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T-cell Receptor CDR3 Sequence but Not Recognition Characteristics Distinguish Autoreactive Effector and Foxp3+ Regulatory T-cells

Xin Liu

University of Tennessee Health Science Center

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T-cell Receptor CDR3 Sequence but Not Recognition Characteristics Distinguish Autoreactive Effector and Foxp3+ Regulatory T-cells

Abstract

Regulatory T-cells (Treg) play an important role in maintaining immune tolerance to self-antigens and in suppressing excessive immune responses in the host. The source, specificity, and plasticity of the forkhead box transcription factor 3 (Foxp3⁺) Treg and conventional T (Tconv) cell populations active at sites of autoimmune pathology are not well characterized. To evaluate this, we combined global repertoire analyses and functional assessments of isolated T-cell receptors (TCR) from TCRA retrogenic mice with autoimmune encephalomyelitis (EAE). Treg and Tconv cell TCR repertoires were distinct, and autoantigen-specific Treg and Tconv cells were enriched in diseased tissue. Autoantigen sensitivity and fine specificity of these cells intersected, implying that differences in responsiveness were not responsible for lineage specification. Notably, autoreactive Treg and Tconv cells could be fully distinguished by an acidic versus aliphatic variation at a single TCR CDR3 residue. Our results imply that ontogenically distinct Treg and Tconv cell repertoires with convergent specificities for autoantigen respond during autoimmunity and argue limited plasticity between Treg and Tconv cells during autoimmune inflammation.

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**T-CELL RECEPTOR CDR3 SEQUENCE BUT NOT RECOGNITION
CHARACTERISTICS DISTINGUISH AUTOREACTIVE EFFECTOR
AND FOXP3⁺ REGULATORY T-CELLS**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Xin Liu
May 2010

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DEDICATION

This dissertation is dedicated to my parents,
Mingxian Guan and Mingfang Liu,
My husband Ziwei Zhang,
My two adorable boys Victor and Derek,
for their love and support

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ABSTRACT

Regulatory T-cells (Treg) play an important role in maintaining immune tolerance to self-antigens and in suppressing excessive immune responses in the host. The source, specificity, and plasticity of the forkhead box transcription factor 3 (Foxp3⁺) Treg and conventional T (Tconv) cell populations active at sites of autoimmune pathology are not well characterized. To evaluate this, we combined global repertoire analyses and functional assessments of isolated T-cell receptors (TCR) from TCR α retrogenic mice with autoimmune encephalomyelitis (EAE). Treg and Tconv cell TCR repertoires were distinct, and autoantigen-specific Treg and Tconv cells were enriched in diseased tissue. Autoantigen sensitivity and fine specificity of these cells intersected, implying that differences in responsiveness were not responsible for lineage specification. Notably, autoreactive Treg and Tconv cells could be fully distinguished by an acidic versus aliphatic variation at a single TCR CDR3 residue. Our results imply that ontogenically distinct Treg and Tconv cell repertoires with convergent specificities for autoantigen respond during autoimmunity and argue limited plasticity between Treg and Tconv cells during autoimmune inflammation.

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CHAPTER 1. INTRODUCTION

1.1 An Introduction to Experimental Allergic Encephalomyelitis (EAE)

Experimental allergic encephalomyelitis (EAE) is a demyelinating disease of the central nervous systems. For over half a century, it has been used as an animal model of human MS. EAE can be induced in different species including mice, rats, guinea pigs, rabbits, macaques, rhesus monkeys and marmosets, among which mice and rats are the most commonly used species due to their low cost, ready availability, lifespan and large number of genetically modified strains. EAE can be an acute, chronic, or relapsing-remitting type disease depending on the induction method and types of animal. For example, C57BL/6 mice develop EAE 8-14 days after immunization and may remain paralyzed for a prolonged period (Mendel *et al.*, 1995). In contrast, SJL mice resemble the relapsing-remitting form of MS, with alternating episodes of paralysis and recovery (Whitham *et al.*, 1991).

EAE is most commonly induced in mice by immunization with CNS-derived auto-antigens in complete Freund's adjuvant (CFA). Myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅), myelin proteolipid protein (PLP₁₃₉₋₁₅₁) and myelin basic protein (MBP₁₋₉) are immunodominant epitopes in the C57BL/6, SJL and B10.PL mice strains respectively (Zamvil and Steinman, 1990). Alternatively, passive transfer of restimulated T-cells that recognize myelin autoantigens can also induce EAE (Yoshizawa *et al.*, 1998; Zamvil *et al.*, 1985). Disease progression can be monitored by clinical observation, as an ascending paralysis or paresis develops in affected animals. The following scoring system is most commonly applied (Table 1-1).

1.1.1 EAE Pathogenesis

In the healthy individual, lymphocytes traffic into the central nervous system (CNS) is tightly restricted by the blood-brain barrier (BBB). After immunization with

Table 1-1. EAE clinical score.

Score	Clinical Symptoms
0	Healthy
1	Limp tail, loss of tail tone
2	Limp tail and hind limb paraparesis
3	Complete hind limb paralysis
4	Complete hind limb and partial front limb paralysis
5	Morbid

MOG₃₅₋₅₅ in CFA, circulating lymphocytes and monocytes traffic across the BBB. T-cells are restimulated by myelin presented by dendritic cells in the CNS, resulting in inflammation and demyelination. The recruitment process is mediated by the sequential interaction of different adhesion molecules on lymphocytes and the inflamed BBB.

During the course of EAE, CNS endothelium upregulates expression of inter-cellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Engelhardt, 2006; Steffen *et al.*, 1994). Activated myelin-specific T-cells upregulate adhesion molecules and chemokine receptors, allowing them to adhere and transmigrate through the endothelium (Alt *et al.*, 2002). Blocking antibodies against VCAM-1 or its ligand, α 4-integrins, inhibits the development of EAE and inflammatory T-cell accumulation in the CNS, implying that the recognition of VCAM-1/ α 4-integrins mediates the adhesion of T-cells to inflamed CNS vessels (Engelhardt *et al.*, 1998). Lymphoid chemokines CCL19 and CCL21 are also found to be expressed in BBB endothelium and CNS venules during EAE (Alt *et al.*, 2002). Their common receptor CCR7 is detected on encephalitogenic T-cells. Functional ablation of CCR7 or blocking CCL19 and CCL21 reduces the accumulation of T-cells to inflamed venules in the CNS, suggesting that CCL19 and CCL21 are involved in the recruitment of encephalitogenic T-cells. Besides, the β 2-integrin LFA-1 expressed on encephalitogenic T-cells was required for transendothelial migration by engaging with endothelial ICAM-1 and/or ICAM-2 (Laschinger *et al.*, 2002; Lyck *et al.*, 2003).

The key histopathologic feature of EAE is the destruction of the axonal myelin sheath in the CNS. This histopathology mimics that seen in MS, with T-cell and macrophage dominated inflammation, extensive demyelination, axonal and neuronal damage. CD4⁺ T-cells, CD8⁺ T-cells, B-cells and activated microglia/macrophages are responsible for the lesion development in patients with MS. One difference in most, but not all mouse systems, is an increased reliance on CD4⁺ T-cells and macrophages, with smaller roles for CD8⁺ T-cells and B lymphocytes, though in some cases polymorphs are also detected in acute EAE lesions (Sobel and Kuchroo, 1992). Most myelin-reactive T-cells are deleted during thymic development because many myelin antigens were expressed in the thymus. However, the deletion cannot be complete, and auto-reactive T-cells escaping negative selection constitute the peripheral T-cell repertoire and must be kept in check by peripheral tolerance. In EAE, auto-reactive T-cells have to be activated by local antigen presenting cells (APCs) and appropriate costimulatory molecules (Chang *et al.*, 1999), then migrate across the BBB into the CNS to initiate inflammation and tissue injury.

1.1.2 TCR Repertoire Development

1.1.2.1 Thymocyte Development

The T-cell receptor (TCR), expressed on the surface of T-lymphocytes, is responsible for antigen recognition. It is a heterodimer composed of two distinct chains,

α and β chain in 95% of T-cells, and γ and δ chains in 5% of T-cells (Davis *et al.*, 1998). The $\alpha\beta$ TCR requires coexpression of invariant CD3 polypeptides, a collection of protein dimers ($\gamma\epsilon$, $\delta\epsilon$ and $\zeta\zeta$). That associate with the $\alpha\beta$ or $\gamma\delta$ chains. Neither CD3 nor $\alpha\beta$ dimer are expressed on the cell surface separately, and the partner proteins are required to prevent the isolated protein from degradation (Manolios *et al.*, 1991). The Ig-like $\alpha\beta$ heterodimer is responsible for antigen recognition. Transfection of cDNAs encoding α and β chains from one T-cell clone into another results in the transfer of reactivity to both peptide antigen and MHC (Dembic *et al.*, 1986). Antibodies directly stimulating the CD3 molecules elicit the same T-cell activation as stimulation with pMHC. The CD3 molecules initiate signal transduction upon antigen recognition. This process relies on the cytoplasmic domains of the CD3 molecules which contain immunoreceptor tyrosine-based activated motifs (ITAMs). Activation of T-cells leads to phosphorylation of these residues and their association with protein tyrosine kinases (PTKs), such as tyrosine-protein kinase (fyn), leukocyte-specific protein tyrosine kinase (lck), and zeta-chain associated protein kinase (ZAP-70), that further propagate the signal into the cell (Figure 1-1).

Similar to immunoglobulin heavy and light chains, all TCR α polypeptides have distinct variable (V) and joining (J) regions. In addition to V and J segment, the TCR β contains diversity (D) segments. During the T-cell development in the thymus, β -chain genes undergo rearrangement. First, a D β locus rearranges to a J β locus, then these associate with a V β . If the rearrangement produces an in frame TCR β , the functional β chain will pair with a surrogate α chain, Pre-T-cell α (pT α), and is expressed on the cell surface along with the CD3 molecules. Failure to successfully rearrange a β chain leads to clonal deletion. The assembly of the CD3: pT α leads to cell proliferation, the arrest of further β chain rearrangement and the expression of CD8 and CD4. In the double positive thymocytes, TCR β rearrangement must be followed by a successful TCR α gene rearrangement within approximately 3 days after the proliferative burst. Otherwise, cells die of “neglect”. The double positive thymocytes express a low level of T-cell receptors and must pass a second TCR dependent checkpoint – positive selection. The criteria here are more stringent than for β selection. They require both a translatable protein coding sequence and recognition of self MHC molecules. If the TCR cannot bind to self MHC molecules, it will be neglected and die. Cells passing positive selection express high levels of the TCR, and undergo negative selection, in which self reactive cells are eliminated. These critical developmental checkpoints yield a TCR repertoire that can respond to a variety of foreign antigens, but minimizes self-reactivity. Only 5% of the double positive cells satisfactorily pass the checkpoints and mature into single positive T-cells that exit the thymus and populate the periphery.

1.1.2.2 TCR Repertoire Diversity

TCR repertoire diversity results from combinatorial diversity and junctional diversity. Combinatorial diversity is generated by somatic recombination from multiple germline gene segments. The mouse TCR α locus has ~100 V α (TRAV) gene, and ~50 J α

Figure 1-1. Signaling cascades stimulated by the T-cell receptor.

Adapted with permission. Razzaq, T.M., Ozegbe, P., Jury, E.C., Sembi, P., Blackwell, N.M., and Kabouridis, P.S. (2004). Regulation of T-cell receptor signalling by membrane microdomains. *Immunology* 113, 413-426.

(TRAJ) gene segments. The TCR β locus has 25 V β (TRBV), 2 D β (TRBD) and 12 J β (TRBJ) gene segments. The maximum number of possible combinations of these gene segments is the product of the numbers of V, J and D segments in each locus. The diversity implied therein would be much less than the actual diversity of antigen receptors in mature lymphocytes. In fact, a large contribution to the TCR diversity is made by the removal or addition of nucleotides between V and D, D and J, or V and J segments at the time of joining, namely, junctional diversity. Nucleotides (P sequences) may be added to broken DNA ends to repair asymmetric RAG-mediated cleavage. Other nucleotides (N regions) may be added to the sites of VD, VJ, or DJ junctions by the action of the terminal deoxynucleotidyl transferase (TdT), generating new sequences that are not present in the germline (Figure 1-2). In addition, random pairing of α and β chains introduces additional diversity. Taking all these factors together, a theoretical maximum diversity is 10^{15} unique TCR. In the thymus, 95% of the precursor cells are eliminated through both positive and negative selection. This still leaves more than 10^{13} possible TCRs that can be produced. The actual diversity of the naive mouse spleen T-cell repertoire is estimated to be $\sim 2 \times 10^6$ using the immunoscope technique, suggesting the average size of a clone expressing a single TCR is ~ 10 cells in spleen (Casrouge *et al.*, 2000).

TCR diversity is localized to complementarity-determining regions (CDRs) on both chains. CDR1 and 2 regions, encoded by the V gene segment, are responsible for interacting with MHC molecules; whereas the CDR3 is the most variable part of the TCR. It is encoded by the juxtaposition of rearranged V-(D)-J or V-J segments. CDR3 forms a flexible loop positioned to associate with the peptide-MHC complexes. Characterization of CDR3 sequence variation therefore provides a good measure of TCR diversity in an antigen selected repertoire.

Because many more TCRs may form than the number of T-cell clones that enter the circulation, there are huge differences between repertoires among individuals. In most T-cell responses, the TCR repertoires consist of TCRs with private specificity, that is TCR only observed within one individual. In addition public or shared TCRs among multiple individuals may be seen. Public TCRs are observed in both acute and persistent pathogen infection and autoimmune diseases such as EAE. Why they exist is unclear. A potential explanation is certain bias promotes selection of certain TCR. That includes unique structural features of TCR or the pMHC complex, biased TdT activity and convergent recombination, and selective expansion in periphery.

1.2 Regulatory T-cells

T-cells are crucial to adaptive immunity and function both as effector cells and helper cells in humoral and cell mediated responses. The activated immune system also requires homeostatic control mechanisms to down-regulate over-exuberant immune responses. Tolerance to self-antigens includes a central and a peripheral component. Central tolerance refers to negative selection of auto-reactive T-cells in thymus. Peripheral tolerance consists of T-cell deletion, T-cell anergy and regulatory T-cell (Treg)

Figure 1-2. Generation of T-cell receptor diversity by somatic recombination of TCR gene segments.

Reprinted with permission. Turner, S.J., Doherty, P.C., McCluskey, J., and Rossjohn, J. (2006). Structural determinants of T-cell receptor bias in immunity. *Nat Rev Immunol* 6, 883-894.

suppression in the periphery (Miller and Heath, 1993; Schwartz, 1990). In mouse neonatal thymectomy (nTx) studies, nTx on the third day of life (d3Tx), but not d1Tx or d7Tx, developed on autoimmune disease that could be prevented by thymus transplant or CD4⁺ T-cell reconstitution from normal adult mice (Nishizuka and Sakakura, 1969). This suggested that autoreactive T-cells were exported from the thymus earlier than the suppressor cells, and that these cells were also CD4⁺ T-cells.

Sakaguchi *et al.* first proposed that a subset of CD4⁺ T-cells, that constitutively express the CD25 antigen (IL-2R α chain), function as regulatory T-cells in the normal mouse (Asano *et al.*, 1996; Sakaguchi *et al.*, 1995; Suri-Payer *et al.*, 1998). Depletion of CD4⁺CD25⁺ T-cells leads to organ specific autoimmune disease in mice, and adoptive transfer of the CD25⁺ cells prevent autoimmunity. CD4⁺CD25⁺ T-cells represent 10%-15% of mouse CD4⁺ lymphocytes, and 5%-10% of CD4⁺ SP thymocytes. Compared with CD4⁺CD25⁻ T-cells, CD4⁺CD25⁺ T-cells express slightly higher levels of CD5, cytotoxic T-lymphocyte antigen (CTLA-4) and glucocorticoid-induced TNF-like receptor (GITR), lymphocyte-activation gene (LAG-3), 4-1BB, CD28, CD134, CD80/86 (B7) as well as intermediate and low levels of CD45RB. However, none of those is restricted to Treg cells (Ndhlovu *et al.*, 2004; Paust *et al.*, 2004; Workman *et al.*, 2009). One unique feature of Treg cells is that they express the X chromosome-encoded forkhead transcription factor 3 (Foxp3), which serves as a key player in the biology of CD4⁺CD25⁺ Treg cells (Brunkow *et al.*, 2001; Fontenot *et al.*, 2003; Hori *et al.*, 2003; Khattri *et al.*, 2003). Expression of Foxp3 is largely restricted to Treg cells and requires Treg cell differentiation (Fontenot *et al.*, 2003; Fontenot *et al.*, 2005b; Hori *et al.*, 2003; Wan and Flavell, 2007). IPEX syndromes (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) in humans and scurfy mice have mutations in the Foxp3 gene. Foxp3 deficient mice show defects in Treg development, and retroviral transduction of Foxp3 into naive CD25⁻ T-cells led to the acquisition of suppressor activity both *in vitro* and *in vivo* (Fontenot *et al.*, 2005b).

1.2.1 Suppressor Mechanisms Utilized by Regulatory T-cells

Defining the mechanisms of Treg suppressive function is crucial in understanding of peripheral tolerance. It has been shown that Treg cells utilize multiple means to limit immune responses, such as suppression by cell contact-dependent cytotoxicity on responder T-cells or APCs, suppression by inhibitory cytokines or deprivation of cytokines, suppression by surface molecules (CTLA-4 or GITR), and suppression by modulation of dendritic cell maturation (Vignali *et al.*, 2008).

1.2.1.1 Role of Cytotoxicity

An early study has shown that CD25⁺ T-cells were unable to suppress the *in vitro* proliferation of responder CD4⁺CD25⁻ T-cells when the two populations were separated by a semi-permeable membrane. This suggests that suppression requires cell contact or close proximity between Treg cells and their targets (Thornton and Shevach, 1998).

Human activated CD4⁺CD25⁺Foxp3⁺ Treg cells express granzyme A and kill activated CD4⁺ and CD8⁺ T-cells in a perforin-dependent, Fas-FasL-independent manner. Activation of mouse Treg cells also results in up-regulation of granzyme B expression (Gondek *et al.*, 2005; Grossman *et al.*, 2004). However, another study demonstrated that highly activated Treg cells could kill APCs but not bystander B-cells or T-cells in a cell-contact-dependent manner (Zhao *et al.*, 2006). Besides granzyme A and B, a recent study showed that galectin-1, which can induce T-cell apoptosis, was up-regulated in human and mouse Treg cells, and galectin-1 deficient Treg cells have reduced suppressive function *in vitro* (Garin *et al.*, 2007). Nevertheless, these studies suggest more work is needed to determine whether Treg cells can develop into cytotoxic cells *in vivo* and the cytolytic mechanisms.

1.2.1.2 Role of Immunosuppressive Cytokines

In vivo studies have shown that the inhibitory cytokines such as IL-10, transforming growth factor (TGF- β) are required for Treg mediated suppression in some disease models. In allergy and asthma models, Treg cells control disease in a manner partially dependent on IL-10 (Hawrylowicz and O'Garra, 2005) or on both IL-10 and TGF- β (Joetham *et al.*, 2007). In colitis studies, administration of a blocking anti-IL-10 receptor mAb abrogated suppression of colitis by CD4⁺CD45RB^{low} T-cells, and Treg cells from IL-10 deficient mice failed to suppress colitis, although they suppress autoimmune gastritis (AIG) (Asseman *et al.*, 1999). In EAE model, Treg cells have been shown to produce IL-10 in CNS during recovery phase (Kohm *et al.*, 2002; Korn *et al.*, 2007; McGeachy *et al.*, 2005). One study showed that Treg cells from IL-10 deficient mice were unable to suppress active EAE (Zhang *et al.*, 2004). However, the Treg cell-specific ablation of IL-10 showed no spontaneous systemic autoimmunity, but result in enhanced pathology in the mucosal surface of the intestine and the lung (Rubtsov *et al.*, 2008). Production of TGF- β by Treg cells is also shown indispensable for suppression of colitis (Fahlen *et al.*, 2005; Zwar *et al.*, 2006). However, other studies have failed to identify the role for TGF- β in Treg-mediated suppression in IBD. In this study, Treg cells from neonatal TGF- β 1 deficient mice can still suppress the incidence and severity of IBD (Kullberg *et al.*, 2005). Taken together, these findings suggest that the importance of IL-10 and TGF- β in Treg mediated suppression is dependent on the environment and experimental setting.

In contrast to the *in vivo* studies, there is little evidence that suppressive cytokines play a role in Treg-mediated suppression *in vitro*. For example, the addition of neutralizing anti-IL-10 or anti-IL-4 antibodies to *in vitro* cultures failed to abrogate suppressive effects of Treg cells. And CD4⁺CD25⁺ T-cells purified from mice deficient in IL-4 or IL-10 showed comparable suppression to wild-type Treg (Thornton and Shevach, 1998). The role of TGF- β in suppression is ambiguous. One study showed that inhibition of *in vitro* suppression was observed in the presence of high concentrations of a neutralizing anti-TGF- β antibody, and the author proposed that Treg cells exert suppression by a cell-cell interaction involving cell surface TGF- β 1 (Nakamura *et al.*, 2001). However, subsequent analyses have shown that responder T-cells from dominant-

negative TGF- β receptor mice were fully susceptible to suppression by Treg cells *in vitro*. Furthermore, Treg cells from neonatal TGF- β 1 deficient mice were as suppressive as Treg from wild-type mice, confirming TGF- β is not critical to the suppression by Treg cells *in vitro* (Piccirillo *et al.*, 2002). Studies demonstrated that the suppressor function of activated Treg cells *in vitro* is antigen nonspecific. When Treg cells from TCR transgenic mice were activated and expanded *in vitro*, the activated suppressors were able to inhibit fresh CD4⁺CD25⁻ T-cells specific for different antigens (Thornton and Shevach, 2000). Collectively, these studies demonstrated that the suppressive effects of Treg cells *in vitro* are less likely cytokine dependent and antigen non-specific.

Recently, a novel inhibitory cytokine, IL-35, has been described that is specifically produced by Treg and is required for maximal regulatory activity (Collison *et al.*, 2007). Ectopic expression of IL-35 confers regulatory activity on naïve T-cells and recombinant IL-35 suppresses T-cell proliferation *in vitro*. A later study showed Treg cells expressed substantial IL-35 following contact with Tconv cells, and this potent suppression was in a cell contact, IL-35 and IL-10 dependent manner (Collison *et al.*, 2009). Although the identification of IL-35 brings new information to Treg cell portfolio, there is much more that remains to be defined, such as its contribution to Treg function *in vivo*, or whether IL-35 plays a role in the development and function of DCs and macrophages.

IL-2 was identified as a T-cell growth factor many years ago (Schimpl and Wecker, 1972). Treg cells do not produce IL-2, but express high affinity IL-2 receptor (CD25). Therefore it was hypothesized that Treg cells may efficiently consume IL-2 and suppress proliferation of responder T-cells by competing for IL-2 in the local environment. However, this proposal is not supported by the Treg cells present in IL-2R α ^{-/-} mice that display similar *in vitro* suppressor activity to the wild-type counterpart (Fontenot *et al.*, 2005a). Therefore IL-2 responsiveness is dispensable in Treg suppression, but is crucial for the maintenance of Treg cells.

1.2.1.3 Role of CTLA-4 and GITR

Cytotoxic T-lymphocyte antigen4 (CTLA-4) is generally expressed on activated CD4⁺ T-cells, and it transduces a negative signal that results in dampening of T-cell activation following binding to CD80/CD86 ligands on APC (Oosterwegel *et al.*, 1999). CD25⁺ Treg cells constitutively express CTLA-4, suggesting its importance in Treg biology. This hypothesis was supported in the study that the addition of anti-CTLA-4 antibody or its Fab fragment abrogated CD25⁺ Treg mediated suppression of responder CD4⁺ T-cell proliferation *in vitro* (Takahashi *et al.*, 2000). However, this result has been difficult to reproduce by other groups (Thornton and Shevach, 1998). The role of CTLA-4 in Treg function is further supported by *in vivo* studies. For example, anti-CTLA-4 treatment abrogated Treg cells suppressing inflammatory bowel disease (IBD) (Read *et al.*, 2000), or induced AIG in BALB/c mice, suggesting that interaction between CTLA-4 and CD80/CD86 is required for Treg suppression effects. Although these findings indicated that the dispensable role of CTLA-4 signaling in CD25⁺ Treg suppressive

function *in vivo*, the action site of anti-CTLA-4 antibody remains unclear, and it is possible that the antibody targeted the activated CD4⁺ T-cells rather than the CD25⁺ Treg cells.

Like CTLA-4, GITR is also expressed on both Treg and activated CD4⁺CD25⁻ T-cells (Shimizu *et al.*, 2002). The ligand for GITR is expressed on endothelial cells, APC and dendritic cells. *In vitro* studies showed that the ability of Treg cells to suppress the proliferation of co-cultured CD25⁻ T-cells was abrogated in the presence of anti-GITR antibody, indicating the important role of GITR stimulation in Treg cell suppressive function *in vitro* (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). The action site of anti-GITR antibody was determined using CD25⁺ Treg cells and responder T-cells from wild-type and GITR deficient mice. Ligation of GITR on CD25⁻ responders, but not CD25⁺ Treg cells, was required to abrogate suppression. Thus the interaction of GITR on effector T-cells with its ligands mediates resistance to suppression by Treg cells.

1.2.1.4 Effects on APC

Suppression may result from down-modulation of the capacity of APC to activate effector T-cells, or competition between CD25⁺ Treg cells and Tconv cells for co-stimulatory molecules expressed on APC. Several studies have reported that Treg cells could down-regulate the expression of co-stimulatory molecules CD80/CD86 by DCs (Cederbom *et al.*, 2000; Houot *et al.*, 2006; Kryczek *et al.*, 2006; Serra *et al.*, 2003). However, activation of CD25⁺ Treg suppressor function did not require co-stimulation by CTLA-4 interaction with CD80/86 (Thornton *et al.*, 2004). CD25⁺ Treg cells were able to suppress CD8⁺ T-cell proliferation in APC-free system *in vitro*, suggesting that APC are not required for suppression and the suppression may involve direct interaction between Treg and effector T-cells (Piccirillo and Shevach, 2001). Therefore competition for co-stimulation is unlikely to be a major mechanism of suppression. Recent studies have shown that lymphocyte-activation gene 3 (LAG-3) may block DC maturation. LAG-3, expressed on CD25⁺ Treg cells, is a CD4 homologue that binds MHCII molecules and has a negative regulatory function which is required for maximal Treg cell suppression (Huang *et al.*, 2004; Workman and Vignali, 2005). Engagement of LAG3 to MHCII molecules expressed by immature DCs induces an immunoreceptor tyrosine-based activation motif (ITAM) mediated inhibitory signaling pathway, that suppresses DCs maturation (Liang *et al.*, 2008). However, more work is required to investigate whether and how Treg mediate suppressive function through DCs.

1.2.2 Thymic and Extrathymic Origin of Foxp3⁺ Treg

1.2.2.1 Thymic Development of Foxp3⁺ Treg Cells

In 1969, it was observed that neonatal thymectomy at certain period of time (day three after birth) results in autoimmune damage of various organs (Nishizuka and

Sakakura, 1969). Subsequent studies showed that d3Tx induced autoimmunity can be prevented by transfer of CD4⁺ T-cells or CD4⁺ CD8⁻ thymocytes from syngeneic adult mice (Fowell and Mason, 1993; Sakaguchi *et al.*, 1982), or more specifically, by transfer of CD25⁺ Treg cells isolated from normal adult mice (Asano *et al.*, 1996). This suggests that d3Tx mice intrinsically lack CD25⁺ Treg and provide a clue that Treg cells may originate in the thymus. It is found that nTreg develop in fetal thymic organ cultures (FTOC) (Carter *et al.*, 2005; Kim and Rudensky, 2006). And GFP-Foxp3 knock-in mice revealed selection of T-cells into the Treg lineage at the transition from DP to SP thymocytes (Hsieh *et al.*, 2006). Treg cells migration from the thymus to the periphery was documented by injection of FITC intrathymically (Papiernik *et al.*, 1998). Furthermore, Itoh *et al.* found that CD25⁺CD4⁺CD8⁻ thymocytes showed similar *in vitro* functional characteristics as their peripheral counterparts, such as anergic and suppressive responses to conventional T-cells (Itoh *et al.*, 1999). In addition, CD25⁺CD4⁺CD8⁻ thymocytes display a similar phenotype – expression of CTLA-4, GITR and OX40, as peripheral Treg cells (Itoh *et al.*, 1999; Shimizu *et al.*, 2002). Taking together, these data support the thymus is the site of nTreg generation. Mice deficient in all class II molecules did not develop CD4⁺CD25⁺ T-cells, while mice expressing MHC class II molecules only in the thymic cortex did (Bensinger *et al.*, 2001). This indicates that the development of Treg cells requires interaction with self-antigen-MHCII complexes on the thymic APC.

It is not known whether Treg cells undergo positive and negative selection as conventional T-cells do. The induction of Foxp3 in nTreg requires CD28 signaling induced by CD80 and CD86 expressed on antigen presenting cells, as Treg cells were markedly decreased in CD28 deficient and CD80/86 deficient mice (Salomon *et al.*, 2000; Tai *et al.*, 2005). Additionally, the Ick-binding domain of CD28 cytoplasmic tail is critical for the induction of Foxp3, therefore suggesting that CD28 signaling is important in Treg differentiation in thymus (Tai *et al.*, 2005). IL-2, IL-2R α (CD25) and IL-2R β (CD122) are important in Treg development. Mice which lack any of them showed a significantly reduced number of thymic and peripheral CD25⁺ Treg cells and developed autoimmunity (Almeida *et al.*, 2002; Malek *et al.*, 2002; Papiernik *et al.*, 1998). The autoimmune diseases could be controlled by reconstitution of these mice with normal CD25⁺ T-cells (Malek and Bayer, 2004). This suggests that IL-2 signaling was required for thymic development of CD25⁺ T-cells. Whether TCR on Tregs has a higher affinity for self-antigen-MHC complexes than conventional T-cells is still controversial and is discussed below.

1.2.2.2 Peripheral Generation of Foxp3⁺ Treg Cells

One important question is whether thymus is the only place for the generation of Foxp3⁺ T-cells. A number of studies (both *in vivo* and *in vitro*) have revealed that Foxp3⁺ T-cells can also be generated extrathymically from peripheral naive CD4⁺ Foxp3⁻ T-cells, called adaptive Treg (iTreg). A variety of conditions favor iTreg conversion. For example, *in vitro* antigen-stimulation of naive T-cells in the presence of TGF- β will induce iTreg (Apostolou and von Boehmer, 2004; Chen *et al.*, 2003; Kretschmer *et al.*,

2005). In TGF- β dependent *in vitro* Treg induction, IL-6, an acute phase protein induced during inflammation, dampens the conversion from naive T-cells and facilitates T-cell differentiation to the Th17 lineage in mice (Bettelli *et al.*, 2006; Veldhoen *et al.*, 2006). Genetic deletion or antibody blockage of IL-2 also promotes the differentiation of naive CD4⁺ T-cells into Th17 cells, suggesting that IL-2 facilitates the Treg differentiation but inhibits Th17 cell differentiation via signal transducer and activator of transcription 5 (STAT5) (Laurence *et al.*, 2007). In addition, the vitamin A metabolite retinoic acid (RA), which is produced by a subset of DCs in the gut-associated lymphoid tissue, inhibits IL-6 mediated Th17 cell induction. In the presence of TGF- β , RA facilitates the differentiation of naive T-cells to Foxp3⁺ Treg cells (Benson *et al.*, 2007; Coombes *et al.*, 2007; Mucida *et al.*, 2007; Sun *et al.*, 2007). Furthermore, naive monoclonal HA-specific CD4⁺ T-cells were induced to express Foxp3 under the chronic low dose antigen (HA) stimulation. This suggests that suboptimal co-stimulation is critical for differentiation of iTreg (Apostolou and von Boehmer, 2004; Kretschmer *et al.*, 2005). Gut-associated lymphoid tissue is a prominent *in vivo* site for iTreg generation because it is enriched in commensal bacteria and food antigens, and CD103⁺ DCs, which produce RA and TGF- β , are potent inducers of iTreg. These dendritic cells are abundant in lamina propria (LP) and in the mesenteric lymph node (LN), helping to limit gut inflammation through the generation of iTreg.

Early evidence of peripheral conversion of naive CD4⁺ T-cells into Foxp3⁺ T-cells originated from adoptive transfer of polyclonal CD4⁺CD25⁻ naive T-cells into lymphopenic mice. Part of the donor population became CD25⁺ Foxp3⁺ and acquired suppressive activity (Curotto de Lafaille *et al.*, 2004). However, these CD25⁺ Foxp3⁺ Treg cells might have originated from CD25⁺Foxp3⁺ cells present in the initial population. To avoid this issue, mature TCR transgenic (Tg) Foxp3⁻ T-cells on a Rag^{-/-} background which lack a Foxp3⁺ component, were transferred into Rag^{-/-} recipients that express agonist peptide under control of the Ig promoter. The injected cells segregated into CD25⁺ (43.5%) and CD25⁻ (56.5%). Both populations were anergic and suppressive (Apostolou *et al.*, 2002). This suggests that mono-specific naïve T-cells can differentiate autonomously into antigen-specific Treg cells in peripheral lymphoid tissue.

However, the extent to which Foxp3⁻ T-cells are converted to Foxp3⁺ Treg in both normal circumstances and autoimmunity remains unclear. In unimmunized mice, TCR repertoire analysis showed distinct populations among Treg and Tconv TCR (Hsieh *et al.*, 2006; Lathrop *et al.*, 2008; Wong *et al.*, 2007b). Tracing the Foxp3⁻ Tconv cells which were adoptively transferred into normal adult hosts confers different results. In one study, 5-12% of transferred CD4⁺CD25⁻ T-cells express CD25 (Liang *et al.*, 2005), indicating a significant contribution of conversion to maintain the peripheral Treg population. However, another study showed that average of 0.5% CD4⁺Foxp3⁻ T-cells converted into Foxp3⁺ T-cells, arguing that peripheral conversion plays a relatively small role in Treg pools (Lathrop *et al.*, 2008). In autoimmune circumstances, sequencing of CDR3a motifs from Treg and Tconv cells in BDC2.5/NOD mice, which is a diabetes model, indicated that little or no conversion occurs in response to pancreatic auto-antigen (Wong *et al.*, 2007a). Foxp3⁻ splenocytes adoptively transferred into congenic hosts followed by EAE induction did not convert into Foxp3⁺ T-cells in spleen and CNS, suggesting conversion

was unlikely to occur following immunization of MOG/CFA (Korn *et al.*, 2007). Collectively, the role of conversion to contribute to the peripheral Treg population is still ambiguous, and we will address this question using repertoire analyses (in Chapter 4) and studying specificity of a cohort of individual TCR (in Chapter 5).

1.2.3 *Specificity of Regulatory T-cells*

Affinity based model of Treg development propose that the increased strength of TCR and self-peptide-MHC complex favors the selection of Treg and these Treg cells have an increased affinity for self antigens. This is supported by studies with double transgenic mice (HA28 x TS1). When TCR transgenic mice bearing a TCR specific for epitope S1 derived from influenza hemagglutinin (HA) were crossed with mice expressing HA transgene, the S1-specific T-cells were not deleted and a large proportion expressed CD25 and functioned as Treg (Jordan *et al.*, 2001). Furthermore, thymocytes bearing TCRs with low affinity to S1 did not develop into Treg, which suggested that the selection of Treg appeared to require a TCR with high affinity for self-antigen-MHC complex (D'Cruz and Klein, 2005; Jordan *et al.*, 2001; Kawahata *et al.*, 2002; Lohr *et al.*, 2003; Walker *et al.*, 2003). Hsieh *et al.* directly tested the specificity of Treg cells *in vivo* and *in vitro*. He retrovirally transfected different TCR α genes into conventional CD4⁺ T-cells of TCR β transgenic x Rag^{-/-} mice (TCR $\alpha\beta$ TCR is specific for foreign antigen human class II-associated Ii peptide), and thus recreated the TCRs derived from either CD25⁻ or CD25⁺ population. The T-cells expressing Treg TCRs proliferated more rapidly than the transductants with Tconv TCRs upon adoptive transfer into lymphopenic hosts or in co-cultures with autologous APCs (Hsieh *et al.*, 2004). Moreover, when the TCR β transgenic mice were crossed with Foxp3 deficient mice, the autoreactive Foxp3⁻CD25⁺ T-cells, but not Foxp3⁻CD25⁻ cells, preferentially used TCRs found in the Treg cells in Foxp3 sufficient TCR β transgenic mice (Hsieh *et al.*, 2006). Taken together, these results implies that Treg cells express TCRs with increased affinity to self, and these TCRs use an overlapping repertoire with pathogenic autoimmune T-cells.

However, an alternative explanation of the Treg percentage increase in the HA28 x TS1 double transgenic system could be that Treg cells were more resistant to negative selection than their Foxp3⁻ counterparts (Bonasio *et al.*, 2006; Liston *et al.*, 2003; van Santen *et al.*, 2004). In experiments where the transgenic TCR was confronted with different levels of expression of tetracycline-regulatable neo-self antigen (MCC), the proportion of Treg cells increased without a change in absolute numbers. Thus, selective survival might be an alternative explanation for Treg differentiation. In addition, Treg cells can recognize foreign antigen derived from bacteria, viruses and parasites and neo-antigens. And analyses of T-cell hybridomas derived from Treg and non-Treg TCRs found no evidence that Treg hybridomas preferably recognize self-antigens. Instead, Treg hybridomas recognized foreign MHC-peptide complexes as often as non-Treg cells (Pacholczyk *et al.*, 2007). In conclusion, whether Treg cells possess higher affinity to self antigens than Tconv cells is still controversial, and requires further studies to determine the affinity range of Treg cells and self reactive Tconv cells.

Another important issue is whether antigen specificity of Treg cells is required for suppressive function. *In vitro* studies indicate that CD25⁺ Treg cells, once activated, are capable of suppressing responder T-cells of disparate antigen specificity (Takahashi *et al.*, 1998; Thornton and Shevach, 1998). However, *in vivo* studies link Treg specificity with their activity. Myelin basic protein (MBP) specific TCR transgenic Treg cells are more potent than non-transgenic Treg cells in EAE protection (Hori *et al.*, 2002). Similar findings have been seen in nonobese diabetic (NOD) mice. In this study, *in vitro* expanded BDC2.5 TCR transgenic Treg cells that are specific for an islet antigen expressed in the granules of β cells, suppressed diabetes in NOD Treg deficient mice better than expanded polyclonal NOD Treg cells (Tang *et al.*, 2004). A similar result was seen in an autoimmune gastritis (AIG) model, in which antigen-specific induced Treg (iTreg) completely prevent AIG while polyclonal iTreg did not (Huter *et al.*, 2008). In contrast, studies of a model of autoimmune uveitis failed to identify tissue specificity among involved Treg cells (Grajewski *et al.*, 2006).

1.2.4 Regulatory T-cells in MOG-EAE

During autoimmunity, Treg cells substantially accumulate in affected organs. Early studies showed that reconstitution of nude mice with CD4⁺ T-cells depleted for the CD25⁺ subpopulation resulted in the spontaneous development of autoimmune disease (Sakaguchi *et al.*, 1995), while replenishment of the CD4⁺CD25⁺ T-cells prevented this. In EAE, CD25⁺ T-cells from naive C57BL/6 mice can inhibit both IFN- γ production and proliferation of encephalitogenic cells from MOG₃₅₋₅₅ immunized mice *in vitro*. Adoptive transfer of these cells can ameliorate or abrogate the severity of EAE (Kohm *et al.*, 2002; Mekala *et al.*, 2005; Selvaraj and Geiger, 2008; Tang and Bluestone, 2006; Zhang *et al.*, 2004). On the contrary, depleting functional Treg with anti-CD25 antibody (PC61) prior to immunization increases the severity of disease (McGeachy *et al.*, 2005; Reddy *et al.*, 2004; Stephens *et al.*, 2005).

During the course of EAE, Treg cells have been identified in the inflamed CNS by flow cytometry and histological analysis (Kohm *et al.*, 2006; Korn *et al.*, 2007; Liu *et al.*, 2006; McGeachy *et al.*, 2005). Importantly, the accumulation of Treg cells peaks at the recovery phase. About 30% of these cells can produce IL-10 and these cells displayed potent suppressive capacity *ex vivo* (McGeachy *et al.*, 2005). The origination of these CNS-infiltrating Treg cells is still a puzzle. One study demonstrated that transferred pathogenic T-cells could convert to Foxp3⁺ phenotype once localized in the CNS (Liu *et al.*, 2006). However, tracking of transferred MOG-specific Tconv cells in a passive model, and tracking of transferred polyclonal CD4⁺Foxp3⁺ T-cells in an active model failed to identify the conversion (Davidson *et al.*, 2007; Korn *et al.*, 2007). The previous conversion phenomenon might come from expansion of an already existing Foxp3⁺ population within the transferred “pathogenic” T-cells. Thus, whether Tconv cells can convert to Treg during EAE is still controversial. Another feature of these CNS-infiltrating Foxp3⁺ Treg cells is their remarkable proliferative capacity, and this striking proliferative state is observed in CNS but not in draining lymphoid tissues

(O'Connor *et al.*, 2007). Treg cells are usually anergic when cultured *in vitro*, and it seems the inflammatory environment really promotes their expansion.

Another important question is what antigens CNS-infiltrating Treg cells recognize. Isolated Treg cells quickly revert to the classic anergic state *in vitro*, making antigen identification technically challenging to address. Although some studies showed Treg cells may recognize foreign antigens (Pacholczyk *et al.*, 2007), substantial evidence indicates that the repertoire also bears an overrepresentation of self-specific TCRs (Andersson *et al.*, 2007; Hsieh *et al.*, 2004; Hsieh *et al.*, 2006; Jordan *et al.*, 2001; Romagnoli *et al.*, 2002). Similarly, MOG-specific Treg cells were identified by tetramer staining in Foxp3-GFP reporter mice (Korn *et al.*, 2007; Reddy *et al.*, 2004; Reddy *et al.*, 2005). Likewise Foxp3⁺ cells derived from the CNS of MOG immunized mice with EAE expanded greatly after the addition of MOG-peptide to the culture (O'Connor *et al.*, 2007). Those studies suggest that CNS-infiltrating Treg cells in MOG-EAE contain at least a portion of MOG-reactive cells.

Cross-reactivity is also common in T-cell receptors. For example, EAE spontaneously developed in MOG deficient mice expressing a MOG-specific TCR (2D2). This paradox was explained by the finding that 2D2 cells are cross reactive with a neuronal cytoskeletal self antigen, neurofilament-M (NF-M) (Krishnamoorthy *et al.*, 2009). Even though 2D2 cell belongs to Tconv family, it is possible that CNS-infiltrating Treg cells in MOG-EAE also have cross-specificity for additional antigens.

1.2.5 Repertoire Analysis of Treg Cells

Although Treg cells are only a small fraction of CD4⁺ T-cells (5-10%), they can respond to a broad spectrum of antigens, both self and foreign. How does such a limited population recognize so many different specificities? Analyses of $\alpha\beta$ TCR diversity on CD4⁺CD25⁺ T-cells showed that their repertoire is diverse. Early studies showed $\alpha\beta$ TCR repertoire of CD4⁺CD25⁺ T-cells is more diverse than the repertoires of natural killer T-cells (NK T-cells) (Olivares-Villagomez *et al.*, 2000; Takahashi *et al.*, 1998). The comparison of the use of TCR V β or V α on Tconv and Treg cells revealed similar contributions of variable subfamilies to the two subpopulations of T-cells in both mice and humans (Fujishima *et al.*, 2005; Kasow *et al.*, 2004; Pacholczyk *et al.*, 2002; Romagnoli *et al.*, 2002; Takahashi *et al.*, 1998). Further analysis of CDR3 size distribution showed that the diversity of Treg CDR3 β was as broad as Tconv subsets (Fujishima *et al.*, 2005; Kasow *et al.*, 2004).

Since each individual can generate millions of different TCRs, CDR3 length distribution analysis is not sensitive enough to determine the identity of individual TCRs. A more detailed analysis is to sequence individual TCR CDR3 α or β regions amplified by reverse transcription-polymerase chain reaction (RT-PCR) (Apostolou *et al.*, 2002; Hsieh *et al.*, 2004; Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006; Wong *et al.*, 2007b). Most CDR3 sequence analyses were performed on TCR β transgenic mice, in which all T-cells express a single rearranged TCR β chain paired with variable TCR α chains. Therefore,

the CDR3 α can be used as an identifier of individual T-cell clones. The direct comparison of CDR3 α sequences among thymic or peripheral Treg and Tconv cells suggest that both populations have very diverse repertoires. Interestingly, reduction in the diversity of peptide-MHC complexes presented in thymus did not restrict the Treg repertoire diversity (Apostolou *et al.*, 2002; Pacholczyk *et al.*, 2006; Wong *et al.*, 2007b). This correlated with previous work showing that the proportion of CD4⁺CD25⁺ cells among CD4⁺ SP thymocytes remains constant in mice expressing different diversities of peptides bound with MHCII molecules (Pacholczyk *et al.*, 2002). This might suggest that the selection of Treg cells is not exclusively dependent on specific high-affinity to particular peptide-MHC complexes but rather Treg are selected more loosely as natural CD4⁺ T-cells. Or alternatively, the diversity of Treg repertoire is reduced in these mice with limited thymic antigen diversity, but is still too diverse to be detected by current techniques. Because fewer DP thymocytes will pass through positive selection as a result of the reduced diversity of peptide-MHC complexes, not only the Treg repertoire but also Tconv repertoire will shrink, probably to the same extent, which leads to a similar proportion of Treg cells developing.

Analyses of Treg and Tconv repertoires also demonstrates that the Treg and Tconv repertoires have partial sequence overlap, ranging from 10 to 42%, depending on different experimental strategies (Hsieh *et al.*, 2004; Hsieh *et al.*, 2006; Lathrop *et al.*, 2008; Pacholczyk *et al.*, 2006; Pacholczyk *et al.*, 2007). In mice expressing unmanipulated polyclonal self antigens on MHCII molecules (A^{bwt}), the overlap is approximately 10-20% (Apostolou *et al.*, 2002; Hsieh *et al.*, 2004; Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006). Whereas in “single-peptide” mice whose MHCII molecules are loaded with exclusively one peptide, the overlap increased to 38% (A^{bEp} mice) and 42% (H2-DM-deficient mice) (Apostolou *et al.*, 2002; Pacholczyk *et al.*, 2006; Wong *et al.*, 2007b). The correlation of increasing overlap with decreasing diversity of selecting ligands implies selection of Treg cells is not directly linked to the high self-reactivity, (Pacholczyk and Kern, 2008) because if Treg cells possess higher self-reactivity, one should expect more limited overlap in “single-peptide” mice.

This type of repertoire analysis can also be used to survey the interconversion between Treg and Tconv cells during the course of autoimmunity. For example, in a diabetes model, only limited Treg and Tconv cell TCR sequence overlap was seen in islets, suggesting that the two cell types were not interconverting at the site of inflammation (Wong *et al.*, 2007a).

Comparison of the TCR repertoire of Foxp3⁺ Treg cells present in the thymus and the ones present in the periphery showed that the peripheral Treg cells resembled those of thymic precursors, and differed from Foxp3⁻ T-cells (Apostolou *et al.*, 2002; Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006; Wong *et al.*, 2007b). These findings support the view that the peripheral Treg cells are derived from thymic precursors. On the other hand, the peripheral Treg repertoire does reshape itself somewhat. For example, TCRs frequently found in the thymus are also represented in the periphery, but their dominance may be altered (Apostolou *et al.*, 2002; Correia-Neves *et al.*, 2001; Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006; Wong *et al.*, 2007b). In addition, peripheral interconversion

between Treg and Tconv cells is observed, suggesting this conversion process could contribute to changes in the peripheral Treg repertoire, compared with thymic Treg repertoire. The peripheral conversion may also play a role in maintenance of the peripheral Treg cell population, and might be an important mechanism limiting inflammation induced immunopathology, especially in gut – associated lymphoid tissues (Coombes *et al.*, 2007; Lathrop *et al.*, 2008; Liang *et al.*, 2005). However, to what extent that the interconversion contributes to peripheral Treg cells in autoimmunity is still uncertain.

1.3 Summary

Regulatory T-cells, which are recognized as CD4⁺CD25⁺Foxp3⁺ T-cells, are critical for immune homeostasis. They are naturally present in the normal immune system as a functionally distinct T-cell subpopulation, and their deficiency produces autoimmune disease (Sakaguchi *et al.*, 1995). Adoptively transferred Treg cells can abrogate or prevent organ specific autoimmunity (Kohm *et al.*, 2002; Mekala *et al.*, 2005; Selvaraj and Geiger, 2008; Tang and Bluestone, 2006; Zhang *et al.*, 2004). Thymus is the major site of producing Treg cells. Besides that, peripheral interconversion between Treg and Tconv cells is also observed (Coombes *et al.*, 2007; Lathrop *et al.*, 2008; Liang *et al.*, 2005), though in different degrees. During EAE, Treg cells are often found in substantial number within affected organs (CNS), and the accumulation is correlated with the recovery phase (Korn *et al.*, 2007; Reddy *et al.*, 2004; Yu *et al.*, 2005). However, the origination of these Treg cells and responsiveness difference to Tconv cells are still unclear. In order to solve this issue, we developed a retrogenic mouse model of EAE, combined both repertoire analysis and studies of a large cohort of individual TCR. We found that Treg and Tconv cell TCR repertoires have limited overlap within the periphery and CNS in mice with EAE, (Chapter 4) and MOG-specific Treg and Teff cells possess distinct CDR3 β characters but similar sensitivity and fine specificity for MOG autoantigen (Chapter 5).

CHAPTER 2. MATERIALS AND METHODS

2.1 Methods of TCR Repertoire Analysis

2.1.1 Vector Construction for TCR α

Generation of MSCV-IRES-GFP vector was described previously (Persons *et al.*, 1997). The GFP was replaced by CFP. To generate different TCR α constructs, the α chain from 1MOG244.2, 2MOG10, 1MOG9, 1MOG23, and 1MOG213 MOG-specific TCR (Alli *et al.*, 2008) were PCR amplified and inserted into EcoRI/XhoI cloning sites in the vector (Figure 2-1).

2.1.2 Generation of 1MOG244.2TCR α Retrogenic Mice

2.1.2.1 Retroviral Production and Cellular Transduction

Ten μ g of the 1MOG244.2TCR α construct DNA and 10 μ g of retrovirus helper DNA constructs PEQPAM were co-transfected into 293T-cells by calcium phosphate precipitation as has been described (Geiger *et al.*, 2001). Briefly, 24 hours prior to transfection, 3×10^6 293T-cells were plated on a 10ml tissue-culture treated Petri dish. On the day of transfection, four hours prior to transfection, cells were approximately 70% confluent. DNA mix was prepared using 10 μ g construct DNA and 10 μ g PEQPAM plasmid, 50 μ l 2.5M CaCl₂ and 450 μ l water at room temperature (RT). This mix was then added to 500 μ l HPBS (pH 7.2) dropwise while vortexing and incubated 1 minute at RT. The whole mixture was added onto 293T-cells and incubated at 37°C. At 16 hours, the cells were washed with PBS to remove Ca²⁺ and cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), 100 U/ml penicillin G, 100 μ g/ml streptomycin, 292 μ g/ml L-glutamine (Invitrogen Life Technologies) at 37°C. At the same time 3×10^5 GP+E86 cells were plated in a 10ml Petri dish. 24 hours later, supernatant from 293T-cells was collected, centrifuged at 3000 rotations per minute (rpm) for 7 minutes and the supernatant was used to infect GP+E86 retroviral producer cells in the presence of 6 μ g/ml polybrene (hexadimethrine bromide; Sigma-Aldrich). 10ml fresh medium was added to 297T-cells, and ready for next time infection. GP+E86 cells were infected twice daily for five days. The GP+E86 cell line was expanded in culture for establishing stable retroviral vector producer lines.

2.1.2.2 Retroviral Transduction and Transplant of Bone Marrow Cells

GFP-Foxp3 gene-targeted mice were provided by A. Rudensky (U. Washington). Tcr α ^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These two strains were bred to generate GFP-Foxp3 x Tcr α ^{-/-} mice. DNA genotyping and flow

Figure 2-1. Structure of retroviral constructs. TCRα cDNA sequences were PCR amplified with primers containing EcoRI and XhoI sites, and subcloned into the MSCV-I-CFP retroviral vectors.

cytometry were performed for screening mice with GFP-Foxp3 gene and TCR $\alpha^{-/-}$ gene. Genome DNA from each mouse was extracted by Gentra Puregene Mouse Tail Kit (Qiagen). Foxp3 gene was PCR amplified with CW78a and CW78b primers (CW78a, 5'- TGCCTCTGACAAGAACCCAATGC; CW78b, 5'- GGCAGGGGGTTCAAGGAAGAAG). Nuclease-free water, dNTP mix, buffer with MgCl₂, Q buffer (Qiagen), Taq polymerase and tail DNA were added to make total of 50 μ l PCR mix. Reactions were carried out using the following PCR parameters: 95°C for initial 3 min, 30 cycles of 95°C for 30 sec, 55°C for 30sec, and 72°C for 2.5 min, followed by 72°C for 6 min. The products were analyzed by 1% agarose gel electrophoresis. The knock-in band is 927bp while the WT is 210bp. To screen TCR $\alpha^{-/-}$ mice, peripheral blood was collected and RBCs were lysed. Isolated cells were stained with anti-TCR β and anti-B220 antibody, and analyzed by flow cytometry.

The production of retrogenic mice has been previously described (Holst *et al.*, 2006a; Holst *et al.*, 2006b). Briefly, bone marrow cells were harvested from the femurs of GFP-Foxp3 x Tcr $\alpha^{-/-}$ mice 48 hr after the administration of 0.15 mg 5-fluorouracil g⁻¹ body weight, cultured in complete Click's medium (Invitrogen) with 20% FCS, IL-3 (20 ng ml⁻¹), IL-6 (50ng ml⁻¹), and stem cell factor (50ng ml⁻¹) for 48 hr and then cocultured for an additional 48 hr with irradiated (1200 rad) GP+E86 retrovirus producer cells. Transduction efficiency was confirmed by flow cytometry with CFP. Transduced progenitor cells were harvested, resuspended in PBS with 2% FCS and heparin (10unit/mouse) in total volume of 500ul/mouse, and injected into sublethally irradiated (450 rad) Tcr $\alpha^{-/-}$ recipients at a ratio of 1.5 recipient mice per bone marrow donor.

To test the engraftment of these retrogenic mice, peripheral blood was collected 6-8 weeks after transplantation. Red blood cells were lysed with Gey's solution (Sigma). Isolated cells were stained with anti-CD4 APC and anti-TCR β PE antibody, and analyzed by flow cytometry.

2.1.3 EAE Induction and Clinical Evaluation

MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by the St.Jude Hartwell Center for Biotechnology and HPLC purified prior to use. 2mg/ml MOG₃₅₋₅₅ was resuspended in water, and mixed with an equal volume of complete Freund's adjuvant (CFA) containing 4mg/ml H37RA. Emulsion was achieved by vortexing the mixture for 1hour at room temperature.

EAE was induced on retrogenic mice by s.c. injection of 100 μ g of MOG₃₅₋₅₅ peptide emulsified in CFA containing 4mg/ml Mycobacterium tuberculosis H37RA (Difco). Two hundred nanograms of pertussis toxin (List Biological Laboratories) was administered i.v. on days 0 and 2. Clinical scoring was as follows: 1, limp tail; 2, hind limb paresis or partial paralysis; 3, total hind limb paralysis; 4, hind limb paralysis and body/front limb paresis/paralysis; 5, moribund.

2.1.4 Cell Isolation

2.1.4.1 Preparation of the Splenocyte Suspension

1MOG244.2 retrogenic mice were sacrificed, spleens were removed and placed individually in 6cm diameter dishes. 5ml PBS with 2% FCS were added and completely covered the bottom of the dish. Cells from the splenic capsule were squeezed through the 70µm nylon mesh by gently mashing spleens with the rubber end of a plunger from a 3cc syringe. A single cell suspension was created and collected into a 50ml conical tube (on ice). 5ml PBS with 2% FCS was added to the dish and the whole process was repeated twice until the splenic capsules were white, indicating complete release of the cells. The splenocytes were washed by adding 20ml buffer and centrifuged at 350g for 7 min at 4°C (Eppendorf centrifuge 5804R with a type A-4-44 swinging bucket rotor).

The supernatant was aspirated and gently resuspended in 3ml/spleen Gey's solution in room temperature for two minutes. The cells were then washed with 10ml PBS buffer and centrifuged again. Splenocytes were resuspended and passed through a 70µm nylon mesh strainer into a new 50ml conical tube. The cell suspension from individual mice was pooled in same experiments. Spleens were prepared individually for TCR Vβ usage analysis, and pooled for TCR CDR3β repertoire survey.

2.1.4.2 Preparation of the Thymocytes and Lymphocytes Cell Suspension

Mice were sacrificed by CO₂ asphyxiation and their thymi, inguinal lymph nodes (LNs), axillary LNs, bronchial LNs, superficial and deep cervical LNs, and mesenteric LNs were collected. Similar to splenocytes, thymocytes and lymphocytes were collected by passing through 70µm cell meshes, and washed twice with PBS. Cell suspensions were centrifuged at 350g for 10 minutes, pellets resuspended in staining buffer, and filtered with a 70µm cell strainer.

2.1.4.3 Isolation of CNS-infiltrating T-lymphocytes

Five to six 1MOG244.2 retrogenic mice were immunized with MOG₃₅₋₅₅ peptide. On day 23-25, the peak phase of EAE disease, the mice were anesthetized with a lethal dose of Avertin (0.5ml of 2.5%) delivered by intraperitoneal injection. When the mice were unresponsive to stimulus (no reflex with paw pressure), they were placed on a polystyrene board and pinned down. The abdomen was opened and spleen was gently removed. Then the heart was exposed by cutting up both sides of the rib cage and the diaphragm. The right atrium was cut open with sharp small scissors, a 27^{1/2} g needle was immediately inserted into the left ventricle and 10ml chilled PBS slowly pushed into the left ventricle. White lungs indicated a successful perfusion. The detached head was opened by peeling each side of skull back away, and brain was gently removed. The

vertebra was dissected and the lumen was opened, and the spinal cord was collected with tweezers.

The CNS tissue was minced and transferred to a 6-well petri dish, digested with 3ml of 2.5mg/ml collagenase (Worthington Biochemical) and 1mg/ml deoxyribonuclease (Sigma) in click's medium for 25 minutes at 37°C followed by mechanical disaggregation. The disrupted CNS was transferred to a 70µm nylon cell strainer and forced through the screen with a 3cc plunge. The suspension was centrifuged at 400g for 10 minutes at room temperature. The pellet was resuspended in 4ml of 80% Percoll (Amersham Pharmacia biotech) and underlayed in 4ml of 40% Percoll. 2ml of 100% Percoll was used to underlay this. This gradient was carefully centrifuged at 1200g for 20 minutes at 20°C. The cells between the 40% and 80% interface were collected, washed twice and resuspended in staining buffer.

2.1.5 Cell Counting

A 10µl cell suspension was mixed with 90µl of PBS, and 100µl trypan blue solution was added and mixed thoroughly. A 10µl sample was transferred to a hemocytometer chamber. All viable cells were counted (non-viable cells stain blue, viable cells remain opaque) in the four corner squares. The cell concentration per ml was determined using the following calculation.

$$\text{Cells per ml} = \text{the average count per square} \times \text{the dilution factor} \times 10^4$$

2.1.6 Flow Cytometry

2.1.6.1 Flow Cytometry (FACS) Analysis of IMOG244.2 Retrogenic Mice

Eight weeks after hematopoietic progenitor cell (HPC) transfer, five to ten retrogenic mice were sacrificed. Thymocytes were isolated and a single cell suspension prepared. 10×10^6 cells were counted and resuspended in 1ml cell staining buffer (500ml PBS and 10ml FCS and 5ml 10% sodium azide). 5µg/ml purified anti-mouse CD16/CD32 antibody specific for Fcγ R III/I (Miltenyi Biotec) was added and incubated for 10 minutes to block nonspecific immunofluorescent staining. The cells were stained with a combination of antibodies, which contained anti-CD4 (clone L3T4), anti-CD8 (clone 53-6.7), anti-TCRβ (clone H57-597), anti-CD44 (clone IM7), anti-CD25 (clone PC61). After 20 minutes incubation on ice, the cells were washed three times with 3 ml of cell staining buffer. The cell pellet was resuspended in 0.5ml of cell staining buffer and analyzed by flow cytometry (BD FACSCalibur).

A similar preparation was performed with retrogenic splenocytes and CNS-infiltrating T-cells, but stained with two sets of antibodies. One set contained anti-CD2 (clone RM2-5), anti-TCRβ (clone H57-597), anti-CD25 (clone PC61), and anti-CD4

(clone L3T4). The other set contained anti-CD69 (clone H1.2F3), anti-TCR β (clone H57-597), anti-CD103 (clone M290), anti-CD5 (clone 53-7.3), and anti-CD4 (clone L3T4).

2.1.6.2 V β Staining of 1MOG244.2 Retrogenic Mice

Splenocytes and CNS-infiltrating T-cells were distributed into 14 wells (96-well round bottom plate). Monoclonal antibody specific for CD4 (clone L3T4) and CD8 (clone 53-6.7) were added equally. TCR β (clone H57-597), TCR V β 2 (clone B20.6), TCR V β 3 (clone KJ25), TCR V β 4 (clone KT4), TCR V β 5.1, 5.2 (clone MR9-4), TCR V β 6 (clone RR4-7), TCR V β 7 (clone TR310), TCR V β 8.1, 8.2 (clone MR5-2), TCR V β 8.3 (clone 1B3.3), TCR V β 10^b (clone B21.5), TCR V β 11 (clone RR3-15), TCR V β 12 (clone MR11-1), TCR V β 14 (clone 14-2) were added sequentially. After incubation and wash, cells were analyzed by flow cytometry (BD FACSCalibur).

2.1.6.3 Cell Separation by Flow Cytometry

At the indicated time points after EAE induction, 5-6 mice/cohort were sacrificed, spleens were removed, the circulation perfused with PBS, and brain and spinal cord removed. T-cells were isolated from CNS tissue by density centrifugation as described above. Isolated cells were stained with CD4-specific Ab and immediately sorted into Tconv (CD4⁺GFP⁻) and Treg (CD4⁺GFP⁺) populations with a MoFlo (DakoCytomation) or iCyt reflection (iCyt, Champaign, ILL) cell sorter.

2.1.7 RNA Isolation and cDNA Transcription

Total RNA was extracted from sorted splenocytes and CNS-infiltrating lymphocytes using RNeasy Protect MiniKit (QIAGEN), according to the manufacturer's instructions. The RNA concentration was determined by NanoDrop ND-1000 spectrophotometer.

cDNA was immediately generated with the Omniscript RT kit (QIAGEN). Oligo dT, RNase inhibitor, Omniscript reverse transcriptase, 10x buffer, dNTP mix, RNase-free water were mixed with RNA, and incubate at 37°C for 1 hour. The cDNA products were kept at -20°C for long term storage.

2.1.8 Polymerase Chain Reaction (PCR) Amplification

cDNA was PCR amplified with either C β - or J β 2.7- specific primers (C β , 5'-CAAGGAGACCTTGGGTGGAGTCACATT; J β 2.7, 5'-TAAAACCGTGAGCCTGGTGCCGG) and a V β 8.2- primer (V β 8.2, 5'-ATGTCTAACACTGCCTTCCCTGACCC). A bulk reagent tube was created and

distributed, which contained nuclease-free water, dNTP mix, buffer with MgCl₂ and primers. Then cDNA and high fidelity polymerase (Roche) were added and tubes kept on ice. Five identical and independent PCR reactions were performed with each sample per primer pair. And a reaction without any cDNA was prepared as a negative control. The PCR program (Eppendorf Mastercycler gradient) was started, and tubes were placed into the machine once the temperature reached 95°C. Touchdown PCR was used to avoid amplifying nonspecific sequences. The programs were as follows (Table 2-1).

To prevent PCR contamination between experiments, cDNA preparation and PCR post-amplification processing were performed in separate areas and using separate equipment. A laminar flow cabinet equipped with UV lamp was also used. Water, dNTP, buffer, tubes, pipette and tips etc were UV irradiated for 15 minutes immediately before use. Fresh gloves, pipette tips with aerosol filters and different sets of pipette were also required. A negative control was always included, in which the reaction mixture did not have DNA template.

2.1.9 Agarose Gel Electrophoresis and PCR Product Purification

A 1% agarose gel was prepared, and ethidium bromide added after the gel solution cooled to 60°C. A 5µl aliquot from each PCR product was mixed with loading buffer and loaded into the gel wells, running at 120V for 30 minutes. The products were examined briefly with a UV transilluminator (Figure 2-2). A positive reaction contained a ~400 bp fragment. Remaining products underwent a second round of gel electrophoresis. The ~400 bp DNA fragments were excised and purified with the QIAquick Gel Extraction Kit, according to the protocol.

Table 2-1. PCR program used to amplify CDR3 region.

Vβ8.2- Jβ2.7	Vβ8.2- Cβ
1. 95°C 2:00min	1. 95°C 2:00min
2. 95°C 0:30min	3. 95°C 0:30min
3. 75°C 0:30min	4. 65°C 0:30min
4. -1 °C 0:00min	5. -1 °C 0:00min
5. 72°C 0:30min	6. 72°C 0:30min
6. Goto 2 repeat 10 cycles	7. Goto 2 repeat 10 cycles
7. 95°C 0:30min	8. 95°C 0:30min
8. 65°C 0:30min	9. 55°C 0:30min
9. 72°C 0:30min	10. 72°C 0:30min
10. Goto 6 repeat 20 cycles	11. Goto 6 repeat 20 cycles
11. 72 °C 10:00min	12. 72 °C 10:00min

Figure 2-2. PCR amplification of CDR3 region. Lane 1 and 12: 1kb plus ladder, lane 3 and 10: 100bp ladder, lane 14-21: amplified with V β 8.2-J β 2.7 primer, lane 11 and 22: negative control.

2.1.10 TA Cloning

A deoxyadenosine (A) was introduced to the 3' ends of the purified DNA fragments. Briefly, gel purified PCR products were mixed with dATP, 25mM MgCl₂ and Taq polymerase (Promega), and incubate at 72°C for 10min. The products are then mixed with salt solution and pCR2.1 - TOPO vector (Invitrogen) and incubated at RT for 5 minutes. 2µl of the TOPO cloning reaction was added into a vial of One Shot Chemically Competent E. coli and mixed gently. After a 30 minute incubation on ice, the cells were heat shocked for 30 seconds at 42°C without shaking. 450µl of pre-warmed S.O.C medium were added to the cells. The tube was shaken (225rpm) at 37°C for 1 hour. 30-60µl medium from each transformation was spread on LB plate containing carbenicillin (1µg/ml). The plates were incubated overnight at 37°C.

2.1.11 Plasmid Isolation

About 50 clones per PCR reaction were picked from antibiotic selection plates described above. They were each inoculated to 1ml LB medium containing carbenicillin (1µg/ml) and incubated overnight at 37°C with vigorous shaking (250rpm). The bacterial cells were harvested, and cDNA-containing plasmid was purified with the Wizard SV 96 Plasmid DNA Purification System (Promega), according to the manufacture's protocol. Plasmid DNA was eluted in 100µl nuclease-free water and stored at 4°C.

2.1.12 CDR3 Sequencing and Analysis

The cloned PCR products were sequenced using a T7 priming site in the cloning vector (T7, 5'-TAATACGACTCACTATAGGG). Capillary sequencing was performed by the St.Jude Hartwell Center for Biotechnology. Vβ, Jβ and CDR3 nucleotide and amino acid sequences were identified with IMGT-V-Quest software (immunogenetics information system; <http://imgt.cines.fr/>). CDR3 charge and hydrophilicity analyses were performed with the innovagen peptide calculator (<http://www.innovagen.se/>).

2.2 MOG-specificity of Transduced 4G4 Hybridomas

2.2.1 Vector Construction

The original XhoI site of 1MOG244.2αβ MSCV-IRES-CFP vector (Alli *et al.*, 2008) was deleted by DNA Polymerase I large (Klenow) fragment (Promega). A new XhoI and BamHI sites were created flanking the CDR3β region without altering amino acid sequence with the Quick change site directed mutagenesis kit (Stratagene) (Figure 2-3). To generate selected Treg and Tconv TCR constructs, the CDR3β region identified during repertoire analyses was PCR amplified with primers incorporating an endogenous Vβ8.2 BamHI site and inserting a XhoI restriction site within the Cβ

Figure 2-3. Structure of modified retroviral constructs containing new BamHI and XhoI sites. CDR3 β region was PCR amplified with primer containing BamHI and XhoI sites, and subcloned into the MSCV-I-CFP retroviral vectors.

(5' primer: GGTGCTGGATCCACTGAGAAAGGAGATATCCC 3' primer: GCCTCGAGAACCGTGAGCCTGGTGC). The PCR products (~200bp) were purified with PCR purification kit (Qiagen), and subcloned into the modified 1MOG244.2TCR $\alpha\beta$ MSCV-IRES-CFP construct, using quick T4 DNA ligase (NEB). The ligation products were chemically transformed into DH5 α TM E. coli. 60-100 μ l transformation products were spread on LB agar plates containing carbenicillin. Plates were incubated at 37°C overnight. ~30 clones per construct were selected and plasmid was isolated. Construct integrity was verified by DNA sequencing using a V β 8.2 primer (V β 8.2, 5'-ATGTCTAACACTGCCTTCCCTGACCC).

2.2.2 Transformation of CD4-4G4 T-cell Hybridoma

Ten micrograms of the TCR $\alpha\beta$ construct and 10 μ g of retrovirus helper DNA constructs were co-transfected into 293T-cells by calcium phosphate precipitation. At 16 h, the cells were washed and cultured in DMEM with 10% FCS, 100U/ml penicillin G, 100 μ g/ml streptomycin, 292 μ g/ml L-glutamine (Invitrogen Life Technologies) for 48 h. Supernatant was collected twice daily and used to infect GP+E86 retroviral producer cells in the presence of 6 μ g/ml polybrene (hexadimethrine bromide; Sigma-Aldrich). Transduced GP+E86 cells were expanded in culture, and supernatant was used to infect murine surface TCR-deficient 4G4.CD4 T hybridoma cells (gift of Dr. Janeway, Yale University, New Haven, CT).

Murine surface TCR-deficient 4G4 hybridoma cells were first transduced with mouse CD4-expressing retrovirus (MSCV-CD4-I-GFP), to produce 4G4.CD4 cells (Alli *et al.*, 2008; Udyavar *et al.*, 2009). To transduce the different TCR constructs, 10⁶ 4G4.CD4 cells were harvested and resuspended in 6-10ml retroviral supernatant. The cell suspension was centrifuged at 1800rpm, 8°C for 90 minutes, in the presence of 6 μ g/ml polybrene. The infected cells were then cultured in complete Click's medium. The transduced cells were flow cytometrically sorted twice for the GFP^{high}TCR⁺ population and expanded. The purity of the GFP^{high}TCR⁺ 4G4 cells was confirmed by flow cytometry (BD FACSCalibur).

2.2.3 Cytokine (IL-2) Analysis by Enzyme-linked Immunosorbent Assay (ELISA)

Cytokine sandwich ELISA is sensitive enzyme immunoassay that can specifically detect and quantitate the concentration of soluble cytokine proteins. The transduced 4G4.CD4 cells (1x10⁵) were cultured in the presence of 3x10⁵ APCs. In one experiment, they were stimulated with 10-fold titrations of MOG₃₅₋₅₅ (100 μ g/ml, 10 μ g/ml, 1 μ g/ml, 0.1 μ g/ml and 0 μ g/ml). In the other experiment, cells were cultured with 100 μ g/ml MOG₃₅₋₅₅ or the indicated alanine-substituted peptides. 100 μ l of supernatant was collected from the cultures 24 hours after stimulation. To measure the IL-2 concentration, purified anti-IL-2 capture antibody (clone MQ1-17H12) was diluted to 2 μ g/ml in binding solution. 50 μ l of the diluted antibody was added per well of a 96-well ELISA plate (Nunc Maxisorb) and incubated at 4°C overnight. Capture antibody was then removed,

and non-specific binding was blocked by adding 200µl of blocking buffer per well (BD Bioscience), and incubated at RT for 2hrs. After wash with PBS/Tween (PBS and 0.1% Tweens) for 3 times, culture supernatants were added to the coated plate, 100µl per well. A series of nine two-fold dilutions of recombination mouse IL-2 standard (from 5000pg/ml to 19.5pg/ml) was added to same wells. The plate was incubated at 4°C overnight. After washing away unbound material with PBS/Tweens, 100µl diluted biotinylated anti-IL-2 (1µg/ml) was added and incubated room temperature for one hour. Again after washing with PBS/Tween for five times, 100µl diluted peroxidase labeled anti-biotin (Vector laboratories, Burlingame, CA) was added and incubated for 30 minutes. The plate was washed for eight times and ABTS substrate solution with hydrogen peroxide was added. After color developed, the OD was read by a microplate reader (Bio-Rad) setting to 405nm.

2.3 Generation of TCR-retrogenic Mixed Chimeras

Retroviral constructs incorporating the TCRαβ from MOG-specific TCRs were generated and transduced into GP+E86 producer cells as described above. These transduced producer cells were used to infect HPCs from B6.129S7-Rag1^{tm1Mom}/J. The transduction efficiency was evaluated by flow cytometry for CFP. The HPCs were mixed at a 1:1 or 1:10 ratio with untransduced, T-cell depleted CD45.1 congenic B6.SJL-Ptprc^aPep3^b.BoyJ-derived bone marrow, and injected i.v into lethally irradiated (1100Rad) B6.SJL-Ptprc^aPep3^b.BoyJ mice.

2.4 Flow Cytometric Analysis of TCR-retrogenic Mixed Chimeras

Eight weeks after transplantation, splenocytes and thymocytes were isolated. Entire splenocytes were stained with a mix of antibodies specific for CD4 (clone L3T4), TCR (clone H57-597) and CD45.2 (clone 104). Entire thymocytes were stained with the mix of antibodies specific for CD4, CD8, and CD45.2. After washing, the cells were fixed and permeabilized by 1ml Fixation/Permeabilization working solution for 30 minutes (eBioscience). Cells were then stained with PE conjugated anti-Foxp3 (clone FJK-16s, eBioscience), and analyzed by flow cytometry.

CHAPTER 3. GENERATION OF 1MOG244.2TCR α RETROGENIC MICE

3.1 Introduction

Immune homeostasis is maintained partially by Treg cells that dominantly control potential hazardous self-reactive T-cells in the periphery. Their suppressive features and direct inhibition of autoimmune diseases attracted a decade of active investigation into their biology. TCR repertoire analyses shed light on the origin of Treg and their relationship with Tconv cells. This chapter describes the development of a mouse model that is not only susceptible for EAE but also facilitated the performance of Treg repertoire analysis.

The diversity of the mature T-cell repertoire is generated by the rearrangement of various gene segments and addition of nucleotides not present in germline to form the α and β chain of the TCR, and the random paring of α and β chains. In theory, more than 10^{15} different possible $\alpha\beta$ TCR may form in mice (Davis and Bjorkman, 1988). But in reality, most (about 95%) of thymocytes die during the development processes as they fail either positive or negative selection. Only 5% survive to become mature T-cells. Casrouge *et al.* has estimated that the actual mouse naïve T-splenocytes repertoire contains 2×10^6 unique clones by the immunoscope technique. This figure, although orders of magnitude smaller than the maximum theoretical diversity (10^{15}), is still large enough to maintain a high functional level of diversity (Casrouge *et al.*, 2000).

The ideal way to analyze TCR repertoire would be identify both α and β chains of each T-cell. However, technical limitations make it impractical to perform this on a large scale. Most current TCR repertoire analyses rely on the use of transgenic mice in which one chain of the TCR is fixed (Hsieh *et al.*, 2004; Malherbe *et al.*, 2004; Wong *et al.*, 2007b; Zehn and Bevan, 2006). The fixed α or β chain limits the repertoire variation to the alternative chain. This technique not only simplifies the complicated system, but also, if the fixed chain has a propensity for auto-reactivity, will result in larger than normal populations of self-reactive cells, thereby facilitating analyses of the self-reactive repertoire. We used lethally irradiated mice reconstituted with bone marrow progenitor cells transduced with a retrovirus incorporating a rearranged TCR α chain, so called retrogenic mice (Holst *et al.*, 2006a). The advantages of the retrogenic system are rapid development (2months) and that they allow analysis of mice expressing different TCR α simultaneously. The major alternative TCR transgenic mice are more costly and time consuming to create, and surveying multiple TCR would be difficult. On the other hand, fewer T-cells develop in retrogenic mice than transgenic mice and they cannot propagate by breeding. Here we developed a retrogenic model of MOG-EAE in which the TCR α chain locus is fixed by the enforced retrogenic expression of a TCR α from a MOG-specific T-cell.

3.2 Results

3.2.1 Retrogenic Mice Expressing a Single TCR V α Chain

To generate a retrogenic mouse model susceptible to EAE, we first generated several different strains of retrogenic mice, each incorporating a different α chain. Briefly, we bred C α KO mice (Mombaerts *et al.*, 1993) with GFP-FoxP3 KI mice (Fontenot *et al.*, 2005b), and transduced the bone marrow of the offspring with retroviral constructs incorporating the α chain from 1MOG244.2, 2MOG10, 1MOG9, and 1MOG213 MOG-specific TCRs. C α KO mice do not express any α chain. That ensures the bone marrow cells only contain the rearranged transduced α chain. Because C α KO mice have normal RAG-recombinase and an intact TCR of β genome, the complete genome of β chains, the engrafted bone marrow progenitor cells will complete β chain rearrangement from genomic V, D, J segments and develop into T-cells with a diverse repertoire. In addition, the GFP-FoxP3 transgene allows the rapid identification of regulatory T-cells (CD4⁺GFP⁺) and conventional T-cells (CD4⁺GFP⁻) by GFP fluorescence.

6 weeks post hematopoietic progenitor cell (HPC) transplantation, the peripheral blood were collected and stained with antibodies specific for CD4 and TCR β . Engraftment was verified by assessing for the presence of CD4⁺TCR⁺ in gated lymphocytes, and this ranged from 1% to 5%. Mice were challenged with MOG₃₅₋₅₅ peptide in CFA, as described in Chapter 2. The retrogenic mice bearing the different specific TCR α chains show inducible EAE, but the severity of disease was strongly influenced by the introduced α chain. Only 1MOG244.2 α retrogenic mice developed EAE with high penetrance in response to MOG₃₅₋₅₅ immunization, with >90% of mice developing typical disease symptoms (Figure 3-1B). Mice expressing other TCR α developed more mild disease (Figure 3-1A). Hence, we chose 1MOG244.2TCR α retrogenic mice as a mouse model to further evaluate the TCR repertoire.

3.2.2 Phenotype of 1MOG244.2TCR α Retrogenic Mice

At approximately eight weeks post-HPC transfer, the retrogenic mice showed good lymphoid engraftment. Thymocyte subsets showed no significant difference in absolute cell number, percent of DN, DP, CD4⁺ SP and CD8⁺ SP subsets compared with C57BL/6 mice, though they trended toward increased double negative (DN) cells (7.0% $\pm$ 6.8% 1MOG244.2 α versus 3.0% $\pm$ 1.3% C57BL/6) and decreased CD4⁺ SP cells (1.7% $\pm$ 1.8% versus 7.0% $\pm$ 3.8%) (Figure 3-2A). Specific analyses of DN3 and DN4 cells subpopulation revealed no statistical difference between retrogenic mice and C57BL/6 mice. Retrogenic mice showed a slightly decreased percent of DN4 subsets among total DN cells (47.93% $\pm$ 5.868% 1MOG244.2 α versus 55.33% $\pm$ 6.886% C57BL/6) (Figure 3-2B-D), though not statistically significant. The DN4 population identified in retrogenic mice demonstrated that a substantial number of DN3 cells pass through β -selection checkpoint while expressing the retrogenic TCR α chain. This is significant as some

Figure 3-1. EAE induction in retrogenic mice. (A) 1MOG244.2, 2MOG10, 1MOG9, and 1MOG213 TCR α retrogenic and wild-type mice (n=4 or 5) were immunized with MOG₃₅₋₅₅. EAE was clinically monitored. Mean clinical score is plotted. (B) EAE was induced on 1MOG244.2 α mice (n=10). Ten of 10 mice developed disease symptoms with a mean $\pm$ 1 s.d. maximal score of 2.6 ± 1.4 (range 1-5). Plot shows mean clinical score $\pm$ 1 s.e.m.

Figure 3-2. Thymocyte reconstitution in retrogenic mice. 1MOG244.2 α retrogenic mice were sacrificed ~8 weeks after HPC transfer, and thymocytes were isolated and quantitatively analyzed for the indicated cell subsets. (A) Percent of DN, DP, or CD4⁺ or CD8⁺ SP T-cells among 1MOG244.2 α or simultaneously analyzed C57BL/6 thymocytes is shown. (B) Percent of DN3 or DN4 cells among CD4⁻CD8⁻TCR⁻ thymocytes. (C and D) Flow cytometric analysis shows DN3 and DN4 cells development in the CD4⁻CD8⁻TCR⁻ gated populations.

reports have shown a decreased DN3 to DN4 transition in TCR α transgenic mice (Baldwin *et al.*, 2005; Borowski *et al.*, 2004; Huang and Kanagawa, 2004; Lacorazza *et al.*, 2001).

Analyses of the periphery showed a mean of $83 \pm 6 \times 10^6$ total splenocytes in retrogenic mice, primarily B-cells (Figure 3-3C). Engrafted T-lymphocytes were decreased compared to wild-type C57BL/6 mice (data available at www.jax.org), and comprised $13.8 \pm 4.9\%$ of lymphocytes in the spleen and $9.7 \pm 2.9\%$ in the lymph nodes (LN) (Figure 3-3A and B). The CD4/CD8 ratio was elevated, 6.2 ± 1.3 in the lymph nodes (LN) and 10.0 ± 2.7 in the spleen compared with ~ 1.6 in C57BL/6 mice (Figure 3-3D), indicating that the 1MOG244.2TCR α developmentally skewed cells to the CD4 lineage. CD4 $^+$ T-cells expressed the GFP-Foxp3 transgene in proportions comparable to that observed in wild-type mouse strains, $12.8 \pm 2.2\%$ in LN and $7.5 \pm 2.4\%$ in spleen (Figure 3-3A and B). Therefore mice expressing the 1MOG244.2 α transgene develop a repertoire that incorporates normal proportions of CD4 $^+$ Treg and Tconv cells and they are susceptible to EAE, though showing decreased representation of CD8 $^+$ T-cells.

3.2.3 *V β Use in 1MOG244.2TCR α Splenic T-lymphocytes*

We first analyzed peripheral TCR repertoire in unimmunized 1MOG244.2 α mice by examining V β chain usage by monoclonal antibody (MAbs) staining (Figure 3-4). These mice, with a fixed V α 5-J α 43 chain, showed different V β use compared with wild-type C57BL/6 mice. A substantially increased usage of V β 14 was seen, $15.2\% \pm 4.1\%$ in retrogenic mice versus $6.49\% \pm 0.48\%$ in wild-type ($p = 0.0128$). Significantly fewer V β 3 ($p = 0.0018$), V β 4 ($p = 0.0348$), and V β 8.3 ($p = 0.003$) T-cells were seen, potentially due to the compensation for the increased V β 14 use. However, V β 8.1/8.2 was the most frequently used TCR among retrogenic mice, and wild-type mice showed a similar percentage.

3.2.4 *Increased TCR V β 8.2 Use in CNS-infiltrating 1MOG244.2 α T-cells*

As a low resolution screen for EAE-associated alterations in the 1MOG244.2 α TCR repertoire, we analyzed V β chain use by staining splenic or CNS CD4 $^+$ GFP-Foxp3 $^-$ and GFP-Foxp3 $^+$ cells with V β -specific Abs. A significant increase in V β 8.1/8.2 $^+$ use in CNS-infiltrating Tconv was seen ($p = 0.018$) compared with paired splenic Tconv or Tconv from unimmunized controls (Figure 3-5A). This is consistent with the documented over-representation of this receptor among MOG₃₅₋₅₅/Ab-specific T_{eff} cells (Mendel *et al.*, 1995; Mendel Kerlero de Rosbo and Ben-Nun, 1996). A trend toward increasing V β 8.1/8.2 TCR use was also seen among CNS Treg (Figure 3-5B), though this did not achieve statistical significance. A decrease in V β 8.3 TCR among Treg cells ($p = 0.048$) was also seen, possibly due to compensation for the increased V β 8.1/8.2 usage. Therefore 1MOG244.2 α mice express a TCR repertoire that is modified by EAE induction. Further, V β 8.1/8.2-expressing T-cells are concentrated in the CNS during EAE, implying increased MOG-reactivity in the infiltrating T-cell population.

Figure 3-3. Lymphoid reconstitution of retrogenic mice. 1MOG244.2 α retrogenic mice were sacrificed ~8 weeks after HPC transfer. Spleen and LN cells were isolated and quantitatively analyzed for the indicated cell subsets. Percent LN (A) and splenic (B) reconstitution with total T-lymphocytes (TCR⁺) and CD4⁺ T-lymphocytes (CD4⁺TCR⁺), and fractional engraftment with CD4⁺TCR⁺ GFP-Foxp3⁺ T-lymphocytes (Foxp3⁺/CD4⁺TCR⁺) are plotted for individual mice. Means are indicated by the horizontal bar. The remaining lymphocytes were composed predominantly of B-cells. (C) Absolute number of total splenocytes and TCR⁺ splenocytes is plotted. (D) The ratio of CD4⁺/CD8⁺ T-cells is plotted.

Figure 3-4. Vβ use by splenic Tconv in 1MOG244.2α and C57BL/6 mice. 8 weeks post transfer of HPCs, 1MOG244.2α (n = 5) were sacrificed and CD4⁺ T-cells were flow cytometrically analyzed using a panel of Vβ-specific antibodies. C57BL/6 mice (n = 4) were analyzed simultaneously. Significant differences between wild-type and retrogenic mice were seen. *, p < 0.05.

Figure 3-5. V β use by retrogenic Treg and Tconv in pre-immune splenocytes and splenocytes and CNS-infiltrating cells from mice with EAE. Preimmune retrogenic mice or mice 17 days after EAE induction were sacrificed and CD4⁺ T-lymphocytes flow cytometrically analyzed using a panel of V β -specific antibodies. Comparisons were made between Tconv (A) and Treg (B) cells from unimmunized and immunized splenocytes and CNS T-cells from mice with EAE. Data show mean + 1 SEM. *, $p < 0.05$ among group by Hochberg-adjusted 1-way ANOVA.

3.3 Summary

TCR repertoire analysis has been used to determine the origins of Treg and their relationship with Tconv cells. This type of study is difficult to perform directly on the naive repertoire due to its high diversity. To circumvent this problem, we developed retrogenic mice expressing a retrovirally transfected TCR α chain. Briefly, α chain isolated from MOG-specific TCR was subcloned into MSCV-IRES-CFP vector. Bone marrow stem cells from Tcr $\alpha^{-/-}$ GFP-Foxp3 mice were retrovirally transfected with α chain, and then transplanted into irradiated Tcr $\alpha^{-/-}$ mice. Several different TCR α retrogenic mice were generated and challenged with MOG₃₅₋₅₅ in CFA. 1MOG244.2 α mice showed similar disease severity as C57BL/6 mice, though the mice experienced later disease onset than wild-type mice.

Analyses of 1MOG244.2 α thymocytes revealed no significant differences compared with C57BL/6 mice. The DN to DP transition was efficient, and the percent of DN3 and DN4 among DN population did not show significant differences to wild-type mice. This suggests that the premature TCR α expression which in some models leads to diminish pT α signaling does not alter the balance of different thymocyte subsets in 1MOG244.2 α mice. Further analyses of peripheral lymphocytes showed a decreased percent of T-cells, which potentially reflects the effects of transplantation into the TCR $\alpha^{-/-}$ mice. The ratio of CD4/CD8 is elevated five-fold versus wild-type splenocytes. This suggests that the enforced α chain expression impacts the peripheral repertoire to some extent, favoring the CD4⁺ lineage commitment or expansion. More importantly, the proportion of Treg cells among CD4⁺ T-cells is normal. Treg lineage assignment in 1MOG244.2 α mice does not appear to be altered by the fixed α chain.

V β usage analysis was performed on unimmunized retrogenic and C57BL/6 mice. V β 8.1/8.2 was the most frequently used TCR β in both of these. However, the percent of V β 14 cells was substantially increased in retrogenic mice, suggesting that the enforced α chain did influence on V β selection. After EAE induction, T-cells bearing V β 8.1/8.2 accumulate in CNS in C57BL/6 mice, and this was similarly seen in the retrogenic mice. Overall, although some difference exist between wild-type and 1MOG244.2TCR α retrogenic mice, this mouse does appear suitable for the analysis of repertoires in EAE.

CHAPTER 4. TCR β REPERTOIRE ANALYSIS OF 1MOG244.2TCR α RETROGENIC MICE

4.1 Introduction

Treg cells can be produced in the thymus as a functionally mature, distinct T-cell population. Sakakura first showed that neonatal thymectomy of female mice at 3 days of age led to autoimmune oophoritis and sterility (Nishizuka and Sakakura, 1969). Subsequent studies showed that other organs could be affected depending on the genetic background of the inbred mouse strain. Disease could be prevented either by thymus engraftment or by transfer of normal day 7 or adult spleen cells (Kojima *et al.*, 1980; Kojima *et al.*, 1976). Efforts to search for more specific markers for Treg identified the CD25 molecule (the IL-2 receptor α chain) as a candidate (Sakaguchi *et al.*, 1995). Subsequently, the transcription factor Foxp3 was shown to be critical for Treg differentiation in thymus (Fontenot *et al.*, 2003; Hori *et al.*, 2003; Khattri *et al.*, 2003). Over-expression of Foxp3 on naïve human CD4⁺ T-cells confers suppressor function (Oswald-Richter *et al.*, 2004; Yagi *et al.*, 2004), and ectopic expression of a transgene encoding Foxp3 in mice (Khattri *et al.*, 2003) or retroviral expression of Foxp3 in CD25⁻ CD4⁺ T-cells results in the acquisition of suppression activity (Fontenot *et al.*, 2003; Hori *et al.*, 2003). Conversely, genetic disruption of Foxp3 in humans and mice leads to autoimmune disease. Foxp3 is currently the most reliable molecular marker for natural Tregs and provides clues to decipher the molecular basis of Treg development and function. Furthermore, studies showing the CD4⁺CD25⁺ T-cells from MHC class II deficient mice do not suppress responder T-cells *in vitro* or *in vivo*, suggested that the development of Treg cells is dependent on MHC class II-positive thymic cortical epithelium (Bensinger *et al.*, 2001).

Despite advances in our understanding of Treg development and function, we still do not know how these cells are generated and the role of TCR specificity in diverting thymocytes into the conventional or regulatory lineage. Sequence analyses of TCR α segments of a particular V α subfamily in Treg cells from TCR β -fixed transgenic mice have revealed strong similarity between the thymic and peripheral Treg TCR repertoire (Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006; Wong *et al.*, 2007b), echoing the view that Treg cells are mainly generated in the thymus (Itoh *et al.*, 1999). However, the peripheral and thymic Treg TCR repertoires were not identical, suggesting that certain TCRs are preferentially enriched or deleted in the periphery, similar to the observations with the total CD4⁺ T-cell population (Correia-Neves *et al.*, 2001). Further analyses of the TCR sequences from fractionated Treg or Tconv cells demonstrated distinct repertoires of the two populations, although with a degree of overlap that differed depending on the experimental setting. These studies were performed with unimmunized adult mice, and the relationship between the two subsets in mice with autoimmune disease like EAE circumstance was still unclear. To clarify the TCR repertoire differences between Treg and Tconv cells in MOG-EAE, we examined J β usage and the CDR3 β sequence of Treg and Tconv cells from 1MOG244.2 α mice with or without EAE.

4.2 Results

4.2.1 TCR J β and CDR3 Use in MOG-EAE

To examine alterations in the Treg and Tconv repertoires at higher resolution, we sacrificed 1MOG244.2 α mice prior to immunization or 23-25 days after EAE induction. Spleen and, for mice with EAE, CNS cells were collected and pooled from 5-6 identically treated mice per group. Pooling was performed due to low post-isolation T-cell yields from the CNS of retrogenic mice. The cells were sorted based on CD4 and the presence or absence of GFP-Foxp3 expression. RT-PCR was performed to analyze the paired β chains in each organ and cell type. V β 8.2-C β or V β 8.2-J β 2.7 primers were used to amplify TCR cDNA. We established a robust system to PCR amplify TCR β cDNA without contamination, and the same experiment was repeated twice. Five PCR reactions were performed on each cDNA to avoid PCR bias. The 400 bp DNA segments were TA cloned into pCR2.1 vector, 50-100 clones were picked and sequenced with T7 primer. Finally, we submitted the sequences in bulk to the IMGT (immunogenetics information system; <http://imgt.cines.fr/>) web server for identifying the TCR gene use. Totally ~4000 sequences were collected. The identical experiment was performed twice.

We initially surveyed V β 8.2-C β sequences to determine the distribution of J β among V β 8.2 $^{+}$ TCR. In total, 1829 sequences were assessed. Twelve different J β regions were found in these TCR sequences. J β used by Treg and Tconv cells showed no significant difference in either spleen or CNS (Figure 4-1C and D). This hinted that Treg cells have a diverse V β 8.2 $^{+}$ TCR repertoire as Tconv cells, and this diversity was not altered by autoimmunity circumstances. Proportions of J β used by either the Foxp3 $^{+}$ or Foxp3 $^{-}$ populations showed no significant differences between the CNS and periphery in mice with EAE ($p > 0.05$, Figure 4-1A and B). Comparison of pre- and post-immune splenocytes also failed to identify changes in J β use among Foxp3 $^{+}$ T-cells and showed a significant difference in Foxp3 $^{-}$ cells only for a single J β , J β 2.4 ($6.2 \pm 0.5\%$ pre versus $14.1 \pm 0.5\%$ post; $p = 0.012$). This indicates preserved diversity in the V β 8.2 $^{+}$ population with EAE induction, especially when comparing the CNS-infiltrating and splenic T-cell populations of diseased mice.

We next surveyed the more circumscribed V β 8.2-J β 2.7 repertoire. V β 8.2 was selected due to the predominate expression of V β 8.2 among MOG-specific T-cells in H-2 b mice (Mendel Kerlero de Rosbo and Ben-Nun, 1996) and J β 2.7 due to its use in the original V β 8.2 $^{+}$ J β 2.7 $^{+}$ TCR β chain partner for the 1MOG244.2 α TCR (Alli *et al.*, 2008). Despite the theoretical potential for formation of $>10^6$ unique TCR sequences with this V β -J β constraint, we therefore knew that V β 8.2 $^{+}$ J β 2.7 $^{+}$ TCR β had the potential to pair with the 1MOG244.2 α to form MOG-specific TCR. An estimated 95% confidence interval for the upper limit of the maximum frequency ($\phi_{\max}$) of unobserved TCR CDR3 sequences for individual cell types/samples in different experiments ranged from 1.4 to 3.1% for different samples and cell types (Table 4-1). The $\phi_{\max}$ confidence interval (CI) refers to the upper frequency of a CDR3 sequence in the V-J analysis for which there is

Figure 4-1. J β used in Tconv and Treg cells. CD4⁺ T-cells from retrogenic mice, preimmune or EAE induction, were flow cytometrically sorted into CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ populations. After a few steps of RT-PCR, TA clone and sequencing, CDR3b regions were identified through IMGT. Comparisons were made between Tconv (A) and Treg (B) cells from unimmunized and immunized splenocytes and CNS T-cells from mice with EAE. Comparisons were also made between Tconv and Treg cells in immunized CNS T-cells (C) and in immunized splenocytes (D). * P < 0.05.

Table 4-1. Isolation of TCR V β sequences from retrogenic mice.

Experiment	Disease scores	T-cell type	Sequence	Isolated sequences		V-J ϕ max 95% CI
				V-C β	V-J β	
1 (Pre-immune)	NA	Spleen Foxp3 ⁻	Total	137	155	
			Unique	131	55	2.25%
		Spleen Foxp3 ⁺	Total	152	183	
			Unique	150	64	1.91%
2 (Pre-immune)	NA	Spleen Foxp3 ⁻	Total	102	161	
			Unique	97	54	2.17%
		Spleen Foxp3 ⁺	Total	128	123	
			Unique	124	62	2.85%
3 (EAE, Day 23)	1,2,3,3,4	Spleen Foxp3 ⁻	Total	159	167	
			Unique	97	52	2.09%
		Spleen Foxp3 ⁺	Total	131	141	
			Unique	57	46	2.48%
		CNS Foxp3 ⁻	Total	207	256	
			Unique	90	42	1.36%
		CNS Foxp3 ⁺	Total	147	226	
			Unique	88	59	1.54%
4 (EAE, Day 25)	1,1,1,2,2,2	Spleen Foxp3 ⁻	Total	160	208	
			Unique	71	48	1.68%
		Spleen Foxp3 ⁺	Total	144	114	
			Unique	82	49	3.08%
		CNS Foxp3 ⁻	Total	207	211	
			Unique	82	45	1.65%
		CNS Foxp3 ⁺	Total	155	207	
			Unique	60	41	1.68%

95% certainty of positive detection. For example, there were a total of 155 amino sequences in splenocyte Treg of pre-immune sample in experiment 1, if the frequency of particular CDR3 sequence in the population is above 2.25%, the chance for it to be detected is 95%. This indicates that the collected sequences were adequate to survey common sequences in each cellular cohort.

Among the V β 8.2-J β 2.7 sequences obtained, 452 unique amino acid sequences were identified. Interestingly, 54 (~12%) of sequences were shared, or public sequences, among the 4 independent experiments. Public sequences were over-represented among more commonly identified sequences, with 74% of public sequences present within the 100 most frequent sequences. An increased probability of identifying sequences in multiple independent samples when their sequence frequency is high is expected, and implies that the observed proportion of public sequences underestimates total representation due to non-identification of low frequency public sequences. 16/54 public sequences were found in TCR exclusively used by Treg, 14/54 in Tconv TCR, and 24/54 in sequences identified both in Treg and Tconv. The CDR3 amino acid sequences of public sequences were diverse and no sequence patterns were identified.

Abundance coverage estimator analysis (ACE) of pooled sequences, an indicator of total sequence diversity, indicated a limited diversity consistent with findings in other systems, with values of 271 and 337 for pre-immune, and 191 and 171 for post-immune V β 8.2⁺, J β 2.7⁺ Tconv and Treg splenocytes respectively. This restricted diversity and the high proportion of public sequences indicates that the retrogenic repertoire is heavily sculpted and that few of the potential sequences that may form from V-D-J rearrangement are realized in the retrogenic mice. Interestingly, the original β chain partner for the 1MOG244.2 α TCR (ASGDAGTGYEQY) was among the public sequences identified in the CNS Foxp3⁻ populations of both EAE induction experiments, though not in pre-immune analyses. This suggests that receptors relevant to EAE in C57BL/6 mice, which served as the source of the 1MOG244.2 hybridoma, play a role in the 1MOG244.2 α retrogenic mice.

Among public TCR, 20 CDR3 sequences were identified as common to both preimmune T-cells and T-cells from mice with EAE. TCR sequences appearing predominantly in Foxp3⁺ or Foxp3⁻ populations preimmune showed the same orientation, Foxp3⁺ or Foxp3⁻, in mice with EAE for 19 of the 20 receptors. This implies that TCR sequence rather than disease status is the primary force governing T-cell Foxp3 expression.

4.2.2 Limited Overlap of Treg and Tconv Repertoires

Comparison of unique CDR3 amino acid sequences for each cellular cohort revealed several notable features. Repertoire overlap was greater among a single class of cells (Treg or Tconv) across different organs (spleen and CNS) than among different classes of cells within an organ (Figure 4-2A). In experiment 3, 32.7% of unique splenic and CNS Treg sequences and 23.9% of unique splenic and CNS Tconv sequences

Figure 4-2. Population differences among TCR subsets in mice with EAE. (A) Unique TCR sequences from the identified experiments (Table 4-1) were compared between population groups, and the frequency of sequences shared by Treg and Tconv cells within an organ (left) or of sequences from a specific cell type, Treg and Tconv cells, shared across organs (right) plotted. (B) The Morisita-Horn index, a measure of ecologic diversity among populations, is plotted for the same groups as (A). A value of 1.0 indicates population identity. Whereas (A) plots sharing of uniquely identified sequences, (B) measures differences in both sequence diversity and identification frequency between the indicated populations.

overlapped. In contrast, only 11.0% of unique CNS and 10.1% of unique splenic were shared between Treg and Tconv cells. In experiment 4, the values were 20.0%, 27.4% 14.7%, and 6.6%, respectively. In addition, we used the Morisita Horn index to estimate similarity between populations. The Morisita Horn index is a commonly used, unitless quantitative index of the similarity between two populations (a value of 1.0 represents complete identity) that uses information regarding the number of species as well as the abundance of each species. The Morisita Horn index was widely used in T-cell repertoire studies before (Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2002). Here, the M-H index also demonstrated strong dissimilarity in Treg and Tconv cell CDR3 sequences in either spleen or CNS and greater similarity when an individual cell type was compared between spleen and CNS (Figure 4-2B). Taken together, Treg and Tconv TCR repertoires are distinct, though do show some overlap.

To determine whether disease has an impact on the degree of overlap, we also compared the repertoires of preimmune and EAE mice. In preimmune experiment 1 and 2, the percent of unique shared TCRs between splenic Treg and Tconv cells was 3.5% and 6.4% respectively. In EAE experiment 3 and 4, the overlap was 10.1% and 6.6% in spleen, and 11.0% and 14.7% in CNS. Therefore there is a trend toward increasing overlap with disease development. However, this did not achieve statistical significance, ($p = 0.28$ for preimmune versus EAE spleen, and $p = 0.22$ for EAE spleen versus EAE CNS). This implies that autoimmune disease has limited impact on the overlap of Treg and Tconv TCR repertoire, and argues against substantial levels of interconversion during autoimmunity.

Further, among the minority of unique sequences that were shared by Treg and Tconv cells, the isolates of a particular sequence were typically skewed toward a single class of cells, either Treg or Tconv cells. Indeed, the Treg/Tconv cell identification ratio of shared TCR should form a Gaussian distribution, potentially around a ratio of 1, if there was rapid, free and equal interconversion between Treg and Tconv cells. Yet 73% of shared CDR3 isolates were skewed by a ratio $\geq 2:1$ toward a single cell class (Figure 4-3A), mean and median for majority Foxp3⁺ TCR: 6.1 ± 7.3 , 2.5; Foxp3⁻ TCR: 9.1 ± 11.4 , 5.1). This was influenced by the presence of CDR3 for which few sequences were isolated, and for which substantial skewing would therefore not be possible. Indeed, for shared sequences independently identified at least 10 times, 84% had a ratio $\geq 2:1$ (Figure 4-3B; mean, median for Foxp3⁺: 9.3 ± 8.4 , 9.1; Foxp3⁻: 13.4 ± 13.8 , 10.0). Skewing of CDR3 toward a single cell class, Treg or Tconv cells, was also readily apparent in graphical mapping of the distribution among Foxp3⁺ or Foxp3⁻ cells of the most common sequences isolated in individual experiments (Figure 4-4 and 4-5).

Comparative analyses were further performed on three structural features of the identified CDR3, length, charge, and hydrophilicity as potential correlates of specificity. The six cohorts of unique CDR3 sequences, acquired from postimmune CNS, postimmune spleen, and preimmune spleen Foxp3⁺ and Foxp3⁻ cells were compared via the Kruskal-Wallis test and significance was observed only for charge ($p < 0.0001$). With the Wilcoxon rank-sum test to compare any two cohorts within this group, the charge of the CNS Foxp3⁻ CDR3 showed significant differences with each of the five other

Figure 4-3. Distribution of shared CDR3 sequences. Numbers of CDR3 amino acid sequences identified in both Foxp3⁺ and Foxp3⁻ populations at the indicated ratio is shown. Left sides of plots indicate sequences that were primarily identified among Foxp3⁺ cells, and right sides indicate sequences primarily identified among Foxp3⁻ cells. A 1:1 ratio indicates equal numbers of sequences were identified among Foxp3⁺ and Foxp3⁻ populations. (A) Data is plotted for all shared sequences identified. (B) Data is plotted only for those shared sequences identified 10 or more times.

Figure 4-4. Frequency representation of the most common splenic V β 8.2⁺J β 2.7⁺ CDR3 isolates. Frequencies of the 10 most commonly isolated splenic CDR3 sequences from GFP-Foxp3⁺ (A) or GFP-Foxp3⁻ (B) T-cells in each of the 4 experiments is plotted (filled bars). CDR3 frequencies are shown as percent values and were defined as $100 * (\text{Foxp3}^+ \text{ or Foxp3}^- \text{ CDR3 isolates with the indicated sequence}) / (\text{total number of Foxp3}^+ \text{ or Foxp3}^- \text{ splenic CDR3 sequences for the indicated experiment})$. Experiments 1 and 2 assayed preimmune mice, and 3 and 4 studied mice with EAE. For sequences shared among Foxp3⁺ and Foxp3⁻ populations, below each filled bar the representation of the identical sequence in the alternative population is plotted, Foxp3⁻ cells in (A) and Foxp3⁺ cells in (B) (unfilled bars).

A Foxp3⁺ TCR

B Foxp3⁻ TCR

Figure 4-5. Frequency representation of the most common CNS-derived V β 8.2⁺J β 2.7⁺ CDR3 isolates. Analyses were performed as in Figure 4-4, but for CNS-derived sequences from experiments 3 and 4.

cohorts (Figure 4-6) ($p \leq 0.02$ for all comparisons after Hochberg's adjustment for multiple tests). A significant difference was seen in only one other comparison group, CNS and preimmune splenic Foxp3⁺ CDR3 ($p = 0.019$). Therefore, CNS-infiltrating T-cells, particularly Foxp3⁻ cells, possess at least one distinguishing CDR3 characteristic, increased negative charge. Of note, the MOG₃₅₋₅₅ epitope bears a potentially complementary positive charge and includes three arginine residues, two of which are predicted to lie in the Ag binding groove with their guanidinium moieties directed toward the TCR (Ben-Nun *et al.*, 2006).

4.3 Summary

We analyzed the Treg and Tconv cell TCR repertoires in EAE utilizing a retrogenic mouse model in which the TCR α chain locus is fixed. The overlap between Treg and Tconv cells was less than the overlap when a single class of cells was compared in two different organs - the spleen and CNS. This demonstrates that Treg and Tconv cells express largely distinct TCRs in the CNS and spleen of mice with EAE. Nevertheless, some overlap (~10%) was seen. This result echoed previous studies performed on unimmunized mice, which saw overlap between Treg and Tconv TCR repertoires, ranging from 10% to 20% (M-H index of 0.1-0.2) (Hsieh *et al.*, 2004; Hsieh *et al.*, 2006; Lathrop *et al.*, 2008; Pacholczyk *et al.*, 2006).

Comparison between preimmune and EAE mice revealed that the degree of overlap was not significantly increased in EAE. Among CDR3 β shared by Treg and Tconv cells, individual sequences tended to be dominantly represented within a single cell type. These results argue against large scale interconversion in an inflammatory environment. It is consistent with previous results, in which little conversion was found after the adoptive transfer of CD4⁺ CD25⁻ T-cells (Lathrop *et al.*, 2008) and in TCR repertoire analysis of NOD mice (Wong *et al.*, 2007a). In contrast it has also been suggested that conversion contributes to ~72% of peripheral Treg cells (Liang *et al.*, 2005).

Public TCRs were identified in the V β 8.2-J β 2.7 repertoire shared in the four independent experiments (preimmune and EAE mice). This is consistent with the previous report that T-cells express V α and V β public repertoires in the CNS of C57BL/6 mice during MOG-EAE (Fazilleau *et al.*, 2006). Further the original 1MOG244.2TCR was among the public sequences identified in the CNS Foxp3⁻ group, implying that this particular TCR is important to EAE in retrogenic and C57BL/6 mice.

In summary, these results and the identification of distinct charge characteristics specifically among CNS-infiltrating Tconv cells implies that Treg and Tconv cells in EAE are distinct populations and do not substantially interconvert.

Figure 4-6. Length, charge, and hydrophilicity of isolated CDR3. Medians $\pm$ 95% CI for length (A), charge (B), and hydrophilicity (C) among unique CDR3 sequences identified in the six indicated populations is plotted. Analyzed sequences were determined as the regions bounded by but not including the conserved C and F residues bordering each CDR3 β . * $p < 0.05$ according to the Hochberg-adjusted Wilcoxon rank sum test.

CHAPTER 5. ANALYSIS OF TCR IN 1MOG244.2TCR α RETROGENIC MICE

5.1 Introduction

T-cell development begins in the thymus, and one of the earliest events is TCR gene rearrangement. The mature thymocyte repertoire is formed based on the ability of the TCRs to react with host MHC molecules, which are mostly occupied with self-peptides (Starr *et al.*, 2003). Continued interactions between TCRs and peptide-MHC molecules contribute to the maintenance and homeostatic expansion of mature T-cells in the periphery (Bender *et al.*, 1999; Ernst *et al.*, 1999), thus reshaping the peripheral T-cell repertoire. These processes allow the immune system to maintain a T-cell repertoire containing sufficient diversity and specificity to respond against an unknown and highly diverse pathogen pool.

In Chapter 4 we showed that Treg repertoire is highly diverse with distinct set of TCRs compared with Tconv cells, though with some overlap between populations both in unimmunized mice and in mice with EAE. However, the role of TCR specificity in directing thymic precursors into the Treg or Tconv lineage remains unclear. Some studies indicated TCR specificity is crucial to Treg differentiation. T-cells exclusively expressing the transgenic TCR appeared incapable of undergoing CD4⁺CD25⁺ Treg cells selection. However, the introduction of cognate peptide into the repertoire of self-peptides can induce the formation of CD4⁺CD25⁺ Treg cells expressing the clonotypic transgenic TCR. For example, Treg cell development was seen when TS1 TCR transgenic mice were mated with mice containing cognate ligands (HA28). The formation of Treg cells has also been described in mice expressing HA under the control of an immunoglobulin promoter in RAG-deficient backgrounds (Apostolou *et al.*, 2002; Jordan *et al.*, 2001). Likewise, when DO11.10 TCR transgenic mice were crossed with RAG^{-/-} mice expressing cognate peptide - ovalbumin (OVA), CD4⁺CD25⁺ Treg cells were also formed (Kawahata *et al.*, 2002; Walker *et al.*, 2003). These findings suggested that the activation by agonist ligands in thymus could promote Treg cell formation. This together with the utilization of different TCRs in Treg than Tconv cells implies that thymocytes possessing self-antigen specificity are more likely to develop into Treg cells. On the other hand, Treg cells can recognize a wide range of foreign antigens, have a diverse TCR repertoire, and no increased reactivity of Treg cells toward self was identified in one analysis of T-cell hybridomas derived from Treg and Tconv cells (Pacholczyk *et al.*, 2007). Therefore, we cannot completely exclude the possibility that TCRs with weak affinity to self or that are not self-reactive will be included in Treg repertoire.

Though TCR repertoire analysis is powerful in picturing the overall relationship between Treg and Tconv cells, it is limited in that inferences are made by population shifts in the absence of specific knowledge about antigen specificity. Likewise, studies of antigen reactivity among Treg and Tconv cells have typically been performed outside of the context of repertoire analyses that illuminate relationships between T-cell populations. To better clarify the specificity and responsiveness of Treg cells, in this

chapter, we focused the studies on a cohort of TCRs identified in Chapter 4, reconstituted into the CD4⁺ TCR $\alpha\beta$ -deficient 4G4.CD4 T hybridoma. We were thereby able to determine the frequency of MOG-specific auto-reactive TCRs in Treg and Tconv cells populating the CNS and spleen. The experiments answered the following questions: Are Treg or Tconv cells present in the CNS during MOG-EAE more prone to be MOG-specific than those in the spleen, and the frequencies of MOG-specific cells differ between Treg and Tconv cells. What are the sensitivity and fine specificity differences among the self-reactive Treg and Tconv TCRs? Are there differences in the CDR3 features of MOG-specific TCR from Treg and Tconv cells? This analysis may give clues as to how the nature of the TCR itself impacts on the fate of Tcells: to become Treg or Tconv? Localize to CNS or periphery?

5.2 Results

5.2.1 *MOG-specificity among Treg and Tconv TCR*

To better delineate the properties of the responding TCR present within the CNS and periphery during EAE, we recreated full-length $\alpha\beta$ TCR cDNAs from 40 identified CDR3 sequences (Table 5-1). The CDR3 were selected from sequences identified at high frequency (all were among the 50 most commonly identified sequences) to provide an approximately equal distribution of predominantly Foxp3⁺ and Foxp3⁻ CDR3 of splenic or CNS origin. As above (Figures 4-3, 4-4 and 4-5), TCR with sequences shared by both Treg and Tconv cells were heavily skewed toward a single cell class (Table 5-1, far right column). A high representation of public TCR was seen among the selected CDR3 (21/40 sequences), reflecting the increased frequency of these sequences among common TCR. Reconstituted TCR were subcloned into a tricistronic retroviral vector essentially as we have previously described (Alli et al., 2008), with TCR α and β chains linked by the Thosa assigna virus 2A sequence to facilitate their stoichiometric expression and an IRES-linked cyan fluorescent protein (CFP). TCR-encoding retrovirus for these and the original 1MOG244.2 $\alpha\beta$ TCR were transduced into the CD4⁺ TCR $\alpha\beta$ -deficient 4G4.CD4 T-cell line (Figure 5-1). The cells were flow cytometrically sorted for similar TCR expression levels and stimulated with MOG₃₅₋₅₅ peptide, and IL-2 production was measured as an indicator of MOG responsiveness.

MOG-specificity varied among the TCR cohorts (Figure 5-2). 2/12 (16.7%) of splenic Foxp3⁻ TCR from immunized mice responded to MOG, whereas 1/8 (12.5%) of splenic Foxp3⁺ TCR responded. In contrast to this more modest representation of MOG-specific cells in the periphery, 8/8 (100%) of CNS Foxp3⁻ and 6/12 (50%) of CNS Foxp3⁺ TCR-transduced cells produced IL-2 in response to MOG. These results therefore demonstrate substantial enrichment of MOG-specificity within CNS-infiltrating Tconv cells of mice with EAE. Further, despite the evidence from our repertoire analyses for limited interconversion between Tconv and Treg cells and the use of a TCR α in the retrogenics derived from a Foxp3⁻ T-cell, a substantial proportion of the Treg cell

Table 5-1. Features of CDR3 β used to reconstitute full-length TCR cDNA.

Clone #	Site	MOG-specific	Foxp3 ⁺	Sequence	Length	Public	Foxp3 ⁺ TCR	Foxp3 ⁻ TCR	Foxp3 ⁺ /Foxp3 ⁻ ratio
4	Spl	N	N	ASGDGGLGGRGEQY	14	Y	0	18	
7	Spl	N	N	ASGEKAEYEQY	11	Y	1	15	0.067
10	Spl	N	N	ASGTGGYSYEQY	12	N	2	82	0.024
17	Spl	N	N	ASGRQSSYEQY	11	N	0	15	
30	Spl	N	N	ASGGGSYEQY	10	N	0	17	
31	Spl	N	N	ASGAPDWGVPEQY	13	N	3	33	0.090
32	Spl	N	N	ASGEKTEYEQY	11	N	2	30	0.067
36	Spl	N	N	ASGDDWGGAYEQY	13	Y	6	14	0.43
37	Spl	N	N	ASGDGLASSYEQY	13	Y	2	20	0.1
47	Spl	N	N	ASGPVEQY	8	Y	1	13	0.077
5	CNS	N	Y	ASGDWDNYEQY	11	Y	35	10	3.5
8	CNS	N	Y	ASGGYEQY	8	Y	33	2	16.5
15	CNS	N	Y	ASGRDRGLEQY	11	N	18	0	
20	CNS	N	Y	ASGDWDVYEQY	11	N	14	0	
34	CNS	N	Y	ASGDGTGGVEQY	12	Y	26	1	26
39	CNS	N	Y	ASGDARLGGREQY	13	Y	20	0	
3	Spl	N	Y	ASGDGEQY	8	Y	86	8	10.8
14	Spl	N	Y	ASGGEVYEQY	10	N	16	0	
24	Spl	N	Y	ASAGWGLYEQY	11	N	7	0	
26	Spl	N	Y	ASGEGGFSYEQY	12	N	20	0	
28	Spl	N	Y	ASGDDWAYEQY	11	N	13	0	
41	Spl	N	Y	ASGRGTGGYEQY	12	Y	19	0	
46	Spl	N	Y	ASGDAWGVYEQY	12	Y	15	0	

Table 5-1. Continued.

Clone #	Site	MOG-specific	Foxp3 ⁺	Sequence	Length	Public	Foxp3 ⁺ TCR	Foxp3 ⁻ TCR	Foxp3 ⁺ /Foxp3 ⁻ ratio
6	CNS	Y	N	ASGEPGQSSYEYQY	13	Y	2	12	0.17
11	CNS	Y	N	ASGDAGISYEYQY	12	Y	7	32	0.22
12	CNS	Y	N	ASGDAGSSYEYQY	12	Y	1	8	0.13
19	CNS	Y	N	ASGDSGGVYEYQY	12	Y	3	15	0.2
21	CNS	Y	N	ASGDAGQAYEYQY	12	Y	1	45	0.022
29	CNS	Y	N	ASGEAGGAYEYQY	12	Y	3	14	0.21
45	CNS	Y	N	ASGDPGSSYEYQY	12	N	0	16	
44	Spl	Y	N	ASGDASRGYEYQY	12	N	0	16	
50	Spl	Y	N	ASGEGGGHYEYQY	12	Y	0	12	
244.2*	Spl	Y	N	ASGDAGTGYEYQY	12	Y	0	7	
1	CNS	Y	Y	ASGASGGLYEYQY	12	N	13	1	13
9	CNS	Y	Y	ASGLTDEYQY	9	N	25	0	
16	CNS	Y	Y	ASGATGAPYEYQY	12	N	16	0	
18	CNS	Y	Y	ASGVPGQGVEYQY	12	N	13	0	
52	CNS	Y	Y	ASGAPGGSYEYQY	12	Y	11	0	
110	CNS	Y	Y	ASGVPGQGGEYQY	12	N	11	0	
35	Spl	Y	Y	ASGAPGHPYEYQY	12	Y	22	0	

Note: Clone #, site of primary isolation, MOG-specificity, association with Foxp3 expression, CDR3 length, isolation from multiple experiments (public) and frequency of identification from Foxp3⁺ and Foxp3⁻ cDNA is indicated. *, original 1MOG244.2TCR.

Figure 5-1. Expression of TCR on CD4.4G4 cells. TCR was retrovirally transduced into CD4.4G4 cells and cytometrically sorted for CD4⁺TCR⁺ population. Sorted cells were expanded for 3-4 days, and TCR expression was analyzed using anti-TCR β antibody.

Figure 5-2. MOG-specificity among isolated TCR. Forty CDR3 β , distributed among Foxp3⁺ and Foxp3⁻ isolates from the CNS or spleen, were used to construct retrovirus incorporating full-length $\alpha\beta$ TCR. Retrovirally transduced 4G4.CD4 T-cells were flow cytometrically sorted for similar surface expression of TCR and stimulated with *Tcra*^{-/-} splenic APC with or without 100 μ g/ml MOG₃₅₋₅₅ peptide or with plate-bound CD3 antibody. IL-2 production was measured in cell-free supernatant at 24 hr by ELISA. Data indicate mean + 1 SEM and are representative of three or more assays for each TCR.

response was directed against MOG. This indicates that regulatory T-cells in MOG-EAE respond to cognate autoantigen.

5.2.2 Segregation of MOG-Specific Treg and Tconv Cell TCR Based on CDR3 β Characteristic

Sequence information for all MOG-specific and nonspecific TCR was compared (Table 5-1). This provided several insights. First, MOG-specific CDR3 β , Foxp3⁺ or Foxp3⁻, bore a consistent length of 12 (11.9 ± 0.8 ; 15/18 [83%] sequences length of 12). The non-MOG-specific TCR showed only slightly decreased average length (11.2 ± 1.6), but many fewer 12 amino acid sequences (5/23; 23%) and more diversity in this measure. MOG-specific TCR further more consistently had a G at the 6th CDR3 position (16/18; 89%) compared with non-MOG-specific TCR (4/23; 17%), implying that the absence of a side chain in that position is important for antigen recognition. More interestingly, among the MOG-specific TCR, CDR3 sequence distinguished TCR derived from primarily Foxp3⁺ and Foxp3⁻ T-cells. Acidic D or E residues were present at position 4 in 11/11 (100%) of Foxp3⁻ but 0/7 (0%) of Foxp3⁺ MOG-specific CDR3. D and E would be anticipated to have a high prevalence at this location; a D is formed with V β 8.2-D β recombination in the absence of junctional mutations or with a C $\rightarrow$ T replacement at the first D-region nucleotide. An E is formed with a C $\rightarrow$ A or G replacement at the same nucleotide. Indeed, 5/10 (50%) of Foxp3⁻ and 8/13 (62%) of Foxp3⁺ nonspecific CDR3 bore a D or E at position 4, making its complete absence more surprising in the Foxp3⁺ MOG-specific population. In contrast, 100% of the MOG-specific Foxp3⁺ TCR bore an aliphatic A (4/7), V (2/7), or L (1/7) residue at that location, residues seen in only 1 of the 34 other CDR3 examined. Therefore, TCR expressed by MOG-specific Treg and Tconv cells comprise distinct families. Implicitly, these cells undergo a distinct selection process in 1MOG244.2 α mice that is either not based on MOG recognition or is based on differential recognition of MOG.

5.2.3 Sensitivity and Fine Specificity of MOG-specific TCR for Auto-antigen

Current models of Treg cell lineage specification suggest that Treg cells possess a proclivity for self-reactivity and that thymocytes with higher affinity for self-antigen are diverted into the Foxp3⁺ lineage (Pacholczyk and Kern, 2008). MOG is expressed in the thymus, though in small amounts (Delarasse *et al.*, 2003). If MOG is responsible for the selection of MOG-reactive Treg cells, then these cells would be anticipated to bear an increased affinity for it. Because the 4G4.CD4 TCR transductants were essentially identical except for the sequence of their TCR CDR3 β , their relative sensitivity to Ag should primarily reflect their affinity. We therefore tested the sensitivity of all MOG-specific TCR to dilutions of MOG₃₅₋₅₅ peptide (Figure 5-3). Interestingly, although Treg cell TCR trended toward increased sensitivity, substantial overlap was observed between the MOG-specific Treg and Tconv cell TCR. In each case, receptors were identified with high, moderate, and low sensitivity for MOG. Moreover, specific pairs of Foxp3⁺ and Foxp3⁻ CDR3 with conserved amino acid sequences showed similar sensitivities for

Figure 5-3. Sensitivity of MOG₃₅₋₅₅-specific TCR for autoantigen. MOG-specific 4G4.CD4 transductants were assayed for relative sensitivity to antigen. Cells bearing Foxp3⁻ (A) or Foxp3⁺ (B) T-cell derived TCR were analyzed as in Figure 5-1 but stimulated with varied doses of MOG₃₅₋₅₅ peptide. All cell lines were simultaneously analyzed for comparability. Data shown is representative of 3 independent experiments with at least 2 independent transductions of each TCR.

antigen (Table 5-1 and Figure 5-3). Clones 19 (Foxp3⁻) and 1 (Foxp3⁺), which besides the variation at position 4 only have a conserved V→L substitution at position 8, both responded vigorously to MOG stimulation. Clones 45 (Foxp3⁻) and 52 (Foxp3⁺), which differ only at position 4 and by a S→G at position 7, also responded similarly and more moderately. Therefore, although Treg cells tend toward increased sensitivity for MOG, this does not seem to be encoded into the position 4 variation and a MOG sensitivity threshold does not appear fundamental to response by Treg versus Tconv cell TCR.

These results suggest that other factors affected by the position 4 variation, for instance differential fine specificity for MOG or sensitivity to an alternative cross-reactive selecting Ag(s), are responsible for Treg cell lineage assignment. To test the former possibility, we examined the fine specificity of recognition of five of the MOG-specific Foxp3⁻ TCR, including the index 1MOG244.2TCR, and four of the Foxp3⁺ TCR. Prior studies with MOG₃₅₋₅₅-specific TCR have identified critical TCR interacting residues, particularly R41, F44, R46, and V47 (Petersen *et al.*, 2004). The different TCR demonstrated variability, through common patterns of reactivity when tested with alanine-substituted MOG₃₅₋₅₅ peptides (Figure 5-4). An R41A substitution did not substantially influence recognition for 1MOG244.2 or most of the other TCR, whereas response was diminished to F44A, R46A, and V47A substitutions. However, with these and alanine substitutions at all residues from V37 to L50, no pattern was identified that distinguished the fine specificity of the Foxp3⁺ and Foxp3⁻ T-cell-derived TCR. Therefore, differential recognition of cognate MOG antigen does not appear to be associated with the binary differentiation of T-cells with acidic or aliphatic residues at CDR3β position 4 alternatively into Tconv or Treg cells. Considering the absence of evidence for affinity or fine specificity-based segregation of Tconv and Treg cell TCR, our data would suggest that a MOG sensitivity threshold is not itself responsible for MOG-specific Treg cell recruitment, and presumably also lineage assignment.

5.2.4 Fidelity in TCR Lineage Specification

The 1MOG244.2α retrogenic mice were produced with *Tcra*^{-/-} HPC donor and recipient mice. We were interested whether the MOG-specific Treg and Tconv cell TCR that we identified in 1MOG244.2α mice (Table 5-1) would similarly guide differentiation in a distinct system where the thymus is from a wild-type and not *Tcra*^{-/-} mouse and most developing T-cells are unmanipulated. To test this, we transduced MOG-specific TCRαβ pairs into CD45.1⁻CD45.2⁺ *Rag1*^{-/-} HPC, which cannot express other TCR. These were diluted with an excess of congenic CD45.1⁺CD45.2⁻ T-cell-depleted bone marrow cells and transplanted into CD45.1⁺CD45.2⁻ mice. Seventeen mice demonstrated detectable CD45.2⁺CD4⁺ T-cell engraftment (range 0.001%–8.5% of CD4⁺TCR⁺ cells), including mice expressing three TCR derived from Treg cells (#1, 9, 18, Table 5-1) and four from Tconv cells (#2, 6, 21, 45) (Figure 5-5). Overall, the Treg cell-derived TCR showed >20-fold increased engraftment into the Foxp3⁺ lineage when compared with the Tconv cell TCR (mean of Foxp3⁺ cells: 21.4% ± 10.6%; of Foxp3⁻ cells: 0.95% ± 1.1%). Therefore, cells expressing TCR derived from Tconv cell TCR in 1MOG244.2α mice differentiate into Tconv and not Treg cells in this system. Cells expressing TCR derived from Treg

Figure 5-4. Fine specificity of Fcγ3⁺ and Fcγ3⁻ TCR. The indicated 4G4.CD4 cell lines expressing Fcγ3⁺ or Fcγ3⁻-derived TCRs were stimulated with irradiated Tcrα^{-/-} APCs in the absence of antigen or in the presence of 100μg/ml MOG₃₅₋₅₅ or the indicated alanine-substituted mutants of this peptide. IL-2 was measured in cell free supernatant at 24 h as an indicator of stimulation. Percent IL-2 production compared with MOG₃₅₋₅₅ stimulation is plotted. Bars indicate from left to right IL-2 production by clones 6,21,29,45,244,2,1,16,18 and 35 after stimulation with the indicated peptide. Mean + SEM is plotted.

Figure 5-5. Foxp3 expression among CD4⁺ T-cells in TCR-retrogenic mixed chimeras. To evaluate the lineage preference of MOG-specific Treg or Tconv TCR identified from 1MOG244.2 α mice in mice with a wild-type thymus and few transgenic precursor cells, we produced mixed chimeras using select cloned TCR. Retroviral constructs incorporating the TCR $\alpha\beta$ from MOG-specific Treg-derived TCR clones 1, 9, and 18, and Tconv derived clones 2, 6, 21, and 45 were transduced into B6.129S7-Rag1^{tm1Mom}/J HPC. The Rag1 deficiency ensures that all T-cells developing from the transduced population express the TCR transgene. An ~1-5% transduction efficiency was obtained. The HPC were mixed at a 1:1 or 1:10 ratio with untransduced, wild-type, T-cell depleted CD45.1 congenic B6.SJL-Ptprc^aPep3^b.BoyJ-derived bone marrow, and transplanted into B6.SJL-Ptprc^aPep3^b.BoyJ mice. At 6-8 weeks after transplant, mice were sacrificed, and splenocytes stained with CD45.2, TCR, CD4, and intracytoplasmic Foxp3 antibodies. (A) Flow cytometric analysis shows Foxp3 expression on CD45.2⁻CD4⁺TCR⁺ or CD45.2⁺CD4⁺TCR⁺ gated populations. (B) Engraftment and percent Foxp3 positivity among TCR-transduced CD45.2⁺ T-cells is indicated. Percent engraftment of CD4⁺CD45.2⁺ T-cells was calculated as 100 x (number of CD45.2⁺CD4⁺TCR⁺ cells identified) / (total number of CD4⁺TCR⁺ cells). Percent Foxp3⁺ T-cells is calculated as 100 x (number of CD45.2⁺CD4⁺TCR⁺Foxp3⁺ cells) / (total number of CD45.2⁺CD4⁺TCR⁺ cells).

cell TCR in 1MOG244.2 α mice show increased Treg cell bias. Although not all of the retrogenic T-cells expressing the Treg cell-derived TCR developed into Foxp3⁺ cells, this is not surprising because niche saturation for Treg cell development may occur even at very small numbers of Ag-specific T-cells (Bautista *et al.*, 2009). Despite heavy dilution with wild-type cells in these mixed chimeric mice, cells expressing the assayed TCR should nevertheless be increased in frequency compared with 1MOG244.2 α mice. Therefore, Treg and Tconv cell lineage assignment in the 1MOG244.2TCR α retrogenic mice is paralleled in a distinct and arguably more natural system, one in which the thymic stroma and the large majority of T-cells are wild-type.

5.3 Summary

In this chapter, we selected 40 high frequency TCRs identified in Chapter 4, transduced them into CD4.4G4 hybridomas, and analyzed their responsiveness to MOG₃₅₋₅₅. We found that autoreactive Tconv cells were highly enriched in the CNS (100% of TCR), but not spleen, of 1MOG244.2TCR α mice with EAE. 50% of CNS-infiltrating Treg cells were also MOG-reactive. This result confirms the presence of auto-antigen reactive T-cells accumulating in CNS of mice with EAE, and indicates that a large percentage of Treg TCR are MOG-reactive.

MOG-specific and non-specific TCRs showed distinct CDR3 β sequence features. MOG-specific TCRs uniformly bear a length of 12 amino acids, and a glycine at position 6. More importantly, among MOG-specific TCRs, Treg and Tconv TCR possess distinct characteristics. Aspartic acid or glutamic acid at position 4 were exclusively seen in Tconv TCR, whereas aliphatic residues – alanine, valine and leucine were found in Treg TCR. This suggests that Treg and Tconv cells during EAE arise from distinct population repertoire.

Analysis of sensitivity differences between MOG-specific Treg and Tconv cells showed similar and overlapping responsiveness. Further differential fine specificity for MOG₃₅₋₅₅ was not identified in Treg and Tconv TCRs. This implies two possibilities; one is that MOG₃₅₋₅₅ is not responsible for T-cells lineage assignment and alternative Ag(s) should be considered, the other is that affinity threshold is not fundamental for Treg cell selection.

To ensure the lineage commitment of Treg and Tconv cells seen in 1MOG244.2TCR α mice can also be observed in a more normal thymic environment, we transplanted highly diluted Rag^{-/-} HPCs expressing MOG-specific Treg or Tconv TCRs into congenic wild-type mice. The same lineage specification was readily observed as in retrogenic mice, suggesting that TCR-driven Treg and Tconv cell differentiation identified in 1MOG244.2TCR α mice is indicative of the differentiation potentials of the same TCR in normal thymus as well.

CHAPTER 6. DISCUSSION

6.1 Introduction

In Chapters 3-5, we demonstrated through studies of repertoire and individual TCR only limited overlap between Treg and Tconv cells responding during MOG-EAE. MOG-specific Treg cells formed a substantial though not exclusive component of the CNS-infiltrating Treg cell repertoire. Examination of MOG-specific Treg and Teff cell TCR with a fixed TCR α and restricted TCR β usage failed to find evidence for sensitivity or fine specificity for MOG influencing Treg versus Tconv cell lineage assignment, but did reveal lineage segregation based on conserved CDR3 features. Our results favor a model in which Treg cell response during EAE is developmentally encoded in the TCR repertoire and argue against anything more than limited plasticity between Treg and Tconv cells during the autoinflammatory response.

In this chapter we will discuss the following problems and future plans: Are 1MOG244.2TCR α mice an optimal model to study T-cell repertoire? Does interconversion play an important role in maintaining Treg population in the inflammatory environment? What is the source of CNS-infiltrating Treg cells in MOG-EAE? Do self-reactive Treg cells show higher sensitivity to self-antigen than Teff cells do? Is MOG the only antigen CNS-infiltrating T-cells recognize in MOG-EAE? Does epitope spread and cross-reactivity influence the regulatory response in MOG-EAE?

6.2 Developing a Retrogenic Model of EAE to Facilitate Repertoire Analysis

6.2.1 *Why Does the TCR α Chain Need to be Fixed?*

The purpose of fixing one TCR chain is to limit TCR variability and help determine the specificity of TCR repertoire. With one TCR chain fixed, it is only necessary to define the second chain's sequence, eliminating the need to clone both chains from a single cell. Previous data showed that TRBV13-2 (V β 8.2) was highly associated with auto-reactivity in EAE, with nearly half of auto-reactive MOG-specific T-cells expressing V β 8.2 (Mendel *et al.*, 1995; Mendel Kerlero de Rosbo and Ben-Nun, 1996; Offner *et al.*, 1992), whereas TRAV use (V α) is much more variable. By fixing the TCR α chain and studying TCR β , we are able to focus on the disease relevant V β 8.2⁺ TCR population. In addition, the CDR3 β region shows greater diversity (V-N-D-N-J) than the CDR3 α (V-N-J), suggesting that the TCR β chain will have increased variability compared with the TCR α . A bias towards particular V β gene families was also seen in virus infection, autoimmunity and alloreactivity models (Aebischer *et al.*, 1990; Baker *et al.*, 2002; Callan *et al.*, 1996; Kedzierska *et al.*, 2004; Oksenberg *et al.*, 1993; Pantaleo *et al.*, 1994; Torres-Nagel *et al.*, 1997; Trautmann *et al.*, 2005). This might be a consequence of the limited number of available mouse TCR V β gene segments (35)

compared with a greater number of mouse V α gene segments (71) (Lefranc *et al.*, 1999). Thus studying TCR β chain repertoire will potentially yield valuable information, particularly on the MOG-specific response.

6.2.2 Does a Fixed α Chain Influence the Course of MOG-EAE Significantly?

One concern in retrogenically fixing TCR α , is that the timing of TCR α chain expression in the thymus is not physiological and may affect the repertoire. In thymic development, TCR β chain rearranges first in DN cells, followed by the formation of TCR β /pT α /CD3/TCR ζ_2 complexes. The pre-TCR triggers the signal transduction cascade by activating the kinase Lck, engaging the adaptor SLP-76 and linker of activated T-cells (LAT), and thus activating protein kinase C, Ras, and MAP kinases (Gartner *et al.*, 1999; Hagenbeek *et al.*, 2004; Iritani *et al.*, 1999; Schmedt and Tarakhovsky, 2001). Comparison of T-cell development in mice with a TCR α transgene and lacking pT α , and mice lacking a transgene but with wild-type pT α suggests that TCR $\alpha\beta$ complexes are less effective than pT α complexes in supporting proliferation, survival, and $\alpha\beta$ T-cell lineage commitment of early T-cell progenitors. This is due to intrinsic differences in signaling capability of the different complexes (Baldwin *et al.*, 2005; Borowski *et al.*, 2004; Huang and Kanagawa, 2004; Lacorazza *et al.*, 2001). To avoid this circumstance, many repertoire analyses have been performed on TCR β transgenic mice, studying the TCR α repertoire (Hsieh *et al.*, 2004; Pacholczyk *et al.*, 2006; Wong *et al.*, 2007b)

For the reasons listed above, we nevertheless selected to fix the TCR α rather than TCR β chain. To evaluate the impact of the retroviral transgene on T-cell development, we conducted flow cytometric analyses of thymocytes from 1MOG244.2 retrogenic and wild-type C57BL/6 mice. No significant difference of DN, DP, SP subpopulations between retrogenic and WT mice were seen between retrogenic and WT mice. More significantly the DN3 to DN4 transition, when pT α signaling appears most important did not appear affected by the 1MOG244.2 α transgene (Figure 3-2). This suggests that the early TCR α expression does not inhibit thymocytes from passing through the DN3 to DN4 checkpoint, or diminish the efficiency of positive selection in 1MOG244.2 retrogenic mice.

We did observe repertoire alterations in the periphery of retrogenic compared with wild-type mice. First, different use of V β segments in 1MOG244.2TCR α retrogenic mice was observed. For example, V β 14 was substantially increased in retrogenic mice ($p < 0.05$), and V β 3, V β 4, V β 8.3 were decreased (Figure 3-4). Second, the peripheral T-cell repertoire in 1MOG244.2TCR α is skewed to the CD4 compartment. The ratio of CD4/CD8 T-cells reaches 5-10 in LN and spleen, implying that the transduced α chain favors CD4⁺ T-cell selection or expansion in the periphery. A similar skewing to the CD4 lineage has been seen in C6 TCR α Tg female mice (Furmanski *et al.*). Third, retrogenic mice with different α transgenes have distinct susceptibilities to EAE (Figure 3-1A). Four different retrogenic mice expressing distinct α chains were challenged with

MOG, and only 1MOG244.2TCR α showed EAE that caused peak disease levels comparable to WT mice, though they have delayed onset of disease. These results suggest that the α transgene has potential impact on T-cell selection and may alter the repertoire to some extent.

Does this change influence our experimental results? It is to be expected that the manipulation inherent in fixing TCR α will limit the repertoire of TCR β , as many rearranged TCR β will not pair with this TCR α . V β distribution in splenic Tconv cells is as diverse as WT mice, and V β 8.1/8.2 is predominately used. After immunization with MOG₃₅₋₅₅ peptide, 1MOG244.2 α develops EAE with similar disease severity as WT, and CNS-infiltrating T-cells predominately use V β 8.1/8.2 as described in C57BL/6 mice (Mendel *et al.*, 1995; Mendel Kerlero de Rosbo and Ben-Nun, 1996). Most importantly, the percent of Treg among CD4⁺ T-cells in thymus, LNs, and spleen is 3.20% $\pm$ 1.06%, 12.84% $\pm$ 2.24%, and 7.50% $\pm$ 2.41% respectively, which is similar to those in wild-type mice. This argues against impaired development of Treg cells. Taken together, the repertoire is altered in 1MOG244.2 α mice, but remains diverse. Further, that diversity is present in both the Treg and Tconv populations. Nevertheless, it is important to experimentally document that the Treg versus Tconv lineage specification we observed in 1MOG244.2TCR α mice would be similarly seen in a more normal situation (Section 5.2.4), and these studies are ongoing.

6.2.3 *Fixing the TCR α*

T-cell receptor (TCR) transgenic mice are widely used and have had a large contribution to understanding of T-cell development and signal transduction. Previous repertoire analyses relied on transgenic mice which had fixed β chain with or without a TCR α chain minilocus (Hsieh *et al.*, 2004; Malherbe *et al.*, 2004; Wong *et al.*, 2007b). However, TCR transgenic mouse production is time consuming and costly.

To avoid this, we utilized retrogenic mice in which specific TCRs are retrovirally transduced into hematopoietic stem cells (Holst *et al.*, 2006b). This approach is substantially faster than making transgenic mice, (6 weeks versus 6 months), and engraftment is reproducible from mouse to mouse. Also, mice containing different fixed α chains can be examined simultaneously. Since these mice are individually generated and each of them is a founder, the potential bias introduced by a founder effect would be limited (Holst *et al.*, 2006b). However, there are still several disadvantages to retrogenic mice. For instance, the number of T-lymphocytes obtained from retrogenic mice is generally 3-4 fold less than that from Tg mice. Pooling mice was necessary to obtain enough CNS-infiltrating Treg cells for RNA extraction and TCR repertoire analysis. Retrogenic mice have to be constantly generated. Also, because T-cells first develop in transplanted adult mice (6-8 weeks) rather than the neonatal environment, an increased memory-like phenotype of peripheral T-cells may result as T-cells form in a lymphopenic environment. Finally, we sub-lethally irradiated recipient mice to facilitate stem cell engraftment, and irradiation itself can generate inflammation and enhanced cytokine secretion that may last up to 6 weeks (Holst *et al.*, 2006b). Although we have not seen

any deleterious consequences per se, these effects can potentially influence experimental results and should be considered in interpreting data from the system.

6.3 Interconversion and Origins of Treg and Tconv Cells in Autoimmunity

The Treg cells largely originate in the thymus. Seemingly, encounter with self-antigens at intermediate affinity induces Treg cell differentiation (Apostolou *et al.*, 2002; Hsieh *et al.*, 2004; Jordan *et al.*, 2001; van Santen *et al.*, 2004). This results in the generation of a largely distinct TCR repertoire in Treg and Tconv cells, as indicated by studies of TCR CDR3 α sequences in transgenic systems. On the other hand, a noticeable overlap, ranging from 10 to 42% between Treg and Tconv is also observed (Hsieh *et al.*, 2004; Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006; Pacholczyk and Kern, 2008; Wong *et al.*, 2007b). One hypothesis is that interconversion may play a role in generating the overlap between Treg and Tconv cells, and this interconversion involves either upregulation of Foxp3 in Tconv or loss of Foxp3 in Treg cells (Hoffmann *et al.*, 2009).

The conversion of Tconv into Treg can be achieved both by TGF β and IL-2 or retinoic acid (RA) exposure during activation *in vitro* (Chen *et al.*, 2003; Wang *et al.*, 2009), or chronic exposure to antigen delivered by osmotic pumps or by antibody mediated targeting *in vivo* (Apostolou and von Boehmer, 2004; Cobbold *et al.*, 2004; Knoechel *et al.*, 2005; Kretschmer *et al.*, 2005). Mechanistic studies have provided a framework to explain the plasticity between Treg and Tconv cells, with selective signaling pathways regulating Foxp3 expression (Coombes *et al.*, 2007; Mucida *et al.*, 2007; Quintana *et al.*, 2008; Selvaraj and Geiger, 2007). In addition, conversion was readily seen in the studies involving adoptive transfer of purified CD4⁺CD25⁻ or CD4⁺Foxp3⁻ T-cells into lymphopenic or lymphoreplete hosts, with conversion varying from 0.3% to 12% (Curotto de Lafaille *et al.*, 2004; Fontenot *et al.*, 2005b; Lathrop *et al.*, 2008; Liang *et al.*, 2005). If one extrapolates the behavior of the adoptively transferred population as representative of their respective T-cell population, 0.4% and 8% conversion in periphery of normal mice would contribute to 3.6% and 72% of the Treg cells, respectively (Lathrop *et al.*, 2008). This is a very large range and the higher numbers are not consistent with other repertoire studies. Thus conversion in the periphery was seen in several studies, though to what extent it maintains the peripheral T-cell population is still controversial.

Whether interconversion influences active immune responses, and more specific cells participating in autoimmunity, is uncertain. Some results suggest that adaptive upregulation of Foxp3 plays an important role in modulating autoimmunity (Manicassamy *et al.*, 2009; You *et al.*, 2007). Other studies failed to identify a role for Foxp3 induction (Korn *et al.*, 2007; Wong *et al.*, 2007a). During EAE, T-cells activated in the periphery migrate to the CNS where they re-engage antigen on local dendritic cells and expand (Bailey *et al.*, 2007). The early CNS response has a low frequency of Foxp3⁺ T-cells, suggesting that substantial numbers of Treg cells are not stimulated to enter the CNS at this time. Yet Treg cells accumulate later in the disease and are associated with resolution (McGeachy *et al.*, 2005). Therefore, either migration or expansion of this

population is delayed or CNS-infiltrating Treg cells are forming through Tconv cell conversion.

There are several possible explanations for how interconversion can influence Tconv and Treg cells in MOG-EAE. First interconversion may be a rare event in lymphoid organs with little target antigen. But once challenged by MOG, the T-cells will migrate to the CNS where the activated T-cells will encounter antigen in varied settings, and interconversion may occur. With this possibility we would expect to observe little overlap of Tconv and Treg in preimmune and little to moderate overlap in postimmune spleen, and much greater overlap in CNS (Figure 6-1A). A second possibility is that interconversion occurs frequently in periphery of normal individuals, and inflammation may or may not change the interconversion rate. In this case we would observe substantial overlap between Tconv and Treg cells in preimmune spleen, postimmune spleen and CNS (Figure 6-1B). A third possibility is that interconversion occurs infrequently in unimmunized organs, and antigen exposure or inflammatory environment does not facilitate interconversion. In this case, we would observe a limited overlap between Treg and Tconv in preimmune spleen, postimmune spleen and CNS (Figure 6-1C). Finally, it is possible that interconversion occurs most prominently with the initial immunization. In this case, we would expect that MOG-specific T-cells would show a large degree of overlap in the postimmune periphery and CNS, but non-specific cells that are not activated with immunization would show lower degree of overlap (Figure 6-1D).

At a population level, our data fail to identify a substantial role for interconversion as a means of locally generating Treg cells in the CNS. First, limited overlap of TCR used by Tconv and Treg cells in CNS and spleen was observed. And comparison of shared CDR3 amino acid sequences for each cohort demonstrated that the resemblance of repertoire among a single cell class (Treg or Tconv) across different organs (spleen and CNS) was greater than among different classes of cells within an organ. This result suggests that a distinct TCR repertoire is used by Tconv and Treg cells. Second, the frequencies of shared CDR3 among unique TCR sequences in preimmune splenocytes (expt 1: 3.5%; expt 2: 6.4%) was only modestly lower than that in postimmune splenocytes (expt 3: 10.1%, expt 4: 6.6%). The difference between postimmune splenocytes and CNS-infiltrating cells (expt 3: 11%, expt 4: 14.7%) was likewise small. These trends may be consistent with limited interconversion between Treg and Tconv cell lineages, but indicates that EAE does not promote substantial interconversion. It is correlated with the finding from NOD mice in which the encounter of cognate antigen in pancreas does not induce Tconv to Treg cell conversion (Wong *et al.*, 2007a). Third, the heavy skewing of the minority of expressed by both Treg and Tconv TCR toward a single cell type argues against extensive plasticity in Foxp3 expression during EAE. Fourth, the acidic/aliphatic variation we observed in position 4 of MOG-specific TCR robustly discriminates between MOG-specific Treg TCR and Tconv TCR; implying auto-antigen specific Treg and Tconv populations are ontogenically distinct. Therefore, our results showed limited interconversion between Treg and Tconv cells both in spleen and CNS during MOG-EAE, and support the third model that inflammatory environment does not promote substantial interconversion between Treg and Tconv populations either in the periphery or the CNS (Figure 6-1C).

Figure 6-1. Models of interconversion between Tconv and Treg cells in MOG-EAE.

6.3.1 Limitations of the TCR Repertoire Analysis

Although our data argue against interconversion, we must point out the limitations of experimental strategy. First, the sequence analysis was performed in two independent experiments. Although similar results were found, it is worthwhile to repeat it multiple times to further confirm the result. Second, the Tconv and Treg populations were separated by flow cytometry, and the sorting process can introduce a low level of error. Even though the purity of cell population was as high as 98%, 2% of cells could come from contamination and because of the larger number of Tconv cells, Tconv cells are more likely misplaced into Treg population, which may potentially exaggerate the overlap. Third, we acquired all the TCR sequence information from isolated cDNA instead of from single T-cell clones. This may introduce bias if different T-cells express different amounts of TCR. However, in regards to this it is important to note that our conclusions were identical based on analyses of unique TCR rather than total TCR isolates. We also had to pool 5-6 mice together to harvest enough brain Treg cells for PCR. Therefore representation of TCR from different mice may differ and this could skew the repertoire assessments if they had different quantities of infiltrating T-cells. This limitation may lead to decreased similarity between the two populations, and an underestimation of the degree of overlap. However, we observed a dramatically increased overlap of single cell types across organs compared with different T-cell types within one organ (~30% versus ~10%), and the result was reproducible, suggesting that skewing from pooling of organs did not alter our conclusions.

Another difficulty in whole repertoire analyses is that all TCR are analyzed, including cells that are not specific to MOG₃₅₋₅₅. Tetrameric pMHC-MOG complex staining may be a better technique to directly sort out TCRs with high-affinity to MOG₃₅₋₅₅. However, autoreactive T-cells, including MOG-specific T-cells have low affinity, and the MOG-A^b tetramer only stains a minority of these cells. We attempted to stain 1MOG244.2 and other T-cells with this tetramer, but signal was not adequate for analyses (R. Alli and T.L.G., unpublished observations). We got around this problem by analyzing specific TCR for MOG reactivity. When we characterized the properties of selected TCRs *in vitro*, 100% of Tconv and 50% of Treg TCR from cells infiltrating the CNS were MOG-specific, much higher than the published data of about 10% of MOG-specific CNS-infiltrating T-cells using a MOG-A^b tetramer (Korn *et al.*, 2007). This may be due to that study's inability to detect low affinity but pathogenic TCRs, or our repertoire analyses only including high frequent TCR. It is possible that frequent T-cells may have a higher tendency toward MOG-specificity than low frequent T-cells. Nevertheless, despite its limitations, the approach we have used has a strong track record (Lin and Welsh, 1998; Pannetier *et al.*, 1995; Sourdive *et al.*, 1998), and has yielded new insights into the study of other repertoires.

6.3.2 Future Studies

To solve the problem generated by pooling cDNA, single-cell RT-PCR can be considered to acquire sequences of individual Tconv and Treg cells. This will also

circumvent the problem of pooling mice to get enough T-cells from CNS, though it is technically more challenging and smaller numbers of sequences may be obtained. Alternatively, next-generation DNA sequencing can be used to confirm the limited conversion of Tconv and Treg cells (Freeman *et al.*, 2009; Shendure and Ji, 2008). With the help of massive parallel DNA sequencing, one can interrogate the repertoire more thoroughly and rapidly. The whole analysis of 1MOG244.2 α EAE could be repeated at higher resolution with different primer sets, such as V β 14-C β and V β 6-C β to confirm our results. These experiments could also be performed at the different time point of disease – early onset or recovery phase, to see if there are temporal changes in interconversion between Foxp3⁺ and Foxp3⁻ cells. However, this scenario would seem unlikely considering our results indicating that interconversion between Foxp3⁻ and Foxp3⁺ cells is minimal. Additionally, the 1MOG244.2TCR α thymocytes repertoire has not been studied yet, comparing Treg and Tconv TCR in thymus, spleen and CNS will give more information and allow us to trace TCR clonotypes to the thymus.

Furthermore, experiments on the V β 8.2 repertoire of C57BL/6 mice with EAE can investigate the trend of interconversion in spleen and CNS in wild-type mice. It would be interesting to know whether the D/E to A/V/L variation we found in retrogenic mice is also seen in wild-type mice with multiple α chains. Finally, repertoire analyses on peripheral T-cells from MS patients have not been performed, and such analyses may test whether results in EAE model is correlated with MS. One caveat here is that the cell surface markers for isolation of human Treg are imperfect, and isolation of pre Treg and Tconv populations in this setting will be a challenge.

6.4 Segregation of Tconv and Treg Cells Based on CDR3 β Sequence

Analysis of selected receptors identified in our repertoire analyses provided insight into the composition of the auto-reactive Treg and Teff cell responses. MOG-specific Teff cells were highly enriched in the CNS of 1MOG244.2 α mice with EAE (100% of TCR). More interestingly, we identified a concentration of MOG-reactive TCR among the CNS-infiltrating CD4⁺Foxp3⁺ population (6/12 versus 1/8 among CD4⁺Foxp3⁺ splenocytes). This functionally confirms the presence of cognate autoantigen-reactive Treg cells in the CNS of mice with EAE at high frequency. Unlike Teff cells, which were uniformly MOG-specific, 50% of V β 8.2⁺J β 2.7⁺ Treg cell TCR tested did not recognize MOG. These cells may be bystanders or effectors specific for alternative antigens. Indeed, Treg cells need not to be specific for cognate autoantigen, and response toward tissue-specific or even tissue-unrestricted self antigens released during inflammation may potentially stimulate regulatory functions.

Among the MOG-specific T-cells, a length of 12 amino acids and a G at position 6 in the V β 8.2⁺J β 2.7⁺ CDR3 β was highly correlated with MOG-specificity. Importantly, MOG-specific Teff cells during EAE were fully distinguished by a D or E at position 4, and Treg cells by an aliphatic residue, most commonly an A at the same site. Implicitly, Treg and Teff cells during autoimmunity arise from distinct population reservoirs, identifiable in 1MOG244.2 α retrogenic mouse EAE by the variation at residue 4.

Additional structural criteria overlaid on this must be met for MOG-specificity. It is likely that these distinct populations arise from differences in the origin and selection of the T-cells, studies of developing thymocytes may help clarify this origin.

Recognition of the acidic versus aliphatic variation in CDR3 β of Tconv and Treg cell was possible only by examining a narrow slice of the repertoire, one with a fixed TCR α and limited to V β 8.2 and J β 2.7. This selective repertoire analysis required the production and study of a manipulated mouse strain, the 1MOG244.2 α retrogenic mice. The consistent identification of specific public TCR within Treg or Tconv cell repertoires in these mice indicates consistency in T-cell differentiation patterns within them. However, the 1MOG244.2 α mice do use a *Tcra*^{-/-} stem cell recipient, whose thymi developed in the absence of normal T-cell maturation. Further, premature expression of TCR α in transgenic or retrogenic systems, such as 1MOG244.2 α mice, can influence pT α signaling and decrease the efficiency of the DN to DP transition, and thus potentially altering the balance of different thymocyte subsets (Aifantis *et al.*, 2006; Borowski *et al.*, 2004; Huang and Kanagawa, 2004). We do not find evidence for these alterations influencing Treg cell lineage assignment. Grossly, proportions of Treg cells are normal in 1MOG244.2 α mice, and overlap among Treg and Tconv cell TCR is similar to that observed in other systems (Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006). More importantly, precursor cells retrogenic for specific Treg or Tconv cell TCR, when heavily diluted with wild-type bone marrow and transplanted into wild-type mice, show the same lineage predilection as in 1MOG244.2 α mice. This suggests that TCR-driven Treg and Tconv cell lineage specification in 1MOG244.2 α mice is indicative of the potentials of the same TCR in normal thymi as well.

6.5 Future Plans to Confirm the Segregation of Treg and Tconv Based on TCR Sequence

If the hypothesis that the acidic versus aliphatic variation fundamentally influences T-cell lineage assignment is true, exchange of acidic to aliphatic residues or vica versa at position 4 may lead to T-cell lineage re-commitment. We have mutated a D or E at position 4 to an A in MOG-specific Tconv TCR, and an A, V, L at same position to a D in Treg TCR. The mutations were confirmed by sequencing. The idea is to use these TCR to repeat the experiment shown in Section 5.2.1. Specifically the TCR will be retrovirally transduced into HPCs from CD45.2⁺Rag^{-/-} mice, then transplant into sublethally irradiated congenic CD45.1⁺ mice after dilution with CD45.1⁺ HPCs. After 8 weeks, we will analyze Foxp3 expression among CD45.2⁺ T-cell population, and the mice with original TCR will serve as control. If the hypothesis is correct, Foxp3 expression will be elevated in mice with Tconv TCR mutated to express an A at position 4 compared with mice containing original TCR. On the other hand, Foxp3 expression will be diminished in mixed chimera with Treg TCR mutated to express a D at position 4.

6.6 Affinity in Treg Responses

6.6.1 Models of Treg Repertoire Selection

Treg cells have been alternatively described as recognizing self-antigens with a relative higher affinity than Tconv cells, having increased or moderate avidity for self, and overlapping in recognition characteristics with pathogenic auto-reactive T-cells. Experiments with TCR transgenic mice suggest that precursors develop into the Treg lineage when higher avidity interactions with selecting ligands were found (Apostolou *et al.*, 2002; Jordan *et al.*, 2001; Kawahata *et al.*, 2002; Walker *et al.*, 2003). Subsequent studies proposed a two-step model that TCR-dependent signals followed by TCR-independent IL-2 signaling and activation of Stat5 signals (Burchill *et al.*, 2008; Lio and Hsieh, 2008; Liston and Rudensky, 2007). In this model, commitment of Treg cells is driven by higher affinity interactions between the TCR and MHC/self-peptide complexes, followed by accessory signals (IL-2, B7 and APC etc). Consequently, Tconv and Treg cells express different sets of TCRs that have different affinity to self-antigens (Figure 6-2A). However, the actual range of this “higher” affinity for self antigens has never been determined, especially in the polyclonal Treg repertoire. Possibly affinity or avidity alone is not an exclusive determinant of Treg lineage assignment. Even TCRs with weak affinities to self antigen may possibly be included in the Treg repertoire.

An alternative presumption is the role of TCR-affinity dependent signals may be complementary rather than dominant in Treg lineage selection. In this model, the commitment of Treg precursors occurs prior to thymic selection by TCR, and the TCR-dependent and/or TCR-independent signals may further complete the Treg lineage commitment. Therefore the final Treg repertoire can be enriched in self-reactive and non-reactive TCRs, and the separation of Treg and Tconv cells is achieved by both TCR-dependent and TCR-independent signals (Figure 6-2B). This model would explain several findings. First, Foxp3 expression can occur in pre-selected CD4⁺CD8⁻ thymocytes (Pennington *et al.*, 2006). Second, Treg cells can recognize a wide range of foreign antigens and have a diverse repertoire. Third, increased overlap between Tconv and Treg cells was seen in “single-peptide” transgene mice, where all MHCII molecules are occupied by one peptide (Pacholczyk and Kern, 2008).

6.6.2 Sensitivity and Fine Specificity of MOG-specific Treg and Tconv TCR

Expression of high-affinity cognate antigen on thymic stroma promotes enhanced Treg cell differentiation for some TCR (Picca *et al.*, 2006). MOG is expressed at low levels in the thymus (Delarasse *et al.*, 2003). If MOG sensitivity guides Treg cell development, then according to the first (two-step) model introduced above, MOG-specific Treg should be more sensitive to MOG than specific Tconv cells. Overall, Treg cell TCR showed higher MOG sensitivity than did Teff cell TCR. However, the substantial overlap in Treg and Teff cell TCR sensitivity implied that this was not fundamental to their selection (Figure 5-3). Further, differential fine specificity for MOG

Figure 6-2. Generation of specificities of the Treg repertoire. Reprinted with permission. Pacholczyk, R., and Kern, J. (2008). The T-cell receptor repertoire of regulatory T-cells. Immunology 125, 450-458.

was not identified in Treg and Teff cell TCR.

There are several explanations for the similar MOG response yet different and conserved CDR3 features of Treg and Teff cell TCR. One is that lineage selection for the corresponding cell types is not MOG-dependent, but rather that an alternative Ag(s) promotes selection, which we will further discuss in Chapter 6.6.1. Second, the antigen sensitivity we measured in the 4G4 system is not the actual affinity to MOG-peptide. Theoretically it should be correlated with affinity because of identical CD4 and TCR expression on the 4G4 cells, essentially these cells only differed in their CDR3 β . However, direct measurement of affinity should be considered to confirm the results from the CD4.4G4 cells, such as by Surface Plasmon Resonance (SPR). A third possibility is that Treg TCR do not necessarily show higher affinity to self-antigen, as described above (Figure 6-2B). That would explain the observation of the low sensitivity of some Treg TCR (#9 and #52) in our experiment, and overlapping sensitivity to MOG-peptide among Treg and Tconv TCRs. In addition, when Treg TCRs (#1, #9 and #18) from different “sensitivity windows” were tested for giving rise to Treg cells in mixed-BM chimeric mice, Treg cells developed with all three TCRs, up to ~30% at very low frequencies. No deletion was seen in #1 receptor, which showed highest sensitivity and maximal response in CD4.4G4 cells.

It will also be important to determine how the sensitivity or affinity of Treg TCR influences their protective abilities. To address this, further *in vivo* studies should be performed on the Treg TCRs. To determine their actual function in EAE, we will generate mixed-BM chimeras with low or high-sensitivity receptors, and challenge them with MOG peptide, to see whether there are differences in the ability of these receptors to protect mice from EAE.

6.7 Antigen Specificity in Regulatory T-cell Responses

6.7.1 *Does MOG-specific T-cell Cross-react with Alternative Self-antigen Which Mediates Thymic Selection?*

In order to mount a rapid and efficient immune response against invading pathogens, TCRs have evolved a high level of antigen cross-reactivity and recognize multiple different peptides bound to MHC molecules. Cross-reactivity is necessary for T-cell development and is critical given the fixed size of the T-cell repertoire relative to the vast universe of potential antigens (Bhardwaj *et al.*, 1993; Evavold *et al.*, 1995; Mason, 1998; Maverakis *et al.*, 2001; Wilson *et al.*, 2004; Wucherpfennig and Strominger, 1995). Mason *et al.* estimated that each T-cell is capable of reacting with ~10⁶ different MHC-associated peptide epitopes (Mason, 1998), and Hiemstra *et al.* further confirmed the estimation through deduction of effective concentrations of agonist ligands in a peptide-“gemisch” (Hiemstra *et al.*, 1998). Although the possible antigens are non-self peptides, based on these estimates it must be considered that any particular TCR will likely be specific for more than one self-antigen. Molecular mimicry and cross

reactivity is frequently found in autoimmunity. An elegant example is that mice developed EAE after immunization with peptide of hepatitis B virus, which shared a sequence of six consecutive amino acids in common with MBP (Wucherpfennig and Strominger, 1995). Subsequent studies showed that only a few critical amino acids serving as contact residues were needed to be shared by two antigenic peptides in order to elicit cross-reactivity (Evavold *et al.*, 1993; Kersh and Allen, 1996; Madrenas *et al.*, 1995).

In one study, MOG-specific 2D2 TCR transgenic mice developed EAE in the absence of MOG because 2D2 T-cells cross react with neurofilament-M (NF-M₁₈₋₃₀, TETRSSF_{SR}/SGS), which shares crucial contact amino acids with MOG₃₅₋₅₅ (MEVGWYRSPFSR/VHLYRNGK) (Krishnamoorthy *et al.*, 2009). We hypothesized that the Treg cell identified here could be selected by NF-M₁₈₋₃₀ in thymus originally, and could cross react with MOG₃₅₋₅₅. As a first step to test this hypothesis, we transduced the original MOG-specific TCR and their counterpart mutations into CD4.4G4 cells, then examined their responsiveness to NF-M₁₈₋₃₀ and MOG₃₅₋₅₅ by IL-2 production. If NF-M₁₈₋₃₀ is responsible for Treg selection, Treg TCRs recognize NF-M and may have higher sensitivity than Tconv TCR. Further, the D/E versus A/V/L variation is responsible for T-cell lineage assignment, mutation in Treg TCR should lead to the decreased response to NF-M₁₈₋₃₀, whereas mutation in Tconv TCR may enhance their recognition to NF-M₁₈₋₃₀. So far pilot experiments were performed on 12 receptors. 4 out of 6 mutated Tconv TCR showed increased sensitivity, and 4 out of 6 mutated Treg TCR showed decreased sensitivity to NF-M. We will confirm this result by testing more receptors.

6.7.2 What Do Non-MOG-specific Treg TCR Recognize?

Epitope spreading is when the specificity of T and B-cells in the immune response spreads or switches from the initiating epitope to other epitopes, including self epitopes, (Vanderlugt and Miller, 2002). This phenomenon has been observed in PLP₁₃₉₋₁₅₁ induced EAE in the SJL mouse, where after priming SJL mice with PLP₁₃₉₋₁₅₁, the immune response switches from anti-PLP₁₃₉₋₁₅₁ to anti-PLP₁₇₈₋₁₉₁ and anti-MBP₈₄₋₁₀₄ during the course of EAE (McRae *et al.*, 1995; Vanderlugt *et al.*, 2000). These findings were based on total CD4⁺ T-cells, mainly effector T-cells. In MOG-EAE epitope spread has not been seen in the Teff response. Whether epitope spreading exists in Treg cells in MOG-EAE, however, remains uncertain.

In the CNS of 1MOG244.2 α mice, we identified six receptors (50%) from the Treg population that were not MOG-specific. Kuchroo *et al.* showed that ~7% and 34% CNS-infiltrating Treg are MOG tetramer-positive in the peak and recovery phase, respectively, and concluded that the rest of Treg are bystanders (Korn *et al.*, 2007). Why do these “bystander” Treg cells accumulate in CNS? Do they recognize other self-antigen(s) because of epitope spreading? To answer this question, we can test whether these receptors react with NF-M₁₈₋₃₀ using CD4.4G4 hybridomas.

The second question is whether these non-MOG-specific Treg cells play a role in EAE protection. We tried to generate retrogenic mice expressing a single TCR, but the number of monoclonal Treg cells acquired from single TCR-retrogenic mice was very low presumably because of niche saturation. We also tried to convert Tconv cells isolated from these retrogenic mice into Treg with TGF- β and high dose of IL-2 and injected the induced Treg cells into Rag^{-/-} mice, to test EAE protection. Unfortunately, it was much more difficult to expand small numbers of clonal cell from retrogenic mice than Tconv cells from wild-type, and we also found the converting efficiency was receptor dependent and often poor. Further studies will be needed to optimize this. Hsieh's group created mixed-bone marrow chimeras with varying ratios of polyclonal progenitor cells and progenitor cells transgenic for Treg cell-derived TCRs. We will also generate similar mixed-bone marrow chimeras with retrovirally transduced HPCs expressing "bystander Treg TCR" using HPCs are from Foxp3-GFP x Rag^{-/-} mice. After 8 weeks transplantation, we will examine Treg with transduced TCR in peripheral blood by GFP. Positive mice will be challenged with MOG peptide and EAE will be followed to determine if the retrogenic Treg diminish disease severity.

In summary, we assessed Treg and Tconv repertoires in MOG-EAE. Under inflammatory conditions, the Tconv used a repertoire distinct from that of Treg, though the repertoires overlapped. The extent of this overlap was not significantly increased with immunization. Both MOG-specific Treg and Tconv with distinct repertoires are concentrated within the CNS during EAE, though infiltrating Treg seemingly possess a broader spectrum of antigen specificities. Examination of MOG-specific Treg and Teff cell TCR with a fixed TCR α and restricted TCR β usage failed to find evidence for sensitivity or fine specificity for MOG influencing Treg versus Tconv cell lineage assignment, but did reveal lineage segregation based on conserved CDR3 features. Our results imply that ontogenically distinct Treg and Tconv cell repertoires during MOG-EAE and argue against more than limited plasticity between Treg and Tconv cells during the autoinflammatory response.

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VITA

Xin Liu was born in Heilongjiang Province at P.R.China in 1979. In 1997, she attended Shanghai Medical University (Merged with Fudan University in 1999), major in Clinical Medicine. After achieving her Bachelor of Medicine in 2002, she joined residency program of OBGYN in International Peace Maternity and Child Health Hospital. In August 2005, she chose to enter the Integrated Program in Biomedical Sciences at the University of Tennessee Health Science Center for Ph.D. studies. In May 2006, she joined Dr. Terrence L. Geiger's laboratory at St. Jude Children's Research Hospital and conducted research in the repertoire analysis of regulatory T-cells in EAE. She received her Doctor of Philosophy degree from University of Tennessee in May of 2010.