoire (Lee et al., 2012a). In this case, the difference in repertoire reflects the fact that Treg cells utilize TCRs that have higher affinity for the same ligand than conventional CD4+ T cells.

In addressing this possibility for RORγt+ Treg and T_H17 cells, segmented filamentous bacteria (SFB) may represent a useful tool. SFB promotes the differentiation of both Foxp3+RORγt+ (Sefik et al., 2015) and T_H17 cells (Ivanov et al., 2009) and comparisons of the TCR repertoires used by these populations in response to SFB could demonstrate whether the two subsets depend on the same TCR sequences to recognize this bacterium. If they do not, SFB-reactive TCRs unique to either RORγt+ Treg or T_H17 cells could be used to investigate the basis of TCR discrimination between these two populations.

Alternatively, the majority of RORγt+ Treg cells might recognize entirely distinct ligands from T_H17 cells. In addition to SFB, Sefik *et al.* identify multiple cultured bacteria that, when used to monocolonize mice, can promote RORγt+ Treg cell development (Sefik et al., 2015). However, as limited information is available regarding individual bacteria capable of inducing RORγt- Treg and T_H17 cells, it is currently difficult to discern how specific these bacteria are to the development of RORγt+ Treg cells compared to other mucosal T cell populations. Moreover, as the commensal microbiota typically contains a vast number of microbial species, it is hard to predict the precise significance of results obtained from mice monocolonized with a single species.

Identifying the antigen to CT2 will be beneficial in determining what unique RORγt+ Treg ligands might look like. CT2 represents a TCR that is highly specific to the Foxp3+RORγt+ T cell population and shows essentially no T_H17 development. Thus, if RORγt+ Treg cells do recognize unique antigens, the CT2 TCR ligand represents an excellent candidate for such an antigen. However, while we have previously shown that CT2 does indeed react to commensally derived antigens (Lathrop et al., 2011), we have yet to determine what the precise source of these antigens are and further study will be needed to identify them.

Antigen-specific γδ T cell responses

Generalized role for γδ T cells in neuroinflammation

While our data and others' suggest that γδ T cells clearly contribute to the pathogenesis of EAE, autoimmune inflammation is certainly not a desirable outcome for a host organism. Therefore, while these studies provide information as to how γδ T cells function, they do not directly help reveal the immunological benefit that has evolutionarily maintained γδ T cells since the emergence of the vertebrate immune system (Criscitiello et al., 2010). One possible explanation of our observations is that the γδ T cell response during EAE may reflect a beneficial response of neuroprotection towards CNS-tropic pathogens. Interestingly there are several CNS viral infections that have noted γδ T cell involvement. For example, γδ T cells alone are sufficient to protect mice from herpes simplex virus (HSV)-1 encephalitis in the absence of αβ T cells, while the absence of both αβ and γδ T cell lineages leads to significant mortality in response to viral infection (Sciammas and Kodukula, 1997). Moreover, γδ T cells can also recognize HSV-1 glycoproteins directly via their TCR (Sciammas and Bluestone, 1998; Sciammas et al., 1994).

γδ T cells play an even more prominent role in West Nile virus (WNV) infection. In contrast to HSV-1, both αβ and γδ T cells are necessary for protection against WNV infection, as mice lacking either populations show increased encephalitis-related mortality (Wang et al., 2003). Moreover, the response of γδ T cells to WNV is dependent on IFNγ (Shrestha et al., 2006) and helps to promote the memory response of CD8+ αβ T cells (Wang et al., 2006). Most interesting is the reciprocal role of Vγ1+ and Vγ4+ γδ T cells during WNV infection. Treatment with anti-Vγ4 antibodies promotes host survival to WNV, while anti-Vγ1 antibody treatment enhances WNV lethality (Welte et al., 2008). As discussed in Chapter 3, Blink *et al.* demonstrated that such antibody treatment results in γδ T cell activation (Blink et al., 2014), suggesting that activated Vγ4+ contribute

towards the protective anti-WNV response, while enhanced Vγ1+ γδ T cell responses are detrimental.

Notably, these results with WNV are the inverse of what has been reported for EAE. Whereas activated Vγ4+ γδ T cells are protective during WNV, they are pathologic in EAE. Similarly, whereas Vγ1+ γδ T cells blunt EAE severity, they enhance the lethality of WNV (Blink and Miller, 2009). A hypothesis consistent with these observations is that the Vγ4+ γδ T cell response is a natural mechanism of protection against CNS-tropic pathogens while Vγ1+ γδ T cells play a more immunomodulatory role. Future studies could utilize our fixed TCRδ-system to assess whether the repertoire of γδ TCRs that expand during the course of WNV infection are similar to those seen during EAE. Moreover, as D3G1-expressing γδ T cells exacerbate EAE pathogenesis, this TCR could be used to test whether γδ T cells that contribute to EAE can expand and contribute towards host survival in the context of WNV infection.

Antigen specificity of D3G1 γδ T cells

As discussed in the Chapter 1, antigen specificity is one of the major ways in which αβ and γδ T cells differ. Whereas αβ T cells almost exclusively recognize peptide antigens in the context of MHC presenting proteins, γδ T cells recognize diverse classes of molecules, largely in the absence of any presenting mechanism. However, for most of the known γδ TCR ligands, the physiological role of that particular TCR:ligand interaction is unclear. For example, one of the most well studied γδ TCRs, KN6, recognizes the MHC-1 like molecules T-10 and T-22 (Ito et al., 1990), yet the biological significance of this interaction is unknown. T-10 and T-22 appear to be upregulated in response to cell stress, but there is no evidence suggesting this upregulation has any effect on the function of KN6 γδ T cells.

Given the role we show for the D3G1 TCR in EAE, this TCR may represent an ideal system to study biologically relevant γδ TCR:ligand interactions *in vivo*. However, a major obstacle that must first be overcome is to identify the D3G1 ligand. Fortunately, there are some clues to what this ligand could be. In the study of γδ T cell response during collagen induced arthritis (CIA), the authors performed γδ TCR sequencing to demonstrate clonal expansion of γδ T cells during disease (Roark et al., 2007). While this data was not gathered in a way that preserves information regarding TCRγ and TCRδ sequencing pairing, the most enriched TCRγ and TCRδ chains during CIA are identical to those of the D3G1 TCR. This suggests that the D3G1 TCR ligand may be shared by CIA and EAE.

The induction of CIA and EAE is similar, both involving the injection of an immunogenic peptide emulsified in complete Freund's adjuvant (CFA). As CFA is shared by both of these models, it represents a natural candidate for a source of D3G1 TCR ligands. Moreover, in the study of CIA, CFA alone, in the absence of collagen peptide, was sufficient to promote γδ T cell proliferation. Interestingly, γδ T cells are already known to recognize antigens from mycobacteria, which represent a principal component of CFA (Happ et al., 1989; O'Brien et al., 1989; Shen et al., 2002). However, we have attempted to use our D3G1 TCR expressing hybridomas to test direct reactivity to CFA *in vitro* without success.

Though binding of the γδ TCR to soluble ligand has been demonstrated for some antigens (Zhang et al., 2010), the interaction of D3G1 may be more complex. Instead the D3G1 TCR may require the presence of a cell surface accessory molecule, such as that seen with butyrophilin-mediated recognition of phosphoantigens by human γδ T cells (Vavassori et al., 2013). Alternatively, the D3G1 ligand may itself be a cell surface molecule, simply upregulated in response to components of CFA. While preliminary experiments using CFA treated cells as a source of D3G1 ligand have yet to identify an antigen, further systematic analysis is needed. In addition, the use of

soluble TCR molecules as an MHC tetramer-like reagent may also be useful in identifying a cell surface ligand.

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