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The Role of TCR Specificity in the Regulation of the Immune Response to Self and Commensal Antigens

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THE ROLE OF TCR SPECIFICITY IN THE REGULATION OF THE IMMUNE RESPONSE TO SELF AND COMMENSAL ANTIGENS

by

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LIST OF ABBREVIATIONS

ACE	abundance coverage estimator
ACP	autoclaved cecum/colon content preparation
ALDH1A1	aldehyde dehydrogenase family 1, subfamily A1
ALDH1A2	aldehyde dehydrogenase family 1, subfamily A2
APC	antigen presenting cell
APRIL	a proliferation inducing ligand
cAMP	cyclic adenosine monophosphate
CD	Crohn's disease
CD4 SP	CD4 single positive
CDR3	complimentarity determining region 3
CFSE	Carboxyfluorescein succinimidyl ester
CLN	cervical LN
CTLA-4	cytotoxic T-lymphocyte-associated gene 4
DC	dendritic cell
DT	diphtheria toxin
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
Flt3L	FMS-like tyrosine kinase 3 ligand
GALT	gut-associated lymphoid tissue
GFP	green fluorescent protein
GITR	glucocorticoid induced TNF receptor
iNKT	invariant natural killer T
IBD	inflammatory bowel disease
IDO	indolamine 2,3-dioxygenase
IEC	intestinal epithelial cell
IEL	intra-epithelial lymphocyte
IFA	incomplete Freund's adjuvant
IgA	immunoglobulin A
IL	interleukin
ILF	isolated lymphoid follicles

IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IRES	internal ribosome entry site
LP	lamina propria
LN	lymph node
MadCAM1	mucosal addressin cell adhesion molecule 1
MLN	mesenteric LN
MAIT	mucosal associated invariant T
MHC	major histoincompatibility complex
NF-κB	nuclear factor-kappaB
NLR	nucleotide-binding oligomerization domain (NOD)-like receptors
NOD2	nucleotide-binding oligomerization domain 2
ODN	CpG oligodeoxynucleotides
OVA	ovalbumin
PBS	phosphate buffered saline
PGE2	prostaglandin E2
PRR	pattern recognition receptors
RA	retinoic acid
rRNA	ribosomal ribonucleic acid
SIGIRR	single-immunoglobulin-domain-containing interleukin-1 receptor-related protein
SPF	specific pathogen free
TCR	T cell receptor
Teff	effector T cell
TGFβ	tumor growth factor beta
TLR	toll-like receptors
TNF	tumor necrosis factor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
UC	ulcerative colitis

ABSTRACT OF THE DISSERTATION

The immune system must be able to fight potentially pathogenic microbial invaders without mounting an attack on its own tissues, food antigens, and harmless or beneficial commensal species. Despite the elimination of many self-reactive T cells in the thymus, some T cells with the ability to recognize self and potentially cause autoimmune disease are present in the periphery. The population of $CD4^+$ regulatory T cells (Treg) expressing the transcription factor Foxp3 are required to actively maintain homeostasis of the immune system, but it is uncertain whether their function is antigen-specific. These studies address the repertoire of the variable T cell receptor expressed on each T cell, in an attempt to understand the role of specificity in the maintenance of tolerance. By sequencing of the TCRα chain on cells expressing a fixed TCRβ chain, we are able to compare the repertoire of TCRs on T cells from different populations.

These studies revealed that Treg use a repertoire of TCRs distinct from that of the $CD4^+CD44^{hi}$ activated/memory or $CD44^{lo}$ naïve T cell subsets, and that it varies considerably depending upon the location of the cells. This data suggests that Treg home to and/or develop in areas in which they recognize antigen, presumably self antigens on the tissues which they protect. Most Treg develop their regulatory phenotype in the thymus; however, it is known that mature T cells can develop into Treg outside of the thymus, a process termed peripheral conversion. While we see evidence of a very small amount of contribution by peripheral conversion to the Treg repertoire of the spleen and lymph nodes, it has been proposed that the gut immune system is a major site of conversion. Therefore we studied the TCR repertoire of the colon, and indeed find that

the colonic Treg TCRs are very unique, and many of them recognize commensal bacterial antigens. We further show that thymocytes expressing these TCRs do not upregulate Foxp3 expression in the thymus, and for two of these TCRs, we show that Foxp3 expression depends upon the composition of the intestinal microbiota. Therefore, our data supports a model in which $Foxp3^{+}$ regulatory cells show antigen specificity for the tissues they protect, and in some cases develop *in situ*, allowing for tolerance to antigens that are not thymically presented.

CHAPTER ONE: INTRODUCTION

$CD4^+$ $Foxp3^+$ Regulatory T Cells Maintain Immune Homeostasis

The need for T cell regulation

An essential feature of the adaptive immune system is the potential of T cells to recognize virtually any antigen. While this allows for the development of an immune response to a wide and constantly changing range of pathogens, it leaves open the possibility of T cells reactive to antigens derived from the self, as well as non-harmful foreign antigens derived from food, non-pathogenic, and beneficial commensal organisms. Therefore, there must exist mechanisms by which an immune response to these antigens is prevented, while still allowing for a response to potentially harmful organisms; the ability to discern self from non-self.

Central tolerance is the first line of defense against self-reactivity. Developing T cells in the thymus express a unique T cell receptor whose amino acid sequence is the product of gene segment rearrangement as well as random nucleotide addition and subtraction. The newly created receptor expressed by the developing T cell is then tested for the ability to recognize self-peptides presented by a major histocompatility complex (MHC). Those T cells expressing a receptor unable to productively interact with available pepetide and MHC complexes (pMHC) do not survive, ensuring that only potentially useful T cells are contributed to the mature T cell pool. However, those T cells whose TCR reacts too strongly to these complexes are eliminated, in a process coined negative selection. This ensures that most T cells released from the thymus do not react strongly to self antigens.

In spite of this process, however, there are T cells in the periphery capable of recognizing self-antigens and contributing to the development of autoimmune diseases [1]. This is attributed to the limitations of thymic negative selection; it is impossible for a

developing T cell to encounter every potential self-peptide/MHC complex that it might later encounter in the body, and not all self peptides are present in the thymus. Therefore, other mechanisms are required to keep these potentially self-reactive T cells in check. These self-reactive T cells can be rendered unresponsive, a state termed anergic, in the periphery or may be sequestered from their cognate antigen and therefore remain ignorant [1].

In addition to these mechanisms, the existence of a "dominant" form of regulation mediated by a population of dedicated regulatory cells was proposed in the early 1970s [2, 3]. Several groups observed that T cells could not only augment but also dampen immune responses, and it was believed that this regulation was mediated by a unique population of T cells coined "suppressor T cells". These cells gained quick acceptance and a flurry of reports on the nature of these cells and their functions were published in the 1970's and early 1980's. However, the development of new molecular biology techniques in the 1980's began to cast doubt on the findings of these reports, particularly as some current beliefs about the structure of the MHC locus were proved to be incorrect [4]. Consequently, the field of "suppressor" T cells lost momentum and the support of most immunologists. However, studies in the 1990's again began to report about a subset of T cells with suppressive capabilities, and to avoid the stigma of the earlier studies these were eventually coined "regulatory" T cells [2, 3]. It is now thought that these cells are one in the same with the suppressor cells of the 1970s, but improvements in technology that allow for better characterization of these cells have led to their wide acceptance, and much has been discovered about their nature.

Foxp3$^+$ CD4$^+$ regulatory T cells

Attempts to identify the population of regulatory T cells grew out of studies of the autoimmunity induced upon the transfer of populations of T cells into lymphopenic SCID or nude mice. Experiments reported in 1985 by Sakaguchi *et. al*. showed that the CD5^{hi}CD4$^+$ T cells contained the ability to prevent autoimmunity [5], and in 1990 it was shown that CD45RC^{lo} CD4$^+$ T cells prevented disease in athymic rats [6]. Other studies identified this population as RT6.1$^+$ T cells [7] or CD45RB^{low}CD4$^+$ T cells [8, 9] in mice. Then, in 1995, CD25 (IL-2 receptor α-chain) was identified as a marker for a subset of CD4$^+$ T cells contained in the CD5^{hi} and CD45RB^{lo} fraction, and depletion of the CD25-expressing cells from a splenic cell suspension before transfer into a nude mouse produced a high incidence and wide range of autoimmune diseases in these recipient mice [10]. Purified CD25$^+$ CD4$^+$ T cells were subsequently shown to be capable of suppressing autoimmunity and effector T cell responses in a wide variety of systems.

While CD25 proved to be a good marker for regulatory T cells and its discovery pushed the field of regulatory T cells forward, its expression is not limited to this cell population alone, as it is transiently upregulated on all T cells upon activation. In addition, these cells were found to express high levels of other molecules previously identified as markers of T cell activation, such as the glucocorticoid induced TNF receptor (GITR) [11, 12], and cytotoxic T-lymphocyte-associated gene 4 (CTLA-4) [13, 14]. Therefore, the search for a more specific indicator of the regulatory T cell lineage continued. In 2001, the transcription factor Foxp3 was identified as the defective gene in the *scurfy* mouse, a spontaneous mutant mouse known to develop severe autoimmunity as a result of an X-linked mutation [15]. Two years later, Foxp3 was found to be expressed

exclusively in the $CD25^+CD4^+$ regulatory T cell subset, and became the first, and to date only, gene known to be exclusively expressed in these cells [16-18]. Identification of Foxp3 as a definitive marker of regulatory T cells greatly facilitated the further study of this population, particularly once identification of these cells was simplified by the engineering of mice co-expressing Foxp3 with a fluorescent protein or a fusion of Foxp3 with green fluorescent protein (GFP) [19-21].

The "Natural" Regulatory T Cell

It is now known that there are several types of lymphocytes possessing regulatory activity [22]. $CD4^+$ regulatory T cells are often designated as "natural" Treg if they express Foxp3 and develop in thymus, and "induced" Treg if they are $Foxp3^-$ and are thought to differentiate in the periphery. Among these "induced" regulatory cells, there are several subtypes, based upon their ability to produce inhibitory cytokines. T cells that are induced in the gut after low doses of orally-administered antigen and produce TGFβ are termed "Th3" cells [23], while cells that produce large amounts of IL-10 are called "Tr1" cells [24]. Tr1 cells were first described after culture of T cells with antigen in the presence of IL-10, but have since been described as prevalent in the gut, particularly the small intestine [25], and also produce TGFβ, calling into question the distinction of their role from that of Th3 cells.

In addition to traditional $CD4^+$ regulatory T cells, regulatory activity has been ascribed to other subsets of T cells, such as certain subsets of $CD8^+$ cells (CD8αβ or CD8αα), γδ T cells, invariant natural killer T (iNKT) cells, and mucosal associated invariant T (MAIT) cells. Most of these have been described as playing a role in gut

tolerance [26]. Although poorly understood, these cells generally express an TCR displaying limited or no variation and recognize non-classical MHC molecules. Their mechanisms of regulation may involve interaction with the epithelial cell barrier or cytotoxic effects on antigen presenting cells.

The importance of Foxp3$^+$ Treg in tolerance

While regulatory cells that do not express Foxp3 have been shown to be involved in the regulation of effector T cell responses, particularly at mucosal sites such as the gut, it has been shown that they are not sufficient to maintain tolerance in the absence of Foxp3$^+$ Treg. The *scurfy* mouse was first described as the result of a naturally-arising X-linked mutation in a strain of mice in the 1950s. This mouse exhibited spontaneous, severe autoimmunity and inflammation which resulted in the death of the animals by about four weeks of age, and resembled the human disease immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome [15, 27]. The CD4$^+$ T cells from these mice were capable of mediating disease upon transfer into lymphopenic SCID or nude mice, much like CD4$^+$CD25$^-$ cells, and were hyperproliferative *in vitro* [28]. It was soon discovered that the mutant protein involved was the transcription factor Foxp3, resulting in the absence of Treg. Mutations in the human equivalent of this transcription factor are also responsible for reduced or absent Treg function in the human IPEX syndrome [29]. Therefore, in both mice and humans, Foxp3$^+$ Treg are absolutely essential for the prevention of widespread autoimmunity. The need for the continual presence of these cells in order to restrain self-reactive T cells was further confirmed by the creation of a mouse that expressed the human diphtheria toxin (DT) receptor on cells expressing

Foxp3, allowing for the deletion of these cells upon administration of DT. At any age, the administration of DT caused a rapid induction of rampant autoimmune syndromes, resulting in the death of the animals within 2-3 weeks [30].

Thymic development of Foxp3$^+$ Treg

As mentioned above, one criteria for the definition of a "natural" Treg is that they develop this phenotype during thymic development. The first clue that Treg indeed develop in the thymus was the model of autoimmunity induced upon thymectomy of mice at neonatal day 3 [31]. It was later confirmed that Treg are slightly delayed in their initial release from the thymus as compared to naïve, non-Treg cells, leaving these mice relatively deficient in Treg and resulting in disease [32]. A comparison of the repertoires of T cell receptor (TCR) sequences found on thymic CD4$^+$Foxp3$^+$ T cells and those found in the periphery (spleen and lymph nodes) showed a high similarity between these populations, suggesting that most or all Treg originate in the thymus [33-35].

During T cell development in the thymus, TCRs, which are the product of randomly rearranged gene segments, interact with self-peptides presented by major histocompatibility (MHC) molecules expressed on thymic stromal cell or thymic dendritic cells (DC). If the TCR is unable to interact productively with the MHC, the developing T cell dies; as long as the interaction is above a certain threshold the cell receives a survival signal, or is "positively selected". This ensures that only T cells capable of productive interactions with the MHC molecules enter the T cell pool. However, if the TCR recognizes the self-peptide/MHC complexes too strongly it receives

a death signal, or is "negatively selected", to prevent this potentially self-reactive T cell from being released from the thymus and possibly causing disease.

Regulatory T cells are thought to be selected in the thymus based upon an affinity to self-peptide/MHC complexes that lies somewhere just below the threshold of negative selection, but higher than most non-regulatory T cells. The theory that a developing Treg must recognize its cognate peptide/MHC complex is supported by studies showing the increased Treg selection in TCR transgenic mice after expression of the antigen recognized by this TCR in the thymus of the mice [36]. An increased prevalence of self-reactive TCRs among thymic Treg TCRs has been demonstrated by the transfer of T cells expressing Treg TCRs vs. non-Treg TCRs into lymphopenic hosts [37]. In these experiments, those T cells expressing Treg TCRs more often proliferated in response to unknown self-antigens encountered in the host animal. It is assumed that recognition of self-antigens by thymically derived Treg protects the tissues expressing those antigens from autoimmune attack.

Peripheral development of Foxp3$^+$ Treg

Although it seems most natural Treg found in the periphery of the mouse were selected into this lineage within the thymus, it has become clear that in certain situations Foxp3$^-$ cells can develop into Foxp3$^+$ T cells which are phenotypically indistinguishable from thymically derived Treg. This process has been coined peripheral "conversion" of non-Treg into Treg. The circumstances under which it has been reported *in vivo* are mostly those of antigen presentation under suboptimal, or "tolerizing" conditions. These include the oral, intravenous, or prolonged subcutaneous administration of cognate

peptide in the absence of adjuvant [38, 39]; transplantation tolerance under the cover of blocking antibodies; direct delivery of antigen to DEC-205$^+$ DCs (using a DEC-205 antibody-antigen fusion)[40]; and the transfer of TCR transgenic (Tg) T cells into a recipient that expresses the cognate antigen [41]. In addition, the transfer of CD4$^+$CD25$^-$ T cells into either a lymphopenic [42] or a non-lymphopenic [43] host will induce the development of T cell population stably expressing CD25.

While these experiments have demonstrated that non-regulatory cells have the ability to become Foxp3$^+$ regulatory cells outside of the thymus, it remains unknown how prevalent this is under steady-state conditions in a normal individual. A few studies have attempted to address this. The aforementioned transfer of CD4$^+$CD25$^-$ T cells into a wild-type congenically marked host reported about 5-12% of transferred cells became CD25$^+$ by six weeks post transfer [43]. The resulting cells were capable of inhibiting proliferation of effector cells in an *in vitro* proliferation assay. However, the use of CD25 as the sole marker for Treg makes it likely that these results could over-estimate the amount of conversion in the system. Studies looking at the TCR repertoires of Foxp3$^+$ and Foxp3$^-$ CD4 single-positive (SP) thymocytes versus peripheral Treg and non-Treg populations showed little evidence of conversion [34]. Additionally, an attempt to track the phenotype of TCR transgenic T cells specific for a pancreatic antigen which also express a secondary TCRα chain did not reveal that TCRα chains seen on the Foxp3$^-$ population in the thymus became part of the Foxp3$^+$ population after exposure to the pancreatic antigen in the periphery[35].

While current data is inconsistent about the contribution of peripheral conversion to the normal Treg repertoire, it is easy to imagine a role for conversion in particular

situations of novel antigen encounter. The peripheral development of regulatory T cells could potentially fill the role of mediating tolerance to food antigen, commensal microbiota, and any other self or harmless foreign antigen which would not be found in the thymus. Indeed, a study of oral tolerance in which Foxp3^{-} OVA-specific TCR transgenic T cells (expressing the Foxp3^{gfp} fusion protein) were transferred into a sublethally irradiated host subsequently fed OVA protein showed the induction of Foxp3 expression on donor T cells in the small intestine lamina propria (LP) and mesenteric LNs [44]. This supports a role for peripheral conversion in the maintenance of tolerance to non-thymic antigens at mucosal sites such as the gut.

It has also been postulated that Treg recognizing foreign antigens routinely develop from activated effector cells during an immune response, in order to dampen down and prevent the response from getting out of control, and as source of replenishment for the Treg pool [45]. A role for Treg in balancing a potentially overzealous immune response has been shown in many types of infections, although the character and source of the Treg involved is unknown [46]. Treg accumulated at the site of infection have been shown to be specific for microbial antigens in *Leishmania major* infection of mice [47], although it is unknown what the source of these Treg is. After adoptive transfer of TCR transgenic T cells specific for a particular peptide/MHC (pMHC), intradermal immunization of a protein containing the peptide was shown to cause the accumulation of donor-derived TCR transgenic T cells of both Treg and IFNγ-producing effector cell phenotypes, both of which were shown to recognize the cognate peptide/MHC complex by tetramer staining [48]. However, the use of incomplete

Freund's adjuvant (IFA) in this study calls into question the relevance of this model to a microbial infection in which antigen would be presented in the context of TLR ligands.

Mechanisms of suppression by Treg

Several mechanisms by which Treg may suppress immune responses have been reported [49, 50]. These can be divided according to whether the suppressive effect is directly on the effector T cells (Teff) in a contact dependent or independent way, or indirect via an alteration of the antigen presenting cells (APC). There seems to be no single, definitive mechanism by which Treg can suppress; it seems, instead, that they rely on a broad combination of actions whose role and importance vary according to the situation.

In *in vitro* suppression assays, contact between the Treg and the Teff cells appears to be required, as separation by a permeable membrane abrogates the suppressive effect [51]. In these studies, the presence of APC in the culture was not required. Contact-mediated effects may involve the delivery of cytoplasmic cAMP from the Treg to the Teff by contact via gap junctions [52], or granzyme B-dependent killing [53]. However, this need for direct contact is not supported by *in vitro* and *in vivo* imaging studies in which the Treg and Teff cells are not shown to interact stably [54]. A recent report argues that the contact with the target T cell may actually provide the Treg with additional suppressive capabilities, such as the ability to secrete cytokines, and after this initial contact it may suppress in a contact-independent manner [55].

Another mechanism by which Treg could suppress is by the production of inhibitory cytokines. While the blockade of the inhibitory cytokines IL-10 and TGFβ was

not reported to affect suppressive function *in vitro* [51], a role for these cytokines has been reported in several *in vivo* systems. The suppressive ability of Treg in a model of airway hyper-reactivity, as well as in various infections, depended upon IL-10, but in these cases the Treg themselves were not the source of the IL-10 [56-58]. However, in models of IBD, IL-10 production by the Treg themselves is absolutely essential for disease prevention. In addition, TGF-β also appears to be required for prevention of IBD, as Teff lacking the TGF-β receptor are refractory to Treg-mediated suppression in models of IBD [59, 60].

Another, more recently discovered Treg-produced inhibitory cytokine is IL-35 [61]. It is preferentially expressed by Treg, and its production is upregulated when they are actively suppressing. Treg deficient in either protein that makes up this heterodimeric cytokine show reduced regulatory activity *in vitro*, and are unable to control homeostatic proliferation or IBD *in vivo*. In addition, ectopic expression of this cytokine by naïve T cells caused them to gain regulatory activity, and addition of recombinant IL-35 can suppress proliferation *in vitro*. Although not much is known about this cytokine, it is likely to be an important player in Treg-mediated suppression.

Treg may also create a local environment that is unfavorable for effector T cell development. Treg may take up the IL-2 in their vicinity via their constitutive expression of the high-affinity IL-2 receptor α chain, CD25, leaving their immediate environment deficient in IL-2. This could cause the apoptosis of target cells as a result of cytokine deficiency, a theory that is supported by reports of Teff apoptosis following exposure to Treg in a process that depends upon expression of the pro-apoptotic factor Bim in the Teff [62]. Additionally, Treg have been shown to express high levels of the ectoenzymes

CD39 and CD73, which catalyze the breakdown of extracellular ATP or ADP into adenosine [63]. Adenosine binds to the adenosine receptor 2A on Teff and can inhibit their function.

Treg may also mediate their suppressive effects by altering the function of the APC, particularly dendritic cells (DC), that present the antigens to the target T cells. Intravital microscopy has revealed direct interactions between antigen-specific Treg and DC *in vivo* within hours after transfer [64, 65], and the presence of Treg shortens the interactions between Teff and DC (65). These interactions may directly inhibit the ability of Teff to interact with DC (by "crowding"), as well as directly alter the characteristics of the DC as to reduce its ability to subsequently present antigen to the Teff, and/or cause it to enhance the proliferation and generation of Treg.

In terms of decreasing the DC's ability to activate a Teff cell, Treg have been shown to condition DC to express indolamine 2,3-dioxygenase (IDO), which can cause suppression by breakdown of tryptophan into pro-apoptotic molecules [66]. Additionally, interaction with Treg has been shown to result in a downregulation of the costimulatory molecules CD80 and CD86 on the DC, thereby inhibiting its ability to activate a naïve T cell [67]. These effects on DC are dependent upon the expression of CTLA-4 by the Treg, which engages CD80 and/or CD86 on the DC; in the absence of CTLA-4 expression or in presence of blocking antibody there was a reduction of Treg-mediated suppression via DCs [13, 68].

It is also possible that the Treg is able to change the phenotype of the DC to become "tolerogenic". Treg have the potential to activate latent TGF-β on the surface of DC by their production of the enzyme furin in response to environmental IL-12, and this

TGF-β may then go on to promote the differentiation of conventional T cells into $Foxp3^+$ Treg [69]. This could help to explain the phenomenon of infectious tolerance, by which Treg with one specificity can go on to promote tolerance to a second antigen presented on the same tissue or cell [70, 71]. In these situations, the original population of Treg is then no longer required to maintain tolerance, suggesting that T cells of a new specificity were generated.

The absence of any one of the aforementioned molecules does not cause a complete of loss of suppressive activity akin to a Foxp3 deficiency; this leads to the theory that all of these mechanisms play but a part in the overall ability of Treg to maintain order. It has been postulated that different mechanisms of suppression come into play during different scenarios; in the most "challenging" situations, all of these mechanisms may need to work together to keep the immune system fully in check [49]. For example, in the case of inflammatory bowel disease (IBD), an array of mechanisms may be required due to the great inflammatory potential of this microbial-laden mucosal surface, while a more slowly progressing autoimmune disease in a sterile organ, such as type I diabetes, may not require as many suppressive mechanisms to control.

T cell receptor specificity in Treg function

There has been some controversy over the need for regulatory T cells to specifically recognize an antigen from the same cells or tissues to which it is preventing an inappropriate immune response. Some reports suggest that the mere presence of a Treg of any specificity will suppress the effector T cells in the vicinity, such as in a model of autoimmune gastritis that was prevented by the transfer of polyclonal, wild-type Treg

[72]. This may be due to the "bystander effect", in which a Treg is activated by recognition of antigen and can then go on to suppress cells of other specificities [73]. However, in a follow-up report the same group reported that antigen-specific Treg were much more efficient at preventing disease [74]. The very opposite has also been reported, in a system where TCR transgenic Treg cells of known antigen specificity only suppressed cells with the same antigen specificity [75]. And while several reports have established a need for TCR engagement for Treg function [51, 76], there is also a report showing that Treg can suppress T cells with a completely different antigen specificity, in the absence of TCR engagement [77].

Evidence for antigen specificity in the Treg population has been observed in models which show a lack of suppressive capability for a tissue or organ which is absent in the animal in which the Treg developed. For example, in the prevention of autoimmune prostatitis due to day 3 thymectomy, T cells isolated from male mice were more effective than those from female mice or from males that had been orchidectomized just after birth [78]. Similarly, in a model of autoimmune thyroiditis in rats, disease was prevented by the transfer of a $CD4^+$ regulatory subset from rats with an intact thyroid but not by cells from rats whose thyroids were ablated *in utero* by ^{131}I treatment [79]. These studies suggest that the development and/or maintenance of Treg capable of controlling autoimmunity of a particular tissue depends upon the presence of that tissue.

A skewing towards self-antigen reactivity has been demonstrated for Treg [37], supporting the hypothesis that Treg maintain tolerance by direct recognition of the antigens of the cells and tissues they are protecting. Additionally, it has been shown that Treg capable of preventing autoimmunity of a particular organ are found preferentially in

the draining LN of that organ [80]. However, another report argues that there are no more self-reactive TCRs found within the Treg repertoire than the non-Treg repertoire, and that most, in fact, could be shown to be non-self antigen responsive [81]. Although this study uses an extremely reduced repertoire of TCRs, and its most compelling data relies on TCRs selected in an environment in which the MHC display only a single self-peptide, it casts doubt on the assumption that the Treg population is highly enriched in self-specific T cells.

Studies of the repertoires of the Treg and non-Treg populations in the peripheral lymphoid organs of mice have reported a varying degree of overlap in the T cell receptors used by, and presumably the antigen specificities of, the two subsets [33-35]. This variability in results may be due to the different systems used by these groups. Additionally, a more recent study suggests that these overlapping TCRs may belong to "unstable" Treg, which tend to express lower levels of Foxp3 and appear to be the result of conversion of non-Treg cells into $Foxp3^+$ T cells [82]. All of these studies, however, agree that the TCR repertoire is not the same between the two populations, indicating that they develop differently.

The highly specialized immune system of the gut

The digestive tract is a portion of the body that presents a unique challenge to the immune system; it must prevent disease caused by infectious organisms at this environmental interface, while maintaining tolerance to the many beneficial and non-harmful organisms that are colonized there. It is estimated that the lower gastrointestinal tract of the mammal is one of the most complex microbial ecosystems on the planet,

harboring over 100 trillion bacteria of 500-1000 different species [83, 84]. The immune system must allow the colonization of beneficial symbionts, which provide host benefits such as supplying essential nutrients, metabolizing otherwise undigestible compounds, and defending against colonization by pathogenic bacteria. On the other hand, it must still be capable of recognizing and fighting pathogenic microorganisms to fight disease. When the ability to appropriately balance these two goals breaks down, the immune system can inappropriately target commensal organisms, resulting in inflammatory bowel diseases (IBD) such as Crohn's disease (CD) or ulcerative colitis (UC).

The commensal microbiota is composed of members from all three domains of life – eukarya, archaea and bacteria. However, the bacteria are the dominant organisms present. The bacteria are acquired during a short period of initial colonization beginning immediately after birth, and by adulthood there is a relatively stable community of bacteria which can vary from individual to individual, in a way that appears to be partly influenced by genetics and partly by environment [85]. The amount of bacteria present depends on the location, with there being very little in the stomach and proximal small intestine, with increasing amounts in the distal small intestine and colon, resulting in a density of approximately $10^9 – 10^{12}$ per ml of luminal contents.

Sequencing of the 16S rRNA of the intestinal microbes has allowed for the identification of the bacteria that typically inhabit the gut, regardless of the ability to culture them in the laboratory. These studies have identified members of the gram-positive, anaerobic phylum *Firmicutes* as the most prominent, with the gram-negative *Bacteroides* as the next most prevalent [86]. The remaining phyla make up less than ten percent of the population, and include Actinobacteria, Proteobacteria, Fusobacteria and

others. The fact that many of these are facultative aerobes may put them at a competitive disadvantage and explain their relatively low abundance in the anaerobic environment of the gut [87].

The barrier which separates the gut lumen and its microbial contents from the body is composed of a single layer of intestinal epithelial cells (IEC) connected by tight junctions. Also included in this epithelial layer are the goblet cells, responsible for secreting the mucin that forms a layer of mucus which coats the lumen, and the Paneth cells, specialized antimicrobial peptide secreting cells found in the base of the crypts of the intestine [88]. The mucus that coats the lumen can extend up to 150μm from the epithelial surface. Its innermost layer is resistant to bacterial penetration and likely also retains secreted antimicrobial peptides, therefore serving as an additional barrier to the bacteria that inhabits the lumen [87].

Also incorporated into the epithelial layer are the specialized microfold or "M" cells, which transporter luminal antigens across the epithelial barrier to the underlying gut associated lymphoid tissues (GALT). This provides a source of antigen for the dendritic cells (DC) of the GALT, which can then present them to the resident T cells. The epithelium also includes a population of specialized T cells, the intraepithelial lymphocytes (IEL), which are largely of the "unconventional" CD8$\alpha\alpha^{+}$ (CD8β^{-}) type, many of which express TCR$\gamma\delta$ and may assist in the clearing of stressed or damaged IEC, and contribute to tolerance by secreting TGFβ upon activation [89]. A population of DC have also been described to form tight-junction-like structures with the IECs and project dendrites into the lumen in order to sample antigens directly [90].

Beneath the epithelium in the lamina propria, the loose connective tissue which supports the delicate epithelium, are most of the more conventional immune cells of the GALT. Organized lymphoid structures include the Peyer's patches of the small intestine and the isolated lymphoid follicles (ILF) of the colon. These serve as the site of T cell interaction with antigen-loaded DC, which are known to acquire antigens from the intestinal lumen [91, 92]. In addition, B cells are located in these follicles, as well as throughout the lamina propria, and produce secretory IgA antibodies which are transcytosed across the epithelium by the IEC [93]. These antibodies help to keep the intestinal bacteria in the lumen, as shown by increased penetration of symbiotic bacteria into the tissues of IgA deficient mice [94, 95].

The intestinal epithelial cell interaction with the commensal microbiota

The microorganisms that peacefully reside in the gut are not just tolerated; they constantly interact with and influence the host. With such a large reservoir of microorganisms in constant contact with host mucosal surfaces, it is not surprising that they have been shown to have an important impact on the immunology of the gut for the benefit of both the host and the resident gut organisms. Studies with germ-free and gnotobiotic mice have shown that recognition of the commensal microbiota is necessary for the normal development of both the mucosal and peripheral immune system [85]. Germ-free mice exhibit poorly formed Peyer's patches, smaller mesenteric lymph nodes (MLN), impaired development of isolated lymphoid follicles (ILFs), altered composition of the $CD4^+$ T cells and IgA-producing B cells in the lamina propria, and an absence of MAIT cells which normally reside in the lamina propria [96, 97]. Additionally, the IEC

exhibit a reduced rate of cell turnover, and altered microvilli formation in germ-free animals [96].

The IEC are the most abundant cells to have direct contact with the microbiota, and they play an important role in maintaining intestinal immune tolerance. Their first role is to provide a barrier between the bacteria of the gut and the immune system of the host. However, the IEC can also sense the bacteria by their expression of pattern recognition receptors (PRR), such as toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which bind to a variety of conserved ligands derived from microorganisms [98]. The hyporesponsiveness of these cells is thought to be a result of several strategies, one simply being the limited contact with bacteria, which find it difficult to penetrate the layer of mucus which lines the lumen. Mice which lack production of mucin suffer from intestinal inflammation, presumably as a result of their inability to form this relatively bacteria-free zone adjacent to the epithelium [99].

In an additional mechanism to minimize reactivity to the bacteria, the expression of TLRs by the IEC is generally quite low, while increased expression is seen in inflamed intestines, such as in patients with Crohn's disease [100]. The TLR expression on the IEL may also be limited in order to minimize engagement of bacterial-derived ligands. For example, it has been shown that TLR5 expression is normally limited to the basolateral side; therefore, TLR5-mediated activation will only occur in response to bacteria that have breached the epithelial barrier [101]. In contrast, TLR9 has been shown to be expressed on both the apical and basolateral sides of the IEC (unlike in immune cells, it does not reside in endosomes), and the response to engagement of the receptor in the two

locations is opposite. When administering CpG oligodeoxynucleotides (ODN), a ligand for TLR9, on the basolateral side of the cell, NF-κB was activated and led to the secretion of the inflammatory cytokine IL-8 [102]. However, when TLR9 was engaged on the apical surface, NF-κB was inhibited. This sort of spatial segregation can emphasize tolerance to bacteria encountered in the lumen, but still allow for a vigorous immune response to bacteria that have breeched the epithelial barrier.

The IEC also appear to actively dampen the response to engagement of TLRs and other PRR when it does occur. For example, IEC express a negative regulator of TLR and IL-1R signaling called SIGIRR (single-immunoglobulin-domain-containing interleukin-1 receptor-related protein), and in the absence of this they express an increased level of pro-inflammatory genes, and increased susceptibility to commensal bacteria-dependent intestinal inflammation [103]. They also express the protein A20, which acts to inhibit NF-kB activation, and mice deficient in A20 develop severe intestinal inflammation [104, 105]. It is also thought that certain commensal bacteria are capable of actively suppressing the immune response by inhibiting NF-kB activation. Both nonpathogenic *Salmonella* strains and *Bacteroides thetaiotamicron* have been shown to interfere with NF-kB activation, although by different mechanisms [106, 107]. In addition, some products of commensal bacteria metabolism are immunomodulatory, such as butyrate that is produced by the digestion of starch, which can inhibit the production of pro-inflammatory cytokines [108].

IECs respond to contact with bacteria in ways which impact and instruct the immune system. Engagement of TLRs can cause NF-kB activation, leading to the increased production of thymic stromal lymphopoietin (TSLP), which is thought to have

a tolerogenic effect on the intestinal DC [109, 110]. TSLP has been shown to restrict the production of IL-12 and promote IL-10 production by DC, and therefore may play a vital role in conditioning lamina propria DC to be tolerogenic. IEC also produce TGFβ, which can inhibit the expression of pro-inflammatory cytokines by macrophages and DC, and inhibit the expression of MHC class II and costimulatory molecules [111, 112]. The production of prostaglandin E2 (PGE2) by the IEC can contribute to the maintenance of tolerance to dietary antigens, by an unknown mechanism involving the inhibition of T cell proliferation [113]. This may be due to the induction of IL-10 and IDO production by DC [114, 115]. Upon TLR engagement IEC can also influence B cells by the production of APRIL, a B cell stimulating factor which can promote plasma cell survival and class switching to IgA [116]. IEC also express MHC class II molecules, and while they lack the costimulatory molecules necessary to prime naïve T cell responses, they are capable of activating antigen-experienced T cells, and may induce anergy in naive T cells due to this lack of costimulation [117]. Antigen processing and presentation by IEC has been shown to occur in a polarized fashion, with antigens obtained on the apical side of the cell and presented on the basolateral side [118].

The Unique Role and Properties of Intestinal Dendritic Cells

Antigen presenting cells are also in contact, both directly and indirectly, with the commensal microbiota and are central to mediating the immune response to it. The dendritic cells (DC) found in the intestine are different in their phenotype and functional properties than those found elsewhere in the body, and multiple types have been identified. These are generally identified by their high expression of CD11c, and then by

the expression of either CD11b, CD8α, or neither, forming three main categories of conventional DC in the gut immune system [119, 120]. These different subsets tend to be found in different locations, with the $CD11b^+$ DC concentrated in the sub-epithelial dome of the Peyer's patch, and the $CD8\alpha^+$ DC in the interfollicular regions. There are also many DC present in the lamina propria of the small intestine, but in the colon most DC are found within the isolated lymphoid follicles (ILF) under steady state conditions [121].

The DCs can obtain antigens from the commensal microbiota in multiple ways. The $CD11b^+$ DC located in the sub-epithelial dome are positioned to receive bacteria and bacterial antigens transported from the gut lumen by M cells. This subset has a higher capacity to produce IL-10 and to prime a T_H2 (rather than T_H1) T cell response to infection, and therefore may help mediate tolerance to the gut antigens constitutively transcytosed by the M cells [120]. Uptake of small numbers of commensal bacteria by this route has been shown to induce production of IgA, which would serve to limit bacterial penetration, and therefore may limit the potential for destructive inflammatory responses to develop to the bacteria [95]. Lamina propria DC expressing the chemokine receptor CX_3CR1 can also form tight-junction-like structures with the IEC and extend dendrites through the epithelial barrier to directly sample luminal contents [90]. This ability to penetrate the epithelium may also allow for DCs to engulf apoptotic epithelial cells, and thereby process and present antigens from them.

DCs which take up antigen in the intestine may constitutively migrate to the MLN, where they can present antigen to T cells. These DC have obtained antigen in close proximity to the IEC, and as discussed earlier, may be affected by the production of tolerogenic molecules by the IEC under non-inflammatory conditions. Intestinal dendritic

cells can be subdivided by their expression of the α_E integrin, CD103. Those which migrate through the lymph to the MLN tend to be $CD103^+$, while the $CD103^-$ subset found in the MLN are thought to be resident DC which have developed from blood-borne precursors. $CD103^+$ DC have the ability to promote expression of the gut-homing receptors $\alpha_4\beta_7$ (which binds to MadCAM1 on the vascular endothelium of the gastrointestinal tract) and CCR9 (whose ligand CCL25 is produced by IEC) on $CD4^+$ T cells, likely including regulatory T cells which are induced in the lymph nodes [122, 123]. $CD103^+$ DC carrying antigens from the intestine are implicated in the development of $Foxp3^+$ Treg from naïve $Foxp3^-$ T cells; $CD103^+$ DC isolated from the MLN were shown to mediate the development of Treg *in vitro* in a TGFβ-dependent manner, while the $CD103^-$ subset did not [124]. In one study, bone marrow-derived dendritic cells (BMDC) were capable of internalizing the commensal bacterium *Lactobacillus rhamnosus* while maintaining an immature phenotype, and were able to prevent intestinal disase upon transfer into a recipient animal by a mechanism that depended upon the presence of $CD4^+CD25^+$ T cells [125]. Presumably, therefore, intestinal DC would be capable of internalizing commensal bacteria in the intestine and trafficking to the MLN, where they could expand and/or convert regulatory T cells, as well as causing them to upregulate gut-homing receptors, therefore sending them to the intestine to mediate tolerance to that bacterium.

The ability of DC to induce B cell class switching to IgA, convert naïve T cells into $Foxp3^+$ Treg, and induce gut-homing molecules may all be due at least in part to the production of retinoic acid (RA) by these DC. Retinoic acid is a product of vitamin A metabolism, and vitamin A deficiency is reported to lead to low levels of IgA [126], and

T cell independent class switching mediated by DCs isolated from the Peyer's patch has been shown to depend upon RA and IL-5 or IL-6 [127]. RA has been shown to enhance TGFβ dependent conversion of naïve T cells into $Foxp3^+$ Treg and to induce gut homing molecules [44, 124, 128-130]. Accordingly, MLN $CD103^+$ DCs express higher levels of one of the enzymes involved in the production of RA from vitamin A, aldehyde dehydrogenase family 1, subfamily A2 (ALDH1A2), than their $CD103^-$ counterparts [124]. Additionally, the IEC have been shown to express the closely related ALDH1A1, so they can also produce RA which can be taken up and transported by DCs [131, 132].

These data suggest a scenario in which the "decision" of whether to respond to the detection of a bacteria rests on the way in which the dendritic cell acquires the antigen. If a DC is in close proximity to the IEC, it can acquire the antigen in a way that promotes a non-inflammatory response; as CD103 expression allows interaction of the DC with E-cadherin expressed on the IEC, it would follow that the $CD103^+$ DC are the ones which have done this. These DC then go on to promote tolerance to these antigens, by inducing and/or enhancing regulatory T cell responses and B cell production of IgA directed at these antigens. On the other hand, if a DC encounters a bacterium in an area that is not in close vicinity to the IEC, such as the lamina propria or MLN, it is likely that the bacteria has actively invaded the epithelium. This DC was not in close contact with the IEC, did not undergo "conditioning", and is still capable of mediating attack to this organism. In the absence of "conditioning", the $CD103^-$ DC would tend to prime a T_H1 response, as this subset has been shown to produce more IFNγ [122]. In addition, IEC would no longer be secreting tolerogenic molecules, in response to the epithelial cell invasion or epithelial layer breech, allowing for engagement of PRR which would normally be

sequestered (such cytosolic NOD2 or basolaterally-expressed TLR5). They would therefore not downregulate DC maturation and inflammatory cytokine production, further driving the immune response.

The loss of tolerance to the commensal microbiota results in inflammatory bowel disease.

In humans, inflammatory bowel disease is a serious clinical problem, affecting 0.1 – 0.2% of the population in the form of Crohn's disease and Ulcerative Colitis [133]. Disease susceptibility, as with most autoimmune disorders, appears to be a combination of genetic predisposition and unknown environmental triggers [134]. There is evidence that disease is dependent upon and affected by the resident bacteria in the gut, as antibiotic treatment often ameliorates disease in IBD patients, and they show higher antibody titers against commensal bacteria than unaffected individuals [135, 136]. Also, individuals with IBD show an altered community of commensal bacteria compared to healthy controls. In most mouse models of IBD disease does not develop in the absence of bacteria.

The prevailing view of IBD is that it is the result of a dysregulated inflammatory response and/or innate immunodeficiency to the commensal microorganisms in the gut [137]. Failures in the mucosal barrier (due to targeting by a pathogen or genetic mutations such as NF-kB deficiency) and defective defensin production by Paneth cells (due to mutations in the intracellular peptidoglycan derivitive detector NOD2 or a defect in autophagy) are failures of the innate immune system which can allow the microbiota to become uncontrolled [138-140]. In addition, a dysregulated response to the sensing of bacteria can cause an inappropriate or exaggerated inflammatory response. This can be

due to defective TLR signaling, which is required to maintain intestinal homeostasis and repair, promotes luminal antigen sampling by DCs and usually causes production of anti-inflammatory cytokines by the IECs [109, 141, 142]. However, inappropriate responses to TLR signals in may also occur, due to a lack of molecules that usually dampen the TLR response in these cells, such as A20 or SIGIRR (mentioned earlier) [103, 104]. A lack of anti-inflammatory cytokines such as IL-10 or TGFβ can also cause intestinal disease, as shown by the spontaneous development of colitis in mice with IL-10, IL-10 receptor, TGFβ, or TGFβ receptor signaling deficiencies [143]. These cytokines have effects both on the activation state of the antigen presenting cells as well as the on the B and T cells directly. The downstream effect of all of these innate immune system deficiencies is a failure to establish or maintain tolerance in the adaptive immune system, resulting in an inappropriately directed adaptive immune response.

While there are many cell types and mechanisms involved in the maintenance of intestinal tolerance, $Foxp3^+$ regulatory T cells are an essential component. One of the earliest and most prevalent disorders seen in the IPEX disorder (resulting from Foxp3 mutations in humans), often leading to the diagnosis, is severe early-onset enteropathy [144]. Crohn's Disease and Ulcerative Colitis are believed to be T cell mediated diseases, and are associated with increased pro-inflammatory cytokines such as TNF and IFNγ. It has been shown that normal mice harbor effector T cells in the MLN and colonic lamina propria which recognize intestinal bacteria, and these are actively restrained by the $CD4^+CD25^+$ cells from the GALT. [145]. This is the basis of a classic model used in the study of colitis first described by Fiona Powrie [146]. In this model, a population of non-regulatory T cells is transferred into a T cell deficient host, where they undergo

homeostatic expansion. This leads to the development of spontaneous colitis upon the arrival of effector T cells into the gut in the absence of Treg. Therefore, disease can be prevented by the co-transfer of a population of Treg, providing a model that allows the requirements for Treg suppressive activity in the gut to be elucidated.

Whether Treg in the gut need to be specific for intestinal (self or commensal-derived) antigens to maintain homeostasis is uncertain. Some evidence suggests that Treg specific for gut antigens are more efficient at preventing disease. In at least one study, Treg cells isolated from germ free mice could not protect from disease in the transfer model of colitis [147]. In another study, germ-free Treg were capable of preventing disease, but not as efficiently as the conventional Treg [148]. This somewhat reduced ability to suppress may rely on Treg that recognize conserved self antigens found in the gut, allowing for their activation and suppressive function even in the absence of bacterial recognition. Alternatively, in the absence of commensal bacteria, the Treg may be less activated, and therefore less suppressive no matter their specificity. Models of oral tolerance, however, have been shown to involve the induction of antigen-specific Treg cells. In these studies, DO11.10 TCR transgenic T cells specific for an ovalbumin (OVA) peptide became $Foxp3^+$ in the MLN and small intestine LP only after feeding the mice OVA [124].

With the importance of Treg in the maintenance of tolerance in the intestines, it seems reasonable that the ability to induce Treg at this location would confer the flexibility that is required to maintain tolerance in a location with such a dynamic antigenic profile. The increased propensity for peripheral conversion of non-regulatory cells into $Foxp3^+$ Treg in the GALT as compared to the "central" immune system has

been reported. As previously mentioned, upon transfer of non-Treg into a lymphopenic host (with co-transfer of whole $CD4^+$ T cells to prevent intestinal inflammation) there were 4-5 times more converted Treg recovered in the MLN and small intestine lamina propria (LP) than in peripheral LN or ear dermis [44]. Additionally, a recent study of the role of the conserved non-coding sequences (CNS) in the *foxp3* gene that one of these sequences was required specifically for conversion of a mature non-regulatory cell into a $Foxp3^+$ regulatory cell *in vitro*. In the mice harboring a deletion in this sequence, a selective decrease was seen in $Foxp3^+$ Treg in the GALT [149]. Most likely conversion is prominent in the gut due to the presence of the $CD103^+$ DC, which have been shown to be efficient at inducing $Foxp3^+$ T cells due to their ability to provide retinoic acid (RA) and TGFβ alongside TCR engagement. These cells have been shown to migrate to the lymph nodes carrying intestinal antigens including bacteria, and are therefore poised to divert T cells with specificity for bacterial antigens into the regulatory lineage.

Summary

The $CD4^+Foxp3^+$ regulatory T cell subset is of vital importance in the maintenance of peripheral tolerance to both self antigens and harmless foreign antigens such as those derived from food or commensal bacteria. The specific mechanisms by which Treg assert their suppressive ability are still unclear, with many different candidate mechanisms having been described. Most likely some combination of these mechanisms is employed, with some variability according to the situation, and some redundancies that highlight the importance of their function. The challenge of maintenaning tolerance while keeping the ability to provide an immune response to pathogens is particularly

challenging in areas of the body in contact with the microorganisms that inhabit our surroundings. The immune system of the gut meets this challenge with a complicated and unique set of solutions, among them the regulatory T cells that are essential for preventing inappropriate responses to the commensal microbiota. What role Treg specificity plays in their function remains undefined, as there are conflicting data concerning their ability to suppress immune responses in an antigen non-specific way.

CHAPTER TWO

Antigen Specific Shaping of the Natural Regulatory T Cell Population

Chapter Two: Preface

The studies described in this chapter were a direct result of previous work done by my thesis advisor, Dr. Chyi Hsieh, while a postdoctoral fellow in the laboratory of Dr. Alexander Rudensky at the University of Washington. It was known that Foxp3$^+$ regulatory T cells emerged from the thymus with this phenotype, but it was not certain whether they were selected on the basis of a unique TCR specificity or were randomly selected. The use of a transgenic mouse expressing a fixed TCRβ chain to sequence a subset of the TCRα chains to get a measurement of the TCR repertoire of the Treg and non-Treg populations proved to be an elegant way to answer this question at the individual sequence level. As was suspected, these populations showed only slight overlap in their TCR usage, suggesting that they recognize different populations of antigens. These studies went on to show evidence that many of these appear to be self antigens, as T cells expressing these Treg-derived receptors expanded preferentially upon transfer to lymphopenic hosts [37].

While these studies showed that regulatory and non-regulatory cells used a different repertoire of TCRs, they left unaddressed the possibility that the regulatory T cells may show similarity to antigen-experienced memory T cells. It has been proposed that upon antigen recognition a naïve T cell may give rise to both effector T cells (a T_H1 cell, for example) and a regulatory T cells, in order to limit the extent of the immune response [45]. The previous studies did not differentiate between non-regulatory cells of a naïve and a memory phenotype; as 90% of the non-regulatory population in these mice is usually of a naive phenotype, the main comparison previously made was between naïve and regulatory T cells.

Additionally, the T cells sequenced in the previous studies were a mix of cells from the spleen and lymph nodes. There have been reports suggesting that Treg act in an antigen-specific manner to prevent autoimmunity, and the presence of a tissue is required to develop and maintain Treg specific for that particular tissue [79]. In addition, data suggested that the Treg would be located preferentially in the draining lymph node of that tissue [80]. Therefore, the studies described in this chapter were designed to answer these questions. The results of this work were published in *The Journal of Experimental Medicine* in 2008 and this chapter is adapted from this manuscript [150].

Chapter Two: Abstract

Although regulatory T (Treg) cells are thought to develop primarily in the thymus, the peripheral events that shape the protective Treg cell population are unclear. Here, we analyzed the peripheral $CD4^+$ TCR repertoire by cellular phenotype and location in mice with a fixed TCRβ chain. We found that Treg ($Foxp3^+$) cells showed a marked skewing of TCR usage by anatomic location in a manner similar to antigen-experienced ($CD44^{hi}Foxp3^-$), but not naïve ($CD44^{lo}Foxp3^-$), cells, even though $CD44^{hi}$ and Treg cells used mostly dissimilar TCRs. This was likely unrelated to peripheral conversion, which we estimate generates only a small percentage of peripheral Treg cells in adults. Conversion was readily observed, however, during the immune response induced by $Foxp3^-$ cells in lymphopenic hosts. Interestingly, the converted $Foxp3^+$ and expanded $Foxp3^-$ TCR repertoires were different, suggesting that generation of $Foxp3^+$ cells is not an automatic process upon antigen activation of $Foxp3^-$ T cells. Retroviral expression of these TCRs in primary monoclonal T cells confirmed that conversion did not require prior cellular conditioning. Thus, these data demonstrate that TCR specificity plays a crucial role in the process of peripheral conversion and in shaping the peripheral Treg cell population to the local antigenic landscape.

Chapter Two: Introduction

The importance of natural Foxp3$^+$ regulatory T (Treg) cells for the maintenance of self-tolerance was recently reaffirmed by the observation that acute depletion of Treg cells creates a state of "immunologic anarchy", resulting in the rapid death of a previously healthy animal [30, 151]. Treg cells are commonly thought to be generated in the thymus due to recognition of self-antigens [36, 152], which is supported by studies using mice with limited T cell receptor (TCR) diversity showing strong similarity between the thymic and peripheral Treg TCR repertoires [33, 34, 153]. However, the peripheral and thymic Treg TCR repertoires were not identical, suggesting that certain TCRs are preferentially enriched or deleted in the periphery, akin to earlier observations in the total CD4$^+$ T cell population [154].

The notion that post-thymic Treg TCR repertoire shaping may play an important role in immune regulation was suggested by a number of studies demonstrating that the presence of an organ is accompanied by functionally enhanced suppression of autoimmunity to that organ [78-80, 155]. Based on these studies, it has been widely hypothesized that tissue-specific Treg cell number is increased due to the presence of the antigen. However, this has not been directly demonstrated, and alternative explanations for these classic observations include altered trafficking patterns [128, 130, 156] and activation status [155, 157] of Treg cells at particular locations. These other possibilities are consistent with reports suggesting that Treg cells may not require tissue specificity for suppression [158-160], nor recognize self-antigens [81]. Thus, an important unresolved question is whether the peripheral Treg TCR repertoire is altered based on the presentation of tissue-specific antigens.

The development of Foxp3$^+$ T cells from mature, naïve Foxp3$^-$ T cells, a process often referred to as peripheral conversion, is another mechanism by which the peripheral repertoire may be altered. This process would allow for the generation of Treg cells specific for antigens not presented in the thymus, such as those from commensal microbiota or sequestered self-antigens [45, 161]. Furthermore, it has been hypothesized that peripheral conversion may also generate Treg cells during the course of an immune response to limit immune pathology. Studies of TCR$\alpha\beta$ transgenic T cells have shown that exposure to cognate antigen under non-inflammatory or tolerogenic conditions in vivo results in the development of suppressive Foxp3$^+$ Treg cells in a portion of the monoclonal T cell population [39, 41, 162]. In polyclonal T cell populations, peripheral development of CD25$^+$ cells characteristic of Treg cells has been observed after adoptive transfer of CD25$^-$ T cells into both lymphopenic and non-lymphopenic hosts [42, 43]. However, a study using endogenous secondary TCRα chains on self-reactive BDC2.5 TCR$\alpha\beta$ transgenic T cells to track T cell fates found no evidence that this TCR facilitates conversion [35]. Thus, the role of peripheral conversion in generating the peripheral Treg cell population remains unresolved.

To address these questions, we analyzed the TCR repertoire based on anatomic location and cellular phenotype by sequencing TCRα chains in mice with a fixed TCRβ chain [37]. We generated a database of nearly 18,000 TRAV14 (Vα2) TCRα chain sequences derived from Treg (Foxp3$^+$), antigen-experienced (CD44^{hi}Foxp3$^-$), and naïve (CD44^{lo}Foxp3$^-$) CD4$^+$ cells isolated from the spleen; and cervical, axillary, inguinal, and mesenteric lymph nodes. We found considerable differences in TCR usage by location in the Treg and CD44^{hi}, but not CD44^{lo}, T cell populations, demonstrating that tissue-

specific antigen recognition dramatically alters the peripheral Treg cell population. Shaping of the peripheral Treg TCR repertoire by location therefore suggests that immune regulation is a tissue-specific activity.

A potential explanation for parallel changes in both the Treg and $CD44^{hi}$ TCR repertoires by location is that peripheral conversion generates a large proportion of the peripheral Treg cell population. However, we found that few cells (~0.03%) became $Foxp3^{+}$ after adoptive transfer of $Foxp3^{-}$ cells into normal adult hosts, resulting in an estimated 3% contribution to the peripheral Treg cell population. By contrast, we found a much higher percentage of conversion during the immune response induced by $Foxp3^{-}$ cells transferred into lymphopenic hosts. Interestingly, there was considerable disparity in TCR usage between the converted $Foxp3^{+}$ and expanded $Foxp3^{-}$ T cell subsets within the same individual host. We excluded an absolute requirement for thymically-derived Treg cell precursors by showing that retroviral expression of particular TCRs on monoclonal peripheral T cells was sufficient to permit peripheral conversion. Thus, these data favor a TCR-specific model of peripheral Treg cell development.

Chapter Two: Materials and Methods

Mice and Reagents

Foxp3^{gfp} reporter knockin, and TCli TCR-β and TCR-αβ transgenic mice were kindly provided by Dr. Alexander Rudensky (U. Washington, Seattle, WA). B6.SJL (CD45.1), B6.PL (Thy1.1), *Tcrb*$^{-/-}$, and *Tcra*$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a specific-pathogen-free animal facility at Washington University and were used according to protocols approved by the Institutional Animal Care and Use Committee. Monoclonal antibodies were purchased from eBioscience (San Diego, CA) and Becton Dickenson (San Jose, CA). Human IL-2 was obtained from the NIH Biological Resources Branch (Frederick, MD).

Cell purification

CD4$^+$ T cells were initially purified from the indicated tissues using the CD4 Untouched kit, which utilizes AutoMACS magnetic separation to remove non-CD4$^+$ cells (Miltenyi Biotech, Auburn, CA). Remaining cells were stained with fluorescently labeled antibodies and sorted by flow cytometry using a Moflo (Dako, Carpinteria, CA) or a FACSAria (Becton Dickenson).

Isolation of T cells for TCR sequencing

CD4$^+$Vβ6$^+$ T cells were purified by FACS into the Foxp3$^+$, Foxp3$^-$CD44^{hi}, and Foxp3$^-$CD44^{lo} subsets from normal mice, and Foxp3$^+$ and Foxp3$^-$ subsets after proliferation and conversion in lymphopenic hosts. CD44 was not used to purify T cells from lymphopenic hosts as the Foxp3$^-$ population was CD44^{hi} after lymphopenia-induced

proliferation. Post-sort purity was > 95%. The percentage of $V\alpha2^+$ cells was found to range from 6-14%, suggesting that the proportion of these cells was relatively stable between mice and experiments. Cloning of TRAV14 TCRα sequences was performed as previously described [33]. As before, we utilized the CDR3 amino acid sequence provided by IMGT/V-QUEST as a unique identifier for individual TCRs [163]. Thymic $Foxp3^+$ and $Foxp3^-$ $CD4^+CD8^-$ data sets have been previously described [164].

Statistical Analysis

The estimated contribution of peripheral conversion to the Treg cell population is a simple extrapolation of the % $Foxp3^+$ in the adoptively transferred $Foxp3^-$ population to the entire $Foxp3^-$ population multiplied by the proportion of $Foxp3^-$ cells of $CD4^+$ T cells, divided by the proportion of Treg cells of $CD4^+$ T cells. Thus, an 8% conversion in normal mice would represent 7.2% of total $CD4^+$ T cells (8% x 90%), which would be 72% the size of the Treg subset (7.2% of 10% Treg cells in the total $CD4^+$ T cell population).

We used the Morisita-Horn index as a statistical measure of similarity between two data sets [165]. This unit-less index ranges from 0 to 1, representing complete dissimilarity to similarity. The Morisita-Horn index takes into account the frequency of each TCR, and is relatively resistant to the sample size, as compared with other similarity indices such as the Jaccard. A sequence found in both data sets at greatly different frequencies would therefore contribute to dissimilarity by this index. For reference, comparison of the first 200 sequences with the last 200 sequences of an ~400 sequence data set results in a Morisita-Horn index of about 0.9, which represents an experimentally

derived value for the maximum expected similarity, incorporating the inherent variability due to random sampling.

The abundance coverage estimator (ACE) was used to estimate the total number of unique species in the population based on the observed number of species in the data set as previously described [37].

To determine whether anatomic location affected TCR distribution, we performed generalized linear model testing using the raw counts of all experiments for each of the phenotypes. We utilized the Bonferroni correction (0.05 divided by the total number of sequences analyzed) to provide the most stringent p-value for rejection of the null hypothesis, that TCR distribution is unaffected by location.

Assessment of Peripheral Conversion in vivo

For non-lymphopenic hosts, 10^7 FACS purified Foxp3$^-$CD4$^+$CD45.2$^+$ T cells from the pooled spleen and lymph nodes of *Foxp3*^{gfp} mice were intravenously injected into congenic Thy1.1 hosts. One to two hundred Foxp3$^+$CD4$^+$CD45.1$^+$ T cells were also co-transferred to assess the contribution of Foxp3$^+$ contaminants in the Foxp3$^-$ population. Acquisition of Foxp3 and other markers by flow cytometry was assessed after 3-4 weeks.

For lymphopenic hosts, $2x10^6$ FACS purified Foxp3$^-$CD4$^+$ T cells from *Foxp3*^{gfp} or TCli TCR-β x *Foxp3*^{gfp} x TCRα$^{+/-}$ were intravenously injected into *Tcrb*$^{-/-}$ hosts, and analyzed for acquisition of Foxp3 and other markers by flow cytometry after 19-21 days. This time point was chosen to diminish the degree of immune activation following transfer of non-Treg cells into lymphopenic hosts seen at later time points, while earlier time points showed a lower frequency of Foxp3$^+$ cells (data not shown). We also verified

that the converted Foxp3$^+$ cells were unlikely to originate from the contaminating Foxp3$^+$ cells in the sorted population using "spike" experiments similar to that in Fig. 2.8 A (data not shown). The percentage of Foxp3$^+$ cells was somewhat higher when TCli TCRβ chain transgenic T cells were used as compared to non-TCR-β transgenic T cells, but the anatomic variation in the frequency of Foxp3$^+$ cells remained consistent (data not shown). This is likely due to the constraint on the TCR repertoire imposed by the fixed TCRβ chain, but should not affect the determination of whether individual TCRs are preferentially found in the converted versus expanded T cell populations.

Facilitation of peripheral conversion by retrovirally expressed TCRα chains

Peripheral T cells from TCli (Vα18, Vβ6) TCR-αβ transgenic x *Foxp3*^{gfp} x *Rag1*$^{-/-}$ mice were depleted by magnetic bead selection of CD25$^+$ cells (Miltenyi), and then retrovirally transduced on days 1 and 2 post activation as previously described [33]. After 7 days, a T cell population containing 4x10^5 transduced Vα2$^+$CD4$^+$ cells was injected intravenously into a *Tcrb*$^{-/-}$ host. Acquisition of Foxp3 on Vα2$^+$ cells was assessed at 2.5-3 weeks by flow cytometry.

Chapter Two: Results

Mapping the peripheral CD4$^+$ TCR repertoire

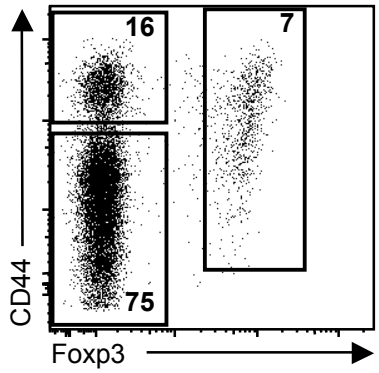
To determine the impact of anatomic location and cellular phenotype on the peripheral CD4$^+$ TCR repertoire, we isolated Treg (Foxp3$^+$), antigen-experienced (CD44^{hi}Foxp3$^-$), and naïve (CD44^{lo}Foxp3$^-$), CD4$^+$ cells from the spleen and cervical, axillary, inguinal, and mesenteric lymph nodes of TCli TCRβ transgenic x *Foxp3*^{gfp} x *Tcra*$^{+/-}$ mice (Fig. 2.1). We considered using CD62L as an additional putative memory cell marker, but found that only a minor subset of CD44^{hi} cells are CD62L^{hi} (data not shown). We obtained TRAV14 (Vα2) TCRα sequences as previously described [33] from four independent experiments; three experiments which each consisted of pooled cells from 3-5 mice, and a fourth experiment in which three mice were individually sequenced. As before, we utilized the CDR3 amino acid sequence as the primary identifier for a unique TCR sequence [33]. Approximately 6,000 sequences were obtained from each of the Treg, naïve, and memory cell populations, for a total of nearly 18,000 sequences.

The Treg TCR repertoire varies by anatomic location.

An important unresolved issue is the mechanism by which tissue-specific antigen presentation results in enhanced protection from autoimmunity, as it could derive from either an increased number or heightened potency of antigen-specific Treg cells. Analysis of TCRs from the various lymph nodes and the spleen revealed that the Treg TCR repertoire varied considerably according to the anatomic location. This could be observed both at the level of individual TCRs (Fig. 2.2, 2.3A), and by a statistical assessment of

Figure 2.1 Generation of the peripheral TCR database (A) FACS purification strategy. Representative flow cytometric plot of CD4+ splenocytes from TCli TCRβ transgenic x *Foxp3*^{gfp} x *Tcra*$^{+/-}$ mice illustrate the three sorted populations: CD44^{hi}, CD44^{lo}, and Foxp3^{+}. Numbers indicate percentages in the gates. (B) Sequence datasets from normal mice. The number of TRAV14 (Va2) TCRa chain sequences from T cell subsets at each location are shown. Experiment numbers 1–3 consisted of cells pooled from three to five mice, whereas experiment 4 consisted of three independently sequenced mice. Cells from axillary and inguinal LNs were not isolated in experiment 4, as indicated by asterisks. Axil, axillary; Cerv, cervical; Ing, inguinal; Mes, mesenteric.

A



B

			Lymph node				
Expt.	Phenotype	Spleen	Mes	Cerv	Axil	Ing	Total
1	Foxp3^{+}	322	298	329	285	357	1,591
	CD44^{hi}	295	330	246	305	291	1,467
	CD44^{lo}	315	306	307	335	274	1,537
2	Foxp3^{+}	330	298	301	333	304	1,566
	CD44^{hi}	268	321	321	321	298	1,529
	CD44^{lo}	333	338	344	344	333	1,692
3	Foxp3^{+}	283	361	284	298	280	1,506
	CD44^{hi}	283	276	365	311	377	1,612
	CD44^{lo}	278	306	278	330	453	1,645
4	Foxp3^{+}	146	161	179	*	*	486
mouse 1	CD44^{hi}	154	198	139	*	*	491
	CD44^{lo}	110	123	167	*	*	400
4	Foxp3^{+}	141	120	164	*	*	425
mouse 2	CD44^{hi}	136	143	116	*	*	395
	CD44^{lo}	113	120	115	*	*	348
4	Foxp3^{+}	122	153	124	*	*	399
mouse 3	CD44^{hi}	161	149	143	*	*	453
	CD44^{lo}	114	152	115	*	*	381
	Total:	3,870	4,124	4,003	2,959	2,967	17,923

Figure 2.1 Generation of the peripheral TCR database.

Figure 2.2 Analysis of the most frequent TCR from each phenotype/location. Using the pooled TCR dataset described in Fig 2.1, we chose the most frequent TCR of a given phenotype (shown at left) at each location (shown on top). To decrease the effect of mouse-to-mouse variability, we chose only TCRs that were found in at least two independent experiments. Because the naïve $CD44^{lo}$ TCRs were fairly uniform across all locations, the three most frequent TCRs irrespective of location are shown. Symbols represent the frequency (as a percentage of total sequences) of the TCR, denoted above by its CDR3 amino acid sequence, within the dataset of that phenotype and the location in each of four experiments.

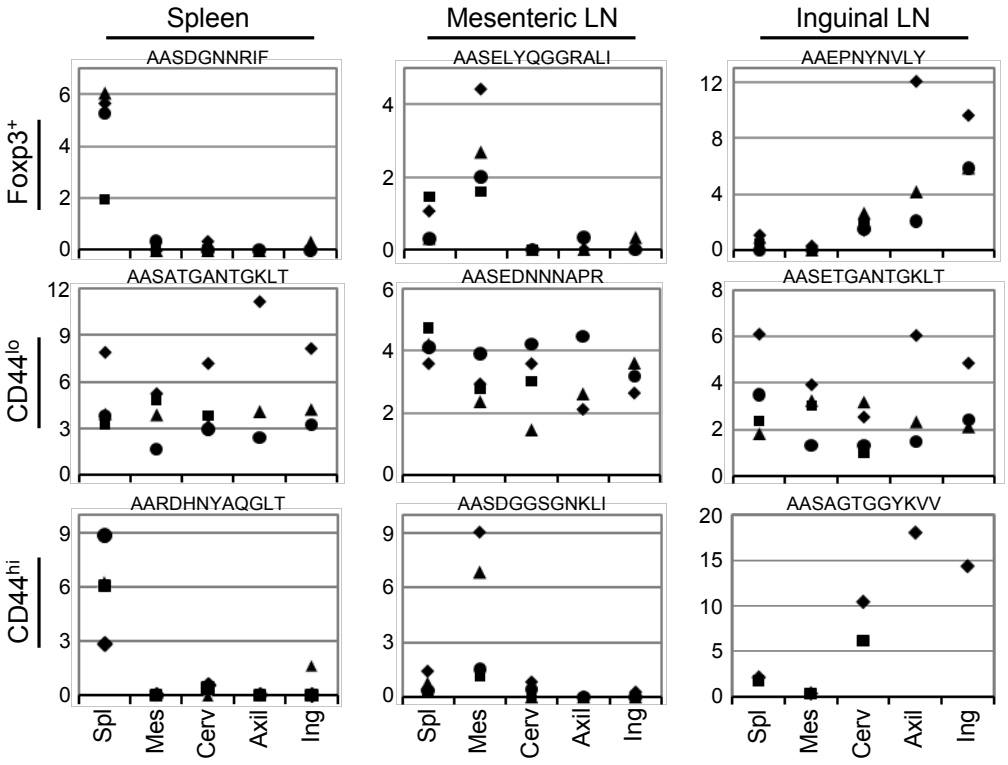


Figure 2.2 Analysis of the most frequent TCR from each phenotype/location

Figure 2.3 The natural Treg and memory CD44^{hi} TCR repertoires vary by anatomical location. (A) Anatomical distribution of the most abundant TCR sequences within each phenotype. Using the pooled TCR datasets from all four experiments, the top 50 TCR sequences by frequency within a given phenotype were selected. For each TCR, the relative frequency with which it is found at each anatomical location was calculated by dividing its frequency at each location by the sum of the frequencies at all locations. The frequency is represented on the heat map by decile, with the scale indicating the color at 100 (red), 75, 50, 25, and 0% (yellow) for a TCR at a given location. The data are clustered such that those TCRs predominantly found in one location are shown together. (B) Statistical analysis of similarity. The mean Morisita-Horn similarity index (±SD;n=3-4 independent experiments) is shown for the comparison betweenthe mesenteric (Mes) LNs versus the spleen, cervical (Cerv), axillary (Axil) and inguinal (Ing) LNs (left), and the inguinal LNs versus the spleen, Mes, Cerv, and Axil LNs (right).

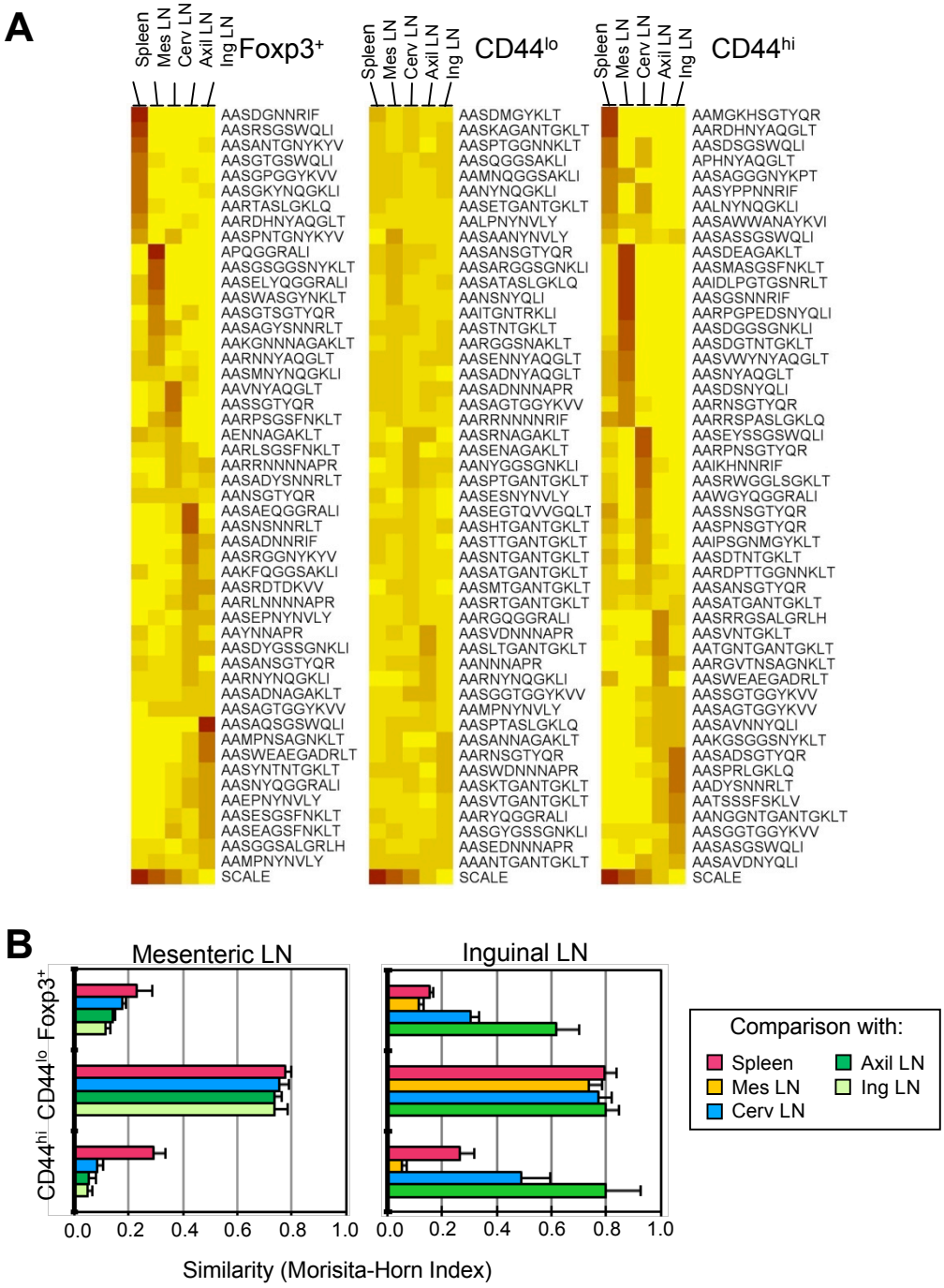


Figure 2.3 The natural Treg and memory CD44^{hi} TCR repertoires vary by anatomical location.

similarity between two populations, the Morisita-Horn index (Fig. 2.3B, see Materials and Methods). By contrast, naïve $CD44^{lo}$ T cells showed little skewing of their TCR repertoire by anatomic location, consistent with the notion that these cells circulate among the secondary lymphoid organs scanning for foreign antigens. Thus, these data provide direct evidence that the peripheral Treg cell population is shaped by local antigen presentation.

Interestingly, the $CD44^{hi}Foxp3^{-}$ TCR repertoire behaves much like the $Foxp3^{+}$ TCR repertoire with respect to repertoire changes based on location (Fig. 2.2, 2.3). For example, we observed that in both subsets the TCR usage in the mesenteric lymph nodes was very different from that in the other lymph nodes, whereas the inguinal and axillary lymph nodes were much more similar to each other (Fig. 2.3B). This shared pattern of TCR localization by the Treg and $CD44^{hi}$ subsets may reflect common recirculation patterns and spectra of antigens in these lymph nodes. For example, one might expect the axillary and inguinal lymph nodes to share a similar antigenic repertoire since they both drain the skin and limbs. We also found, using generalized linear model testing, that of the 15 most prevalent Treg and $CD44^{hi}$ TCRs, 10 Treg and 12 $CD44^{hi}$ TCRs showed a statistically significant level of skewing according to location ($p < 0.05$ with Bonferonni correction, see Materials and Methods), whereas only 1 of the top 15 naïve TCRs did so (Table 2.1). These data therefore suggest that the peripheral Treg population is shaped via a mechanism which is reminiscent of the generation and maintenance of memory cells.

Table 2.1 Distribution of Top TCR from Foxp3^{+}, CD44^{hi} and CD44^{lo} Populations

	CDR3 a.a. sequence	location p-value	Foxp3^{+} Spl	Mes	Crv	Axil	Ing	Foxp3^{-}CD44^{lo} Spl	Mes	Crv	Axil	Ing	Foxp3^{-}CD44^{hi} Spl	Mes	Crv	Axil	Ing	Number of Instances
Top 15 Foxp3^{+} TCRs	AAEPNYNVLY	<0.01	0.6	0.1	1.9	6.1	7.0					0.1						158
	AARPSGSFNKLT	<0.01	0.3	1.7	2.8	0.2							0.1					69
	AASDGNNRIF	<0.01	4.5	0.1	0.1		0.1											64
	AARTASLGKLQ	<0.01	3.0	0.7	0.4	0.3	0.4											63
	AASADYSNNRLT	0.92	0.7	0.4	1.8	0.8	1.6								0.3			67
	AARNYNQGKLI	0.84	0.7	0.9	0.6	1.9	1.5	0.2	0.1	0.5	0.4	0.5	0.2	0.1	0.1	0.4		89
	AASNYQGGRALI	<0.01	0.1	0.1	0.7	2.4	2.8			0.1								61
	AANSGTYQR	1.00	0.9	0.9	0.9	0.9	0.5		0.1		0.1		0.2		0.1	0.1	0.3	61
	AASELYQGGRALI	<0.01	0.8	2.7		0.1	0.1							0.1				51
	AASDYGSSGNKLI	<0.01	0.6	0.2	0.6	1.7	1.6				0.2			0.1				53
	AASGTGSWQLI	<0.01	2.4	0.6	0.1		0.3											45
	AASRGGNYKYV	<0.01	0.2	0.1	0.6	2.1	1.1											41
	AASYNTNTGKLT	0.04	0.3	0.3	0.5	0.9	1.7											39
	AASSGTYQR	0.05	0.2	0.5	1.7	0.2	0.2			0.1	0.1				0.1			41
	AARNNYAQGLT	1.00	0.8	1.3	0.1	0.5	0.2						0.1	0.6				48
Top 15 CD44^{hi} TCRs	AARGVTNSAGNKLT	<0.01				0.4					0.1		2.2	0.1	3.3	19.7	14.4	404
	AASAVNNYQLI	<0.01											1.1	0.2	4.5	6.0	5.6	187
	AARDHNYAQGLT	<0.01	1.3	0.2	0.1	0.4	0.2						6.4		0.3		0.5	120
	AAWGYQGGRALI	<0.01	0.1							0.1			1.9	0.3	3.5	0.5	0.2	85
	AAIPSGNMGYKLT	<0.01											1.9	0.3	2.9	1.0	0.6	82
	AASANSGTYQR	1.00	0.5	0.4	0.4	0.9	0.1	1.9	2.4	1.1	1.3	0.7	1.2	1.3	2.0	0.6	0.6	193
	AASDGGSGNKLI	<0.01		0.1									0.6	4.0	0.3		0.1	71
	AASAGTGGYKVV	<0.01	0.1	0.4	0.4	0.4	0.4	0.7	0.9	0.8	0.7	0.4	0.2	0.3	1.2	2.5	2.4	133
	AATSSSFSKLV	<0.01											0.2		0.1	2.5	4.0	66
	AASPRLGKLQ	<0.01					0.2		0.1				0.2		0.8	1.3	4.0	67
	AAIDLPGTGSNRLT	<0.01		0.1									0.5	3.7				62
	AASMASGSFNKLT	<0.01		0.1									0.5	3.5				57
	AASDTNTGKLT	1.00											1.3	0.5	1.7	0.2	0.3	52
	AARPGPEDSNYQLI	<0.01											0.5	3.0				49
	AARDPTTGGNNKLT	1.00						0.1					0.8	0.2	1.5	1.0	0.6	50
Top 15 CD44^{lo} TCRs	AASATGANTGKLT	<0.01	0.1	0.1		0.1	0.5	4.6	3.9	4.1	5.8	6.5	0.7	0.4	0.8	0.5	0.7	294
	AASEDNNNAPR	1.00		0.1	0.1	0.3	0.1	4.2	3.0	3.0	3.1	3.0		0.1		0.3	0.3	196
	AASETGANTGKLT	0.74		0.2	0.1		0.6	3.3	2.9	2.0	3.3	3.2	0.1	0.1	0.2	0.1	0.4	174
	AASVTGANTGKLT	0.07				0.2	0.1	1.7	2.5	2.8	2.9	3.4		0.2		0.3	0.3	158
	AASQGGSAKLI	1.00	0.1	0.1	0.2	0.1		3.2	2.5	3.2	1.9	1.5	0.1		0.2	0.3	0.1	153
	AASGYGSSGNKLI	1.00	0.1		0.1	0.4	0.6	2.3	2.9	2.3	1.9	2.3		0.2		0.1	0.3	142
	AAANTGANTGKLT	1.00		0.1	0.1	0.1	0.3	2.9	2.4	1.9	1.8	2.6		0.1	0.3		0.5	140
	AASRTGANTGKLT	1.00	0.1		0.1	0.2	1.1	1.8	2.0	2.3	2.1	3.1		0.1	0.3	0.1		134
	AASLTGANTGKLT	1.00		0.1	0.1			2.1	1.6	1.8	2.1	2.8		0.1		0.1		123
	AASGGTGGYKVV	1.00		0.1	0.1	0.3		1.5	1.6	2.7	2.0	1.4	0.3	0.4	0.4	0.3	1.1	112
	AARRNNNNRIF	1.00			0.1	0.1	0.3	1.3	1.6	1.6	1.8	1.9	0.2	0.1	0.2	0.2	0.3	97
	AARGQGGRALI	1.00		0.1			0.1	1.3	1.0	2.2	1.5	1.9		0.1		0.2	0.3	95
	AASKTGANTGKLT	0.87						1.4	1.1	1.2	2.1	2.0			0.1		0.1	91
	AASANSGTYQR	1.00	0.5	0.4	0.4	0.9	0.1	1.9	2.4	1.1	1.3	0.7	1.2	1.3	2.0	0.6	0.6	91
	AASADNNNAPR	1.00	0.1	0.1	0.1	0.2	0.5	1.7	1.9	1.1	1.2	1.6		0.2	0.2		0.1	90

Distribution of top 15 TCRs from each phenotype. The 15 most prevalent TCRα sequences found in the Treg, CD44^{hi}, and CD44^{lo} T cell populations in the data pooled from all four experiments are shown in order of decreasing counts within each phenotype. The number of instances is the total count for each TCR in the pooled dataset. Red TCR sequences showed statistically significant differences in their prevalence at different locations based on the generalized linear model testing described in Materials and methods. We considered the Bonferroni adjusted p-value (second column from the left) significant if it was <0.05. Of note, cell purification typically results in the contamination of minor populations (Treg and CD44^{hi} cells) by the major population (naive cells), with the reverse occurring to a much lower degree. A 95–98% purity of the Foxp3^{+} population generally means that 1 out of 20–50 cells are naive cells, which creates a small degree of false overlap.

The Treg and antigen-experienced CD44^{hi} TCR repertoires are distinct.

One possible explanation for the observed similarity in the pattern of localization between the Treg and CD44^{hi} TCR repertoires is that these cells may co-develop from common precursors. This would be consistent with the hypothesis that peripheral Treg cells normally develop during the course of an immune response to limit pathology [45], and the observation in TCR$\alpha\beta$ transgenic models that peripheral conversion only occurs in a portion of antigen-specific T cells after antigen administration [39, 41, 162]. Thus, it is quite possible that TCRs which select for development into the antigen-experienced CD44^{hi} phenotype may also be found within the Treg cell subset, due to peripheral conversion, and potentially account for the well-described overlap between the Treg and conventional non-Treg TCR repertoires [34, 35, 37].

However, analysis of the fractionated conventional T cell subset revealed that the prominent TCRs in the CD44^{hi} subset were not preferentially found in the Foxp3^{+} subset (Fig. 2.4, 2.5). As expected, the naïve TCR repertoire showed little similarity with either of the CD44^{hi} and Foxp3^{+} TCR repertoires, indicating differential requirements in TCR specificity for the development of these CD4^{+} T cell subsets. These differences between cellular phenotypes were also readily apparent in analyses of the TCRs present in each individual experiment, even though the TCR repertoires varied between experiments somewhat, particularly for the CD44^{hi} population (Fig. 2.6). Thus, in these unimmunized adult animals, we found little evidence to support the hypothesis that peripheral Foxp3^{+} T cell generation is a prevalent occurrence upon antigen encounter.

The strong difference between the TCR repertoires of these three phenotypes is further demonstrated by the cluster analysis of a matrix of Morisita-Horn similarity

Figure 2.4 Frequencies of the top TCR from the Foxp3^{+}, CD44^{hi}, and CD44^{lo} populations. The three most frequent TCRα sequences from each phenotype were selected, and their frequency within each phenotype in each experiment was plotted. Different symbols represent each of the four experiments. Each TCR tends to be prominent in only one of the three phenotypes, as would be expected from the overall dissimilarity.

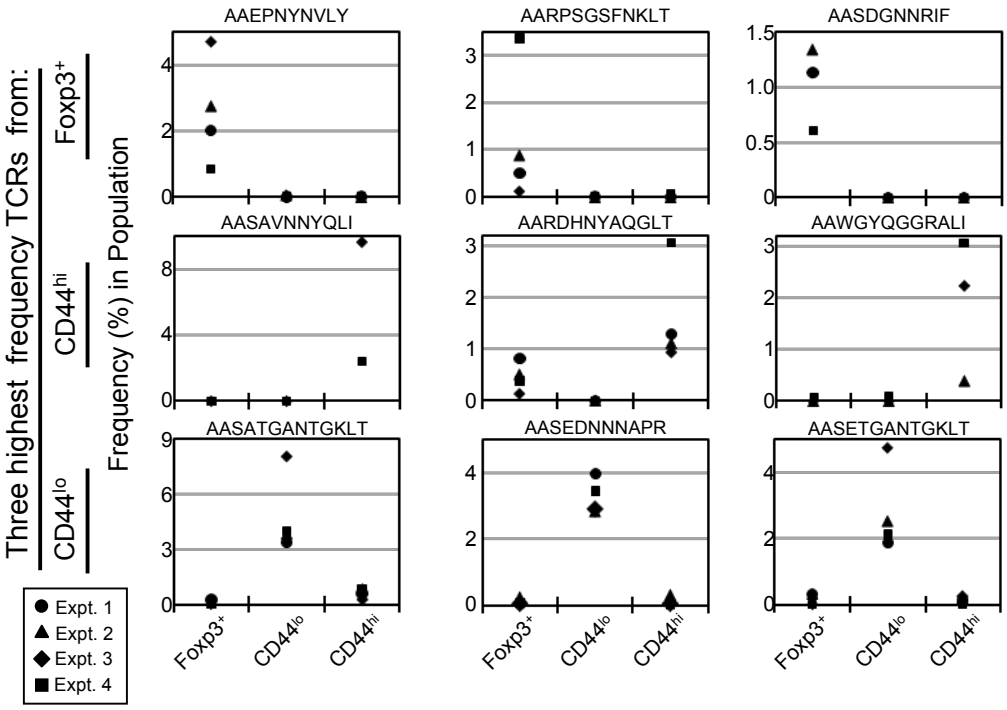


Figure 2.4 Frequencies of the top TCR from the Foxp3^{+}, CD44^{hi}, and CD44^{lo} populations.

Figure 2.5 The CD44^{hi}, CD44^{lo}, and Foxp3^{+} Treg repertoires show little overlap. Comparison of the frequencies of prevalent TCRs between the CD44hi and Foxp3+ or CD44lo datasets. In each graph, the 50 most abundant TCRs from each of the two indicated populations were selected and plotted on the x axis, with the frequency of the TCR in the indicated subset on the y axis. TCRs are arranged in order of decreasing frequency within the CD44^{hi} subset, and increasing frequency for the Foxp3^{+} (top) or CD44^{lo} (middle) subset, or decreasing frequency within the Foxp3^{+} subset and increasing frequency for the CD44^{lo} subset (bottom).

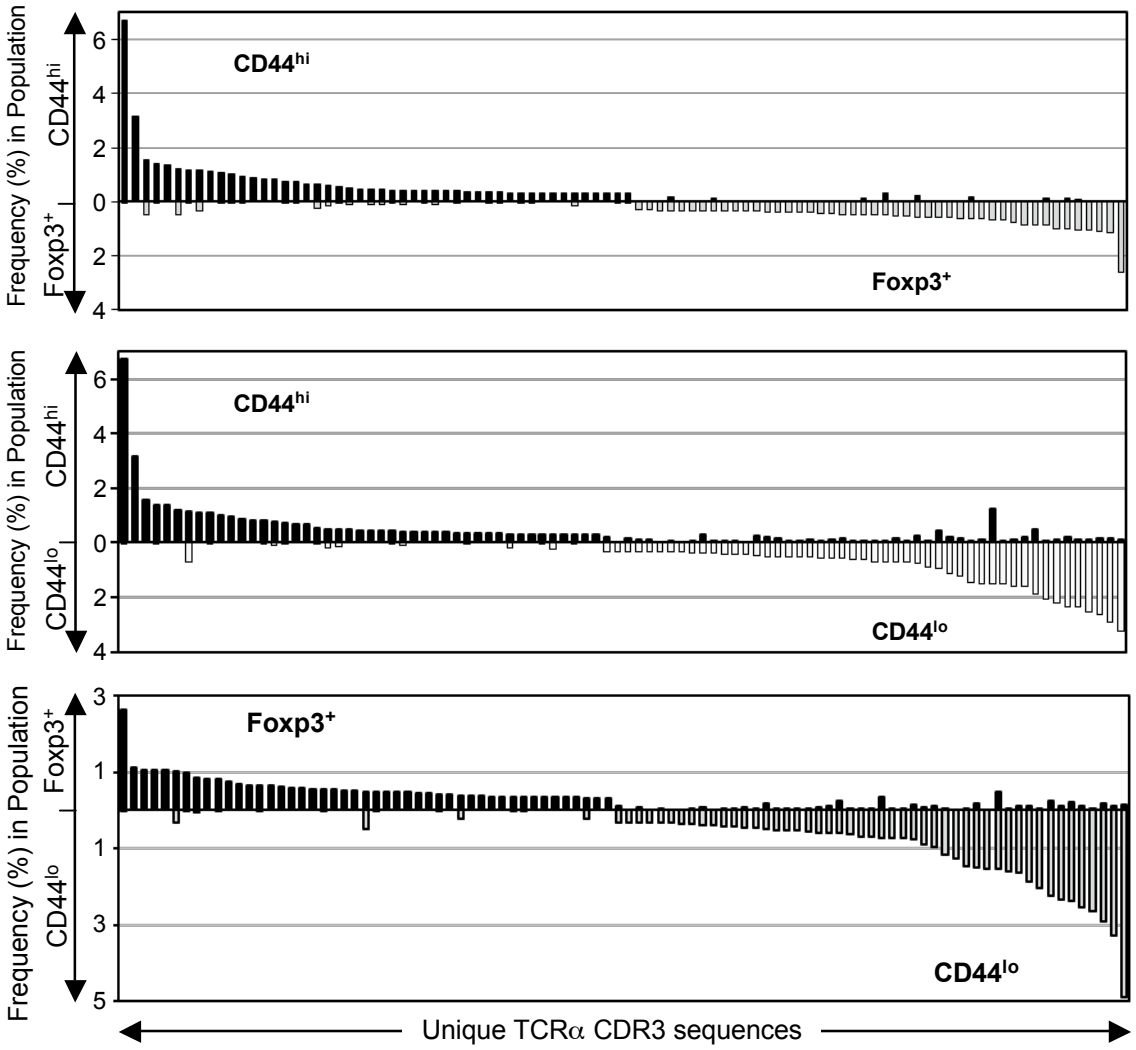


Figure 2.5 The CD44^{hi}, CD44^{lo}, and Foxp3^{+} Treg repertoires show little overlap.

Figure 2.6 Statistical analysis of similarity between different populations. (A) The Morisita-Horn similarity values between the indicated phenotypes within each experiment (left) and between populations with the same phenotype across different experiments (right). (B) Same comparisons as in A, using datasets obtained from three individual animals, showing mouse-to-mouse variability.

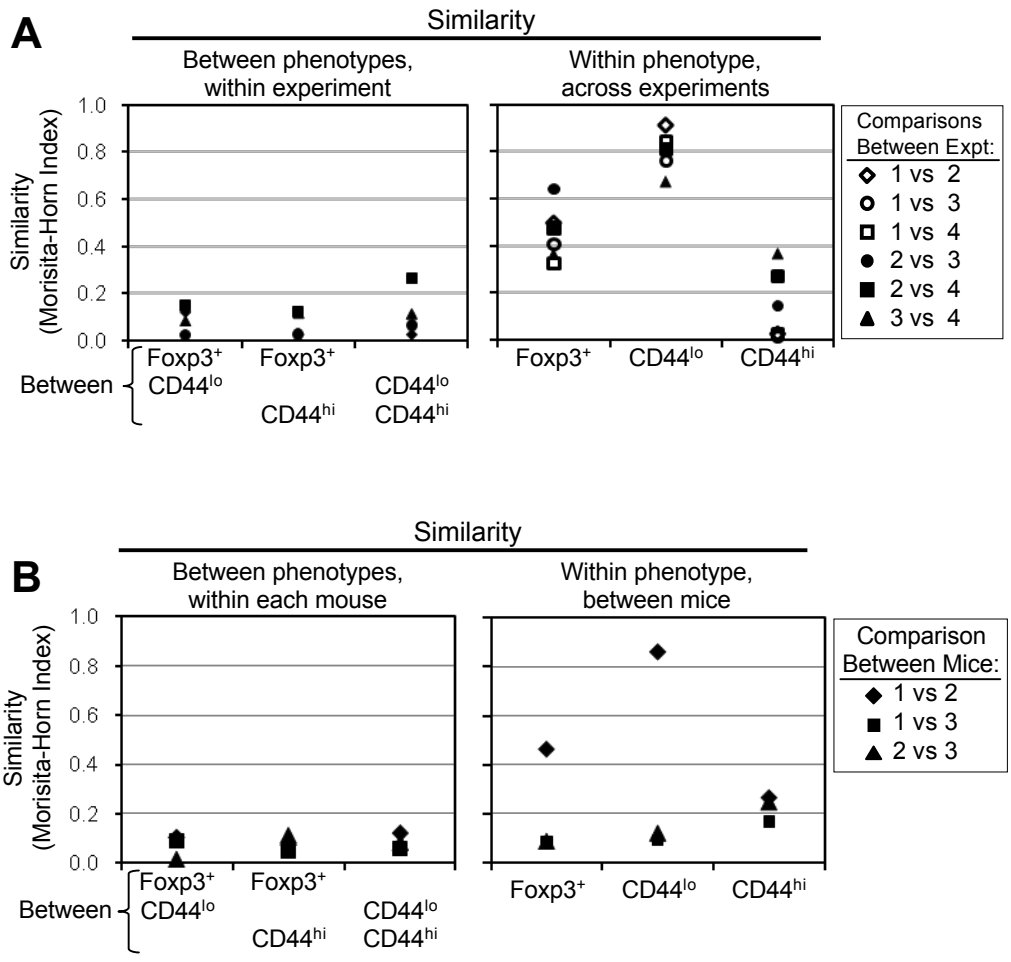


Figure 2.6 Statistical analysis of similarity between different populations**.**

indices, which includes comparisons based on both location and cell phenotype (Fig. 2.7). This plot reveals the dominant effect of the cell type over the location when all data are considered, as the calculated similarities cluster in 5x5 blocks according to cell phenotype first. This analysis also reaffirms the similarity in the naïve T cell repertoires between locations, as shown by the uniformly red 5x5 block representing the naïve subset repertoires, while the other blocks show relatively high similarity between the axillary and inguinal lymph nodes. Thus, these data demonstrate that even though Treg and $CD44^{hi}$ cells share a common behavioral feature at the population level, it is unlikely that they are generated via a common developmental pathway.

A minor role for peripheral conversion in generating the adult Treg cell population.

One explanation for the unexpected lack of similarity between the Treg and antigen-experienced subset is that peripheral conversion is an infrequent process in unimmunized adult animals. Current estimates of the magnitude of peripheral conversion in adult animals are quite divergent. One study suggested that many peripheral Treg cells could arise via conversion, as approximately 8% of $CD25^-$ T cells became $CD25^+$ after transfer into a congenic, non-lymphopenic host [43]. If one extrapolates the behavior of the adoptively transferred population in this study to represent that of the normal non-Treg cell subset at large, conversion would account for approximately 72% of peripheral Treg (see Materials and Methods). On the other hand, it has also been suggested that conversion occurs infrequently based on a study of monoclonal T cells reactive to an islet self-antigen [35].

Figure 2.7 Cluster analysis of the total peripheral TCR dataset. Data shown are a heatmap representation of the Morisita-Horn similarity indices calculated pairwise for all cell phenotype and location combinations using the pooled TCR dataset. Each decile is represented by distinct shades, with the scale indicating the color at 0, 0.5, and 1. The mean linkage algorithm was used to obtain a hierarchical cluster (or dendrogram; right) by sequentially grouping the two most correlated observations on the basis of a distance metric provided by the square root of 1 (Morisita-Horn index).

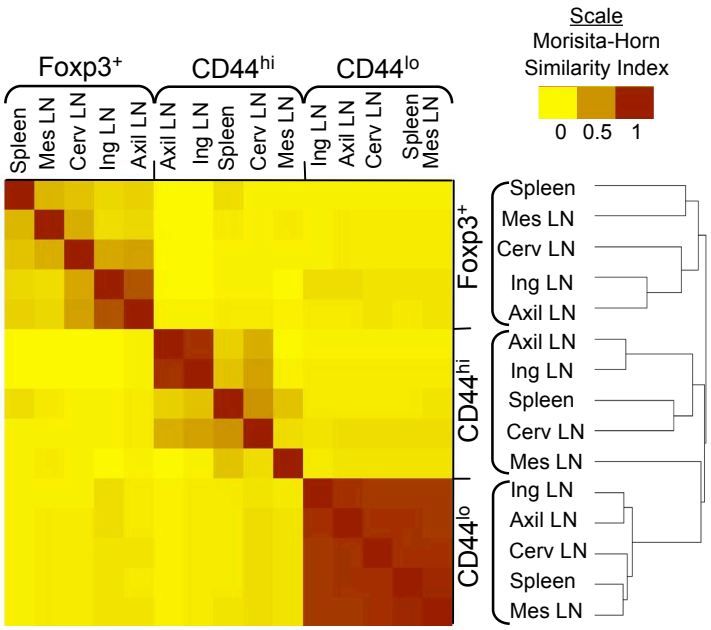


Figure 2.7 Cluster analysis of the total peripheral TCR dataset

To measure the potential for peripheral conversion in a polyclonal T cell population, we used fluorescence activated cell sorting (FACS) to obtain a highly pure population of Foxp3^{-}CD4^{+} donor T cells from *Foxp3*^{gfp} mice. We achieved a purity of > 99.998%, or less than 1 contaminating Foxp3^{+} cell per 50,000 Foxp3^{-} CD4^{+} cells (data not shown). Since Treg cells have been reported to divide at a faster rate than non-Treg cells [166], we also measured the projected expansion of these few contaminating natural Treg cells in this environment by adding approximately 0.001% (100-200 cells per 10^{7}) FACS-purified CD45.1^{+} Foxp3^{+} cells to the donor cell population. In order to detect sufficient numbers of donor cells by flow cytometry 3-4 weeks after intravenous transfer, we analyzed nearly the entire T cell population from the pooled lymph nodes and spleen of each recipient.

We found that the percentage of donor-derived cells that were Foxp3^{+} by three weeks after transfer was quite low–approximately 0.3% of the donor cells analyzed (Fig. 2.8A). Consistent with previous studies, these cells appeared phenotypically similar to natural Treg cells (CD25^{hi}GITR^{hi}, not depicted). As the contribution from contaminating Foxp3^{+} donor cells to the final Foxp3^{+} population was estimated to be about 2%, via analysis of the co-injected CD45.1^{+} cells, we believe that this frequency (0.3%) approximates the extent of conversion from peripheral Foxp3^{-} CD4^{+} T cells.

Recent studies have suggested that the gut environment may preferentially facilitate peripheral conversion [44, 124, 130]. We repeated the experiment described above to compare the frequency of converted Foxp3^{+} cells in the mesenteric LN, spleen, and the pooled cervical, axillary, and inguinal LN. In order to observe sufficient conversion events by flow cytometry, each experiment consisted of tissues pooled from three

Figure 2.8 Peripheral conversion in nonlymphopenic hosts is infrequent. (A) Adoptive transfer of peripheral Foxp3^{-}CD45.2^{+} (10^{7}) and Foxp3^{+}CD45.1^{+} (1-2 x 10^{2}) T cells were intravenously transferred into normal Thy1.1 hosts. After 3-4 wk, acquisition of Foxp3 was analyzed by flow cytometry. Pooled spleen and LN cells were gated on CD4^{+} and Thy1.2^{+} (donor cells; left) to determine the frequency of Foxp3^{+} cells (middle). Within the Foxp3+ cells, the outgrowth of the contaminating cells in the original CD45.2+Foxp3– population was estimated by the spike of Foxp3+CD45.1+ cells (right). Data shown are representative of three independent experiments (n=5 mice), and percentages are shown. (B) The frequency of conversion varies by anatomical location. The experiment was performed as in A, and after 4 wk the LNs or spleens from three animals were pooled for analysis. Data shown are representative of two independent experiments, and percentage are shown. (C) Summary of the flow cytometric data described in A and B. The frequency of Foxp3+ cells is shown. Each symbol represents data from an individual mouse ("pooled"; as in A) or an experiment in which cells from the organs of three mice were pooled (as in B) from the spleen, Mes LNs, and a pool of Cerv/Axil/Ing LNs.

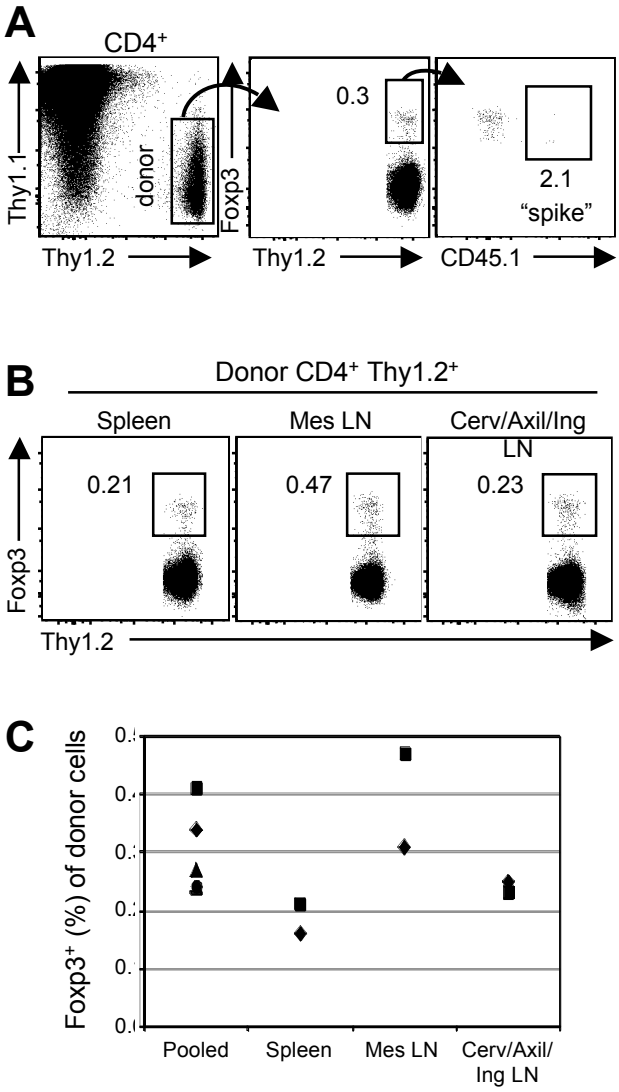


Figure 2.8 Peripheral conversion in nonlymphopenic hosts is infrequent.

individual mice which had received the same preparation of purified donor cells four weeks earlier. Consistent with a previous report [44], we found that the mesenteric LN, in comparison with the peripheral LN and spleen, contained an approximately two-fold higher percentage of converted $Foxp3^+$ cells (Fig. 2.8, B and C). However, converted $Foxp3^+$ cells were found in all locations, at frequencies ranging from 0.2-0.5%.

Because conversion appears to occur naturally in lymphoreplete animals, it was possible that this process would reduce the number of cells that can undergo conversion in the peripheral $Foxp3^-CD4^+$ T cell population used. We therefore assessed the frequency of conversion in immediate precursors to the peripheral T cell population, mature $CD4^+CD8^-$ (CD4 single-positive [SP]) thymocytes. We purified $CD62L^{hi}HSA^{lo}Foxp3^-CD25^-CD4SP$ thymocytes as well as $Foxp3^-CD4^+$ peripheral T cells to enable direct comparison between these populations. 3 wk after adoptive transfer into congenic hosts, we found that, on average, 0.5% of peripheral $CD4^+$ donor cells and 0.8% of thymocytes became $Foxp3^+$ (Fig. 2.9). If one extrapolates this behavior as representative of their respective T cell populations, then peripheral conversion would account for the development of 4-7% of peripheral $CD4^+Foxp3^+$ T cells in a normal mouse (see Materials and methods). Although this is a rough estimate, it suggests that peripheral conversion plays a relatively small role in generating the protective Treg cell population in adult individuals.

Peripheral conversion in lymphopenic hosts is TCR-specific.

The low rate of peripheral conversion in an adult lymphoreplete animal may be caused, in part, to inhibition by the existing Treg cell population. We hypothesized that

Figure 2.9 Peripheral conversion of CD4 SP thymocytes vs. $CD4^+$ peripheral T cells. Mature $HSA^{lo}CD62L^{hi}Foxp3^-CD25^-$ CD4 SP thymocytes or $Foxp3^-CD4^+$ peripheral T cells were adoptively transferred into congenic hosts. The frequency of $Foxp3^+$ cells in pooled spleen and LN preparations was assessed at 3 wk by flow cytometry. Each symbol represents data from an individual recipient from two experiments in which 3.4×10^6 (closed circles) or 9×10^6 (open circles) cells were injected per mouse. The horizontal bars represent the mean values.

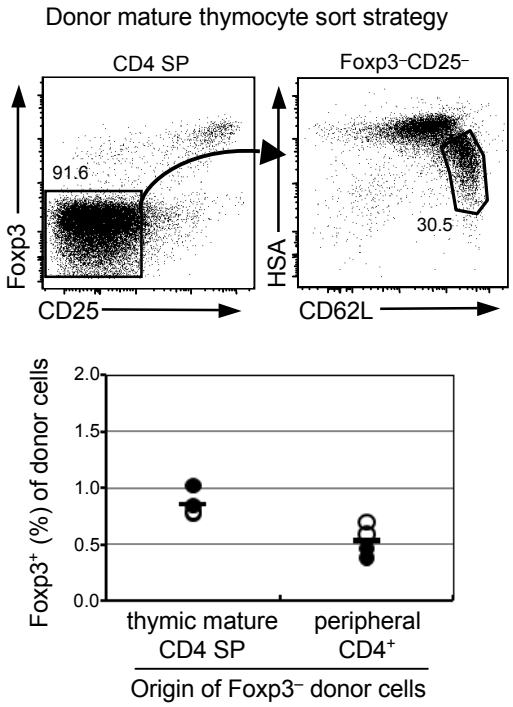


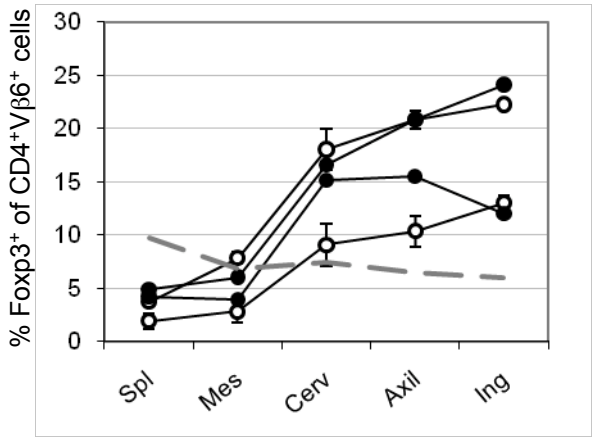
Figure 2.9 Peripheral conversion of CD4 SP thymocytes vs. $CD4^+$ peripheral T cells.

peripheral conversion may play a greater role in a lymphopenic environment, such as that seen in the early ontogeny of the peripheral immune system, which is both lymphopenic [167, 168] and Treg cell deficient due to delayed thymic Treg cell export [32, 169]. Therefore, we used the adoptive transfer of $CD4^+Foxp3^-$ T cells from mice containing the fixed TCRβ chain into lymphopenic hosts as a model of peripheral conversion, which has previously been reported to occur upon the transfer of non-Treg cells into lymphopenic hosts [42, 43].

Consistent with previously published results, we observed peripheral conversion of the highly purified $CD4^+Foxp3^-$ T cells from TCli TCRβ chain transgenic mice 2.5-3.0 weeks after intravenous transfer into *Tcrb*$^{-/-}$ αβ-T cell deficient mice (Fig. 2.10A). The converted cells were phenotypically similar to Treg cells, expressing high levels of CD103, GITR, and CD25 (data not shown). Since the incidence of Foxp3 induction versus subsequent expansion cannot be discriminated, the extent of "peripheral conversion" in this system represents the aggregate outcome. Of note, the percentage of converted $Foxp3^+$ cells was considerably higher after adoptive transfer into lymphopenic (Fig. 2.10A), as compared with non-lymphopenic (Fig. 2.8), hosts, potentially due to the empty Treg cell niche available in *Tcrb*$^{-/-}$ mice. A second difference between the results from these two hosts was the relative frequencies of $Foxp3^+$ cells recovered from the various lymph nodes. Lymphopenic hosts showed a decreased frequency of $Foxp3^+$ cells in the mesenteric relative to other lymph nodes, which may reflect the preferential accumulation of effector cells, as the transfer of $CD4^+$ T cells depleted of Treg is known to result in colitis [170].

Figure 2.10 Peripheral conversion resulting from transfer of Foxp3$^-$CD4$^+$ T cells into lymphopenic hosts. (A) The frequencies of Foxp3$^+$ cells arising from the transferred Foxp3$^-$ T cells are summarized, with each line representing an independent experiment. The open circles represent the mean values (±SEM) from three mice within an independent experiment; the closed circles are values from experiments that consisted of pooled cells. The dashed line is the percentage of Foxp3$^+$ cells found in a normal animal (for comparison). (B) Description of TCRα chain sequence datasets obtained from cells sorted 2.5 wk after adoptive transfer of Foxp3$^-$ cells into *Tcrb*$^{-/-}$ hosts. The total number of sequences by experiment, location (Crv, cervical; Mes, mesenteric; Spl, spleen) and phenotype are shown.

A



B Number of TCR-α sequences obtained after lymphopenia-induced proliferation and conversion

			Lymph node		
Expt.	Phenotype	Spleen	Mes	Crv	Total
1, mouse 1	Foxp3+	159	171	194	524
	Foxp3–	151	147	172	470
1, mouse 2	Foxp3+	162	150	185	497
	Foxp3–	184	180	209	573
1, mouse 3	Foxp3+	176	123	175	474
	Foxp3–	162	170	166	498
1, mouse 4	Foxp3+	184	174	174	532
	Foxp3–	155	200	174	529
2, mouse 1	Foxp3+	404	413	159	976
	Foxp3–	201	154	193	548
2, mouse 1	Foxp3+	367	332	215	914
	Foxp3–	150	248	190	588
					7,123

Figure 2.10 Peripheral conversion resulting from transfer of Foxp3^{-}CD4^{+} T cells into lymphopenic hosts.

Initial experiments analyzing TRAV14 TCRα sequences obtained from FACS-purified Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells pooled from several recipient *Tcrb*$^{-/-}$ mice hinted at a fair degree of mouse-to-mouse variability (data not shown), similar to that seen in the CD44^{hi} T cell subset in normal mice (Fig. 2.6B). We therefore generated a database of over 7,000 Foxp3$^+$ and Foxp3$^-$ TCR sequences from the cervical lymph nodes, mesenteric lymph nodes, and spleens of 6 individual recipient mice from two independent experiments (Fig. 2.10B, Table 2.2).

Based on studies of monoclonal TCR populations showing that the majority of the antigen-specific T cells remain Foxp3$^-$ during peripheral conversion [39, 41, 162], we were surprised to find relatively little overlap in TCR usage between the converted and non-converted T cell subsets (Fig. 2.11A). Most of the prevalent TCRs identified in these experiments showed a marked skewing towards either the Foxp3$^+$ (converted) or Foxp3$^-$ (non-converted) populations, with only a minority (9 of 85) of the TCRs being found more or less equally in both populations **(**Fig 2.11B). We observed considerable differences in the TCR repertoires between the individual mice, even though the donor cell population was identical within each experiment (Fig. 2.11C right), which was suggestive of the preferential expansion of cells with a low precursor frequency. However, the level of dissimilarity between the Foxp3$^+$ and Foxp3$^-$ cells from each animal was equivalent **(**Fig. 2.11C left). Unlike the repertoires in the normal mice (Fig 2.2, 2.3) TCRs in these experiments did not display a skewing of TCR usage according to location (Table 2.3), possibly due to the empty T cell nice in these lymphopenic animals. Although most TCRs were not found in all six individual mice, TCRs recovered from multiple animals were reproducibly found in either the converted, or nonconverted

Table 2.2 Comparison of the normal dataset with TCRs found after adoptive transfer of Foxp3⁻ cells into T cell-deficient hosts.

		Post-transfer		Normal peripheral TCRs			Thymic data set	
TCRα CDR3 Sequence	Conv %	Foxp3+	Foxp3−	Foxp3+	CD44lo	CD44hi	Foxp3+	Foxp3−
AASDYGSSGNKLI	100	263		50	2	1	4	1
AASRPSNNRIF	100	192		2				
AAKGNTGGLSGKLT	100	187						
AARTNYAQGLT	100	185						
AASASGSFNKLT	100	96		4	1	1		1
AASTGTYQR	100	55		4				
AASKDNYAQGLT	100	51						
AARDYGGSGNKLI	100	43						
AARGTNAYKVI	100	37		10			3	5
AASPSGTGSNRLT	100	32						
AAPYQGGRALI	100	30						
AASDGTYQR	100	21		5			4	
AASAPSNNRIF	100	20		1				
AASGGNYAQGLT	100	127		1	4	2	2	1
AAKGNTGGLSDIQN	100	26						
AASAGTYQR	97.6	50	1	16		1	3	1
AATGNYKYV	97.3	132	3	3	10		1	3
AASLSGSFNKLT	97.2	86	2	12	10		1	1
AARHNTNTGKLT	96.6	176	5					
AASSAQVVGQLT	96.3	64	2					
AASWAQVVGQLT	95.8	112	4			1		
AASADAQGLT	95.8	28	1	2	1			
AASPAGTGSNRLT	95.7	27	1					
AASSGTYQR	95.3	25	1	38	2	1	3	
AASAGNYAQGLT	95.1	47	2	5	8			4
AASEDAQGLT	93.9	56	3		1			
AASGSQVVGQLT	93.3	34	2					
AASADNNRIF	93.1	33	2	29	2			
AASEYNYGNEKIT	88.9	205	21					
AASDEGNYKYV	87.6	26	3	1	2			
AASANSGTYQR	87.3	278	33	28	91	74	3	23
AARNYNQGKLI	85.5	180	25	61	19	9	19	13
AASGETGGLSGKLT	85.5	36	5	2				
AASADNAGAKLT	85.4	50	7	21	2	8		1
AASSNSGTYQR	85.1	21	3	7	8	29	1	4
AASEENYNQGKLI	68.9	92	34	2				
AAMGNMGYKLT	66.0	19	8			2		1
AANYNQGKLI	59.2	62	35	7	19	13		3
AASWGNNKLT	57.1	13	8					
AANSNSAGNKLT	43.9	22	23					
AASGYGSSGNKLI	32.7	32	54	13	142	7		2
AASTNTGKLT	30.6	7	13	4	23	17		
AASAGNSNNRIF	26.2	10	23					
AASGAQVVGQLT	23.2	7	19	13				1

		Post-transfer		Normal peripheral TCRs			Thymic data set	
TCRα CDR3 Sequence	Conv %	Foxp3+	Foxp3−	Foxp3+	CD44lo	CD44hi	Foxp3+	Foxp3−
AATSNNNNAPR	20.0	18	59	3		4		1
AASWSNNRLT	18.5	5	18		1			
AASGNNAGAKLT	17.6	6	23		7			1
AASARNYAQGLT	16.0	10	43					
AAYNNAPR	14.8	7	33	33	1	14		
AASPRNSGGSNYKLT	14.3	13	64		3	12		
AASGGNYKPT	11.4	3	19	1	5	1		2
AGNYGSSGNKLI	10.3	6	43	2	11	2		
AASEDNNNAPR	9.6	3	23	7	196	7		13
AASASQVVGQLT	9.3	4	32	1				
AASWVVGQLT	7.2	2	21					
AASGSGSWQLI	6.0	11	141	15		2	3	3
AASDETGNTGKLI	5.9	10	130	11	1	1		
AASVNTGKLT	4.9	4	63	14		39	1	
AASQGGSAKLI	4.6	1	17	6	153	7		4
AASASGNTGKLI	3.9	2	40					
AASDPGYNKLT	3.9	1	20		1	6		
AASENNYAQGLT	3.9	5	101	4	53	5		5
AASPTASLGKLQ	3.6	1	22	2	22	1		4
AASRNSNYQLI	3.5	7	159	1		2		
AASGGTGGYKVV	2.7	1	29	6	112	28		14
AASRSNYAQGLT	1.1	2	154	2				
AASAYRAGNKLT	0.0		61					
AASAGGGNYKPT	0.0		41		4	19		
AASVDNYAQGLT	0.0		40		5	6		2
AASARATGGLSGKLT	0.0		38					
AASAHHTGGLSGKLT	0.0		38					
AANNYAQGLT	0.0		31	2		9		
AASEGGSNAKLT	0.0		31					
AASGPSFSGSWQLI	0.0		30					
AASRNTGKLI	0.0		25	1	4	12		1
AASEPGYNKLT	0.0		22	2		1		
AASADNYAQGLT	0.0		21	9	46	14	3	2
AASTSNNNAPR	0.0		21	2		15		
AASIDNYAQGLT	0.0		20		3			
AASKNSNYQLI	0.0		20		3	3		
AAMNQGGSAKLI	0.0		19	2	42	1		3
AAGDTNAYKVI	0.0		19		5	2		3
AANANNNAGAKLT	0.0		18					
AAMRQGGRALI	0.0		18					
AASQDQVVGQLT	0.0		16					
Sum:		**3417**	**2126**	**467**	**1025**	**379**	**51**	**123**
Total in data set:		**3917**	**3206**	**5973**	**6003**	**5947**	**955**	**1149**

TCRs isolated after adoptive transfer are listed by their ability to facilitate peripheral conversion (Conv %) as in Fig 2.11 B. Rare TCRs found at < 0.5% in the posttransfer Foxp3⁺ and Foxp3⁻ subsets were excluded. The number of times the TCR was found in the posttransfer and normal peripheral and thymic TCR datasets is shown. These more abundant TCRs found in our peripherally converted dataset (Conv > 80%) account for ~5% (302 out of 5,973) of the total sequences in the normal Treg TCR repertoire. However, the appearance of a number of these TCRs in the thymic Treg cell subset suggests that the actual contribution of peripheral conversion may be considerably lower under normal conditions.

Figure 2.11 TCR specificity is important for peripheral conversion in lymphopenic hosts. (A) The converted and nonconverted TCR repertoires differ. TCRα chain sequences were obtained as described in Materials and methods. The 50 most abundant TCRs from each of the converted Foxp3$^+$ and non-converted Foxp3– pooled datasets are shown, with the frequency in the indicated subset on the y axis. The TCRs are ordered from the most abundant in the Foxp3$^+$ subset (left) to the most abundant in the Foxp3$^-$ subset (right). (B) TCRs are preferentially skewed toward either the Foxp3$^+$ or Foxp3$^-$ subset. For prevalent TCRs in the pooled dataset (summed frequencies >0.5%), the percentage of each TCR in the Foxp3$^+$ subset (% Foxp3$^+$/% Foxp3$^+$ + Foxp3$^-$) is plotted in decreasing order. Arbitrary cutoffs (vertical dashed lines) are shown for TCRs highly (> 80% Foxp3$^+$), poorly (< 20%), or not (< 1%) associated with conversion. (C) Assessment of mouse-to-mouse variability. (left) Morisita-Horn index values comparing the Foxp3$^+$ and Foxp3$^-$ TCR datasets within each individual recipient are shown. (right) The Foxp3$^+$ TCR datasets from individual animals within the same experiment are compared in green, and the Foxp3$^-$ datasets are compared in orange. There were four and two recipients for the first and second independent experiments, respectively.

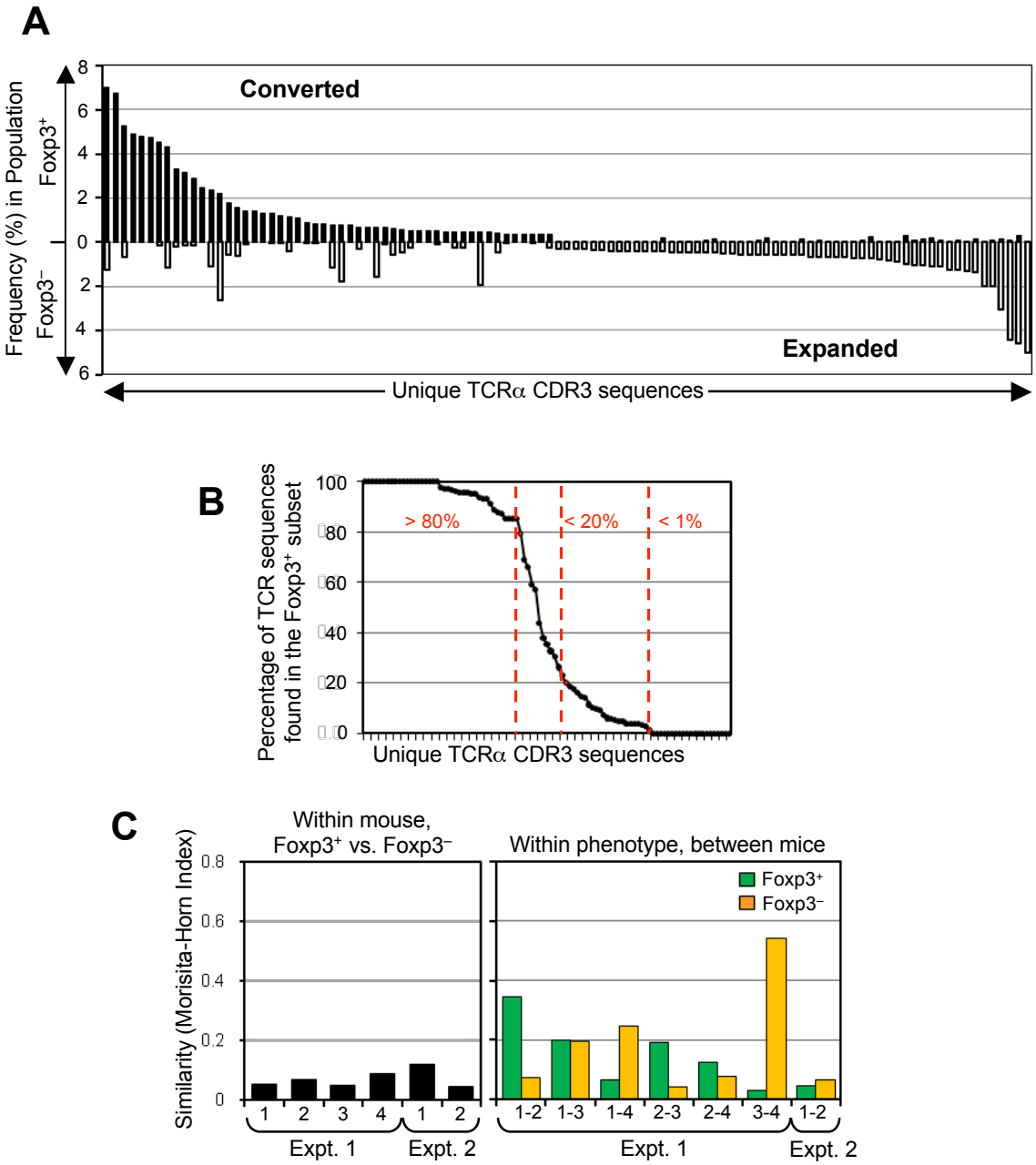


Figure 2.11 TCR specificity is important for peripheral conversion in lymphopenic hosts.

Table 2.3 TCRα distribution after lymphopenia-induced proliferation and conversion

			Foxp3+												Foxp3−											
			mouse 1			mouse 2			mouse 3			mouse 4			mouse 1			mouse 2			mouse 3			mouse 4		
	CDR3 a.a. sequence	Precursor	Spl	Mes	Crv	Spl	Mes	Crv	Spl	Mes	Crv	Spl	Mes	Crv	Spl	Mes	Crv	Spl	Mes	Crv	Spl	Mes	Crv	Spl	Mes	Crv
Top 14 Foxp3+ TCRs	AASANSGTYQR	1.1	7.5	12.3	5.2	46.9	13.3	8.1	2.8	2.4	0.6	9.2	6.3	2.9	2.0	0.7	0.6		2.2	0.5		0.6		3.2	0.5	1.7
	AARNYNQGKLI*	0.2	2.5	4.7	15.5	3.7	19.3	30.8	6.3	8.1	8.6	1.6	2.3		0.7		0.6			0.5		1.2	4.8	0.6	1.0	5.2
	AARHNTNTGKLT											25.5	27.0	47.1										0.6		1.7
	AASWAQVVGQLT					11.1	25.3	30.3																	2.0	
	AASASGSFNKLT		18.9	15.2	20.1	0.6																				
	AASEENYNQGKLI											17.9	20.1	13.8				1.6						2.6	0.5	1.7
	AATGNYKYV	0.2	3.8	6.4	2.6	8.6	9.3	4.3	2.3	6.5	0.6	1.6	7.5	0.6				0.5			0.6	0.6				
	AASLSGSFNKLT	0.2		1.8	3.6	0.6			9.1	22.8	17.7							0.5					0.6			
	AASSAQVVGQLT								18.8	8.1	12.0							1.1								
	AASTGTYQR											20.7	7.5	2.3												
	AASADNAGAKLT	0.2	5.7	5.3	2.6				2.8	4.1	2.3							0.5	0.6							2.3
	AARGTNAYKVI								5.7	8.1	9.7															
	AASGETGGLSGKLT								7.4	3.3	10.9							0.5			0.6	0.6	1.2			
	AASPSGTGSNRLT		11.9	2.9	4.1																					
Top 14 Foxp3− TCRs	AASRNSNYQLI											3.3			0.7	1.4	2.9	0.5		0.5	48.8	10.0	7.8	15.5	2.0	6.3
	AASRSNYAQGLT					0.6		0.5							0.7			34.2	23.9	22.5						
	AASDETGNTGKLI								3.4		1.7				0.7		15.7	0.5		10.5			3.0			5.7
	AASAYRAGNKLT														10.6	0.7	25.6									
	AATSNNNNAPR					3.7		2.2	0.6		0.6	1.1	0.6	1.7				1.1		23.9		0.6	1.8			1.7
	AASGYGSSGNKLI*	4.5	1.3			7.4	7.3	2.2	1.1					0.6	2.0	0.7	2.3	3.3	1.7	5.7	0.6		1.2	1.9	3.5	5.2
	AASPRNSGGSNYKLT		6.3	0.6	0.5				0.6						5.3	1.4	1.7	0.5			8.6	2.4	4.8	1.3		
	AASASGNTGKLI	0.2		0.6									0.6		2.0	4.1		0.5	1.7		1.2	1.8		5.2	7.0	
	AASAGGGNYKPT														9.9	10.9	0.6				0.6	1.8	0.6	0.6	1.0	
	AASARATGGLSGKLT														1.3	6.8	0.6				2.5	5.3			6.0	
	AASAHHTGGLSGKLT																				3.7	9.4		0.6	7.5	
	AASASQVVGQLT					0.6	2.0											0.5	17.2							
	AGNYGSSGNKLI	0.7													0.7									10.3	2.0	6.3
	AASGGTGGYKVV	1.9															0.6		0.6		0.6	9.4	3.6	1.3		1.1

The 14 most prevalent TCR sequences pooled from the four mice in experiment 1 are listed. The 15th most frequent TCR in the Foxp3+ and Foxp3− TCRs are each also found in the opposite dataset, as indicated by asterisks. We did not observe a strong skewing of Treg TCR usage based on anatomical location in these mice, unlike normal mice (Fig. 2.3), possibly as a result of the empty Treg cell niche in lymphopenic animals. Precursor frequency (%) is calculated using 534 TCRα sequences from the sorted CD4+Foxp3− input cell population (note that 0.2% represents a TCR found once in this dataset).

subsets, with few exceptions (Fig. 2.12). Thus, even though both peripheral conversion and lymphopenia-induced proliferation of non-Treg cells occurred within the same animal, these data demonstrate that TCR specificity played an important role in Treg cell fate determination during this immune response.

TCRs that facilitate peripheral conversion are also found within the normal peripheral and thymic Treg TCR repertoire.

Although we were unable to determine which TCRs undergo peripheral conversion in normal (non-lymphopenic) hosts due to the low rate of conversion, we did identify TCRs that facilitate peripheral conversion during the immune response in lymphopenic hosts. As would be expected, these converted TCRs are found in the normal non-Treg cell subsets, since the donor cells for these studies were normal Foxp3^{-} cells (Fig 2.13A). Interestingly, these TCRs were also often found in the normal Treg cell subset, and constitute approximately 5% of the sequences in the normal peripheral Treg cell data set (Table 2.2). Although the extent that these TCRs affect the Treg TCR repertoire in lymphoreplete individuals by peripheral conversion is unknown, these data support our observation that peripheral conversion makes only a small contribution to the normal Treg cell population.

It has been proposed that peripheral conversion may play an important role in generating Treg cells to antigens which are not present in the thymus, such as those found in the gut [44, 124, 130]. This would predict, then, that converted TCRs should generally be found within the peripheral, but not thymic, Treg cell subset. We found that, of the highly converting TCRs that were also present in our thymic sequence data set [164],

Figure 2.12 Reproducibility of TCR-dependent conversion. Foxp3^{-}CD4^{+} cells were transferred into αβ T cell–deficient hosts and recovered 2.5 wk later for TCR sequencing (Figs. 2.10, 2.11). TCRs that were identified at a frequency of >1% in two or more recipients were selected, and the frequencies of the indicated TCR in each of the six individual mice are shown. The frequencies within the Foxp3^{+} (converted) population (green) are shown above the axis, and the frequencies within the Foxp3^{-} (nonconverted) population (orange) are shown below the axis. Ms, mouse.

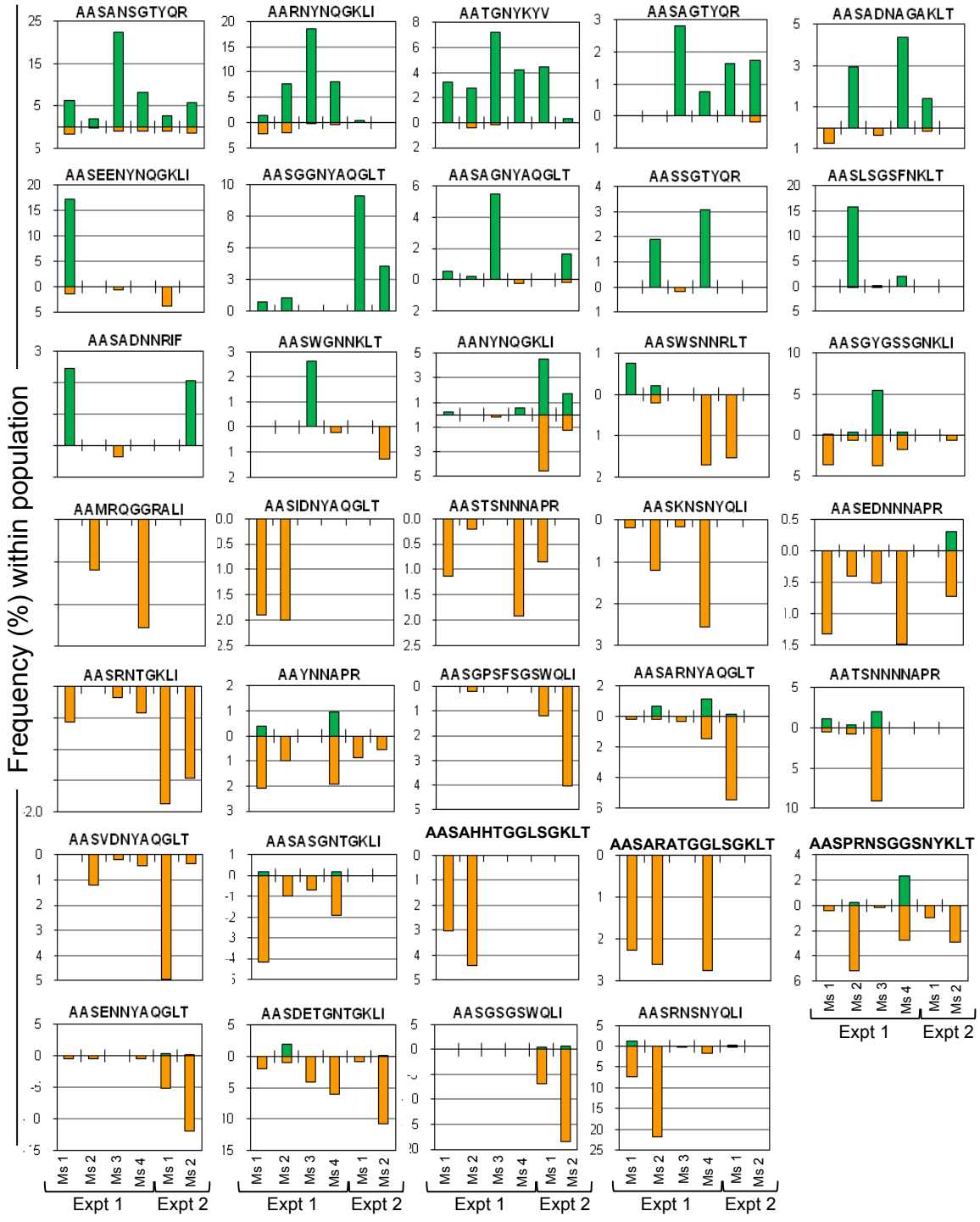


Figure 2.12 Reproducibility of TCR-dependent conversion.

Figure 2.13 TCRs that facilitate peripheral conversion are found in the normal peripheral and thymic Treg cell TCR repertoires. (A) Comparison with the normal peripheral TCR repertoire. Prevalent TCRs recovered after the transfer of Foxp3– cells into lymphopenic hosts that are also found in the normal peripheral dataset (Fig 2.1) at a summed frequency of > 0.15% were identified (Table 2.3). The frequencies of these overlapping TCRs in the normal dataset are plotted. The TCRs are sorted by their ability to facilitate conversion, as designated by the vertical dashed lines on Fig. 2.12. (B) Comparison with the normal thymic TCR repertoire. The frequency of the TCR in the normal thymic dataset [164] is shown as in A.

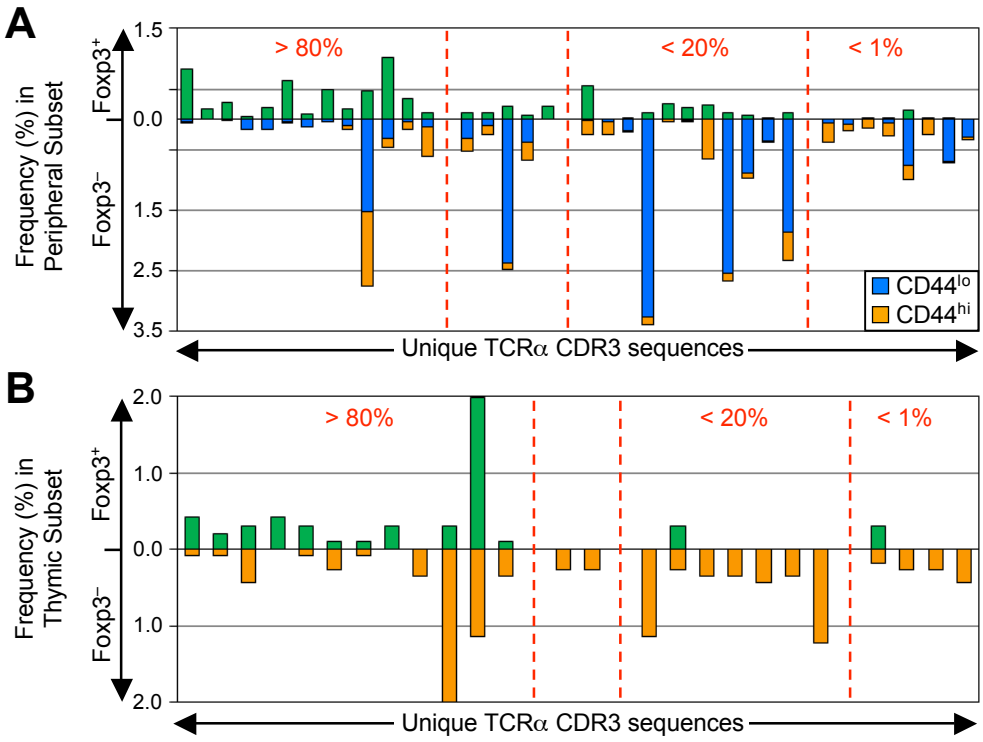


Figure 2.13 TCRs that facilitate peripheral conversion are found in the normal peripheral and thymic Treg cell TCR repertoires.

most (11 of 12) could be found within the Foxp3$^+$ subset (Fig. 2.13B). By comparison, only 2 of 11 TCRs from the poorly converting category were found within the thymic Foxp3$^+$ data set. One potential explanation for this observation is the presence of recirculating mature peripheral CD4$^+$Foxp3$^+$ T cells in the thymus. Although the frequency of recirculating T cells appears low by previous reports [171, 172], a direct assessment of Foxp3$^+$ Treg cell circulation in normal hosts was not performed. To test this possibility, we injected 20 x 10^6 enriched peripheral CD4$^+$ T cells into a congenic host and assessed the percentage of donor cells in the CD4SP thymocyte population after 1 week (Fig. 2.14). If one assumes that the behavior of the donor cells is representative of all peripheral CD4$^+$ T cells, then about 4% of thymic Treg cells arise from recirculation. Because these shared TCRs are not grossly overrepresented in the periphery compared with the thymus, recirculation alone is unlikely to account for the thymic observation of TCRs that facilitate peripheral conversion. Rather, we favor the hypothesis that some TCRs are able to facilitate both thymic and peripheral Treg development. Because we expect primarily self-antigens to be presented in a normal thymus, these data suggest that some self-reactive cells, upon escaping Treg cells selection in the thymus, may get a "second chance" to become Foxp3$^+$ regulatory cells via peripheral conversion.

TCR specificity plays a crucial role in peripheral conversion.

The observation that many TCRs found preferentially on converted Treg cells are also found on normal thymic Treg cells raised the possibility that we were merely observing Foxp3 induction in Foxp3$^-$ Treg cell precursors that arise in the thymus, and are then exported before Foxp3 is expressed [173]. We therefore asked whether TCR specificity

Figure 2.14 Recirculation of peripheral CD4$^+$ T cells to the thymus. 20 million CD4$^+$ T cell–enriched spleen and lymph node cells were injected into normal congenic recipients. 1 wk later, the spleen and thymus were analyzed by flow cytometry to determine the percentage of CD4$^+$ T cells derived from the donor (left), and the percentage of those that are Foxp3$^+$ (right). Data are pooled from two independent experiments (n = 5 mice), summarized in the bottom graphs (closed circle, individual mouse; horizontal line, mean). To estimate the percentage of thymic T reg cells that come from recirculating peripheral T cells, we assumed that the behavior of our adoptively transferred CD4$^+$ cells represented that of the normal peripheral T cell population. Therefore, recirculating cells comprise ~0.8% of the CD4 SP population (0.03% of donor T cells in the CD4 SP thymus times 26.3, the ratio of host:donor cells in the spleen [1:0.038]). 16.4% of recirculating cells are Foxp3$^+$; this suggests that recirculating Treg cells comprise 0.13% of the CD4 SP population. Thus, ~4% of the normal thymic Treg cell subset may arise from recirculation (0.13% of ~3%, the normal thymic Treg cell frequency). If we assume that Treg cells that develop in the periphery recirculate as estimated, then the extent of thymic recirculation is not sufficient to account for the presence of thymic Treg cells with TCRs also found in the converted population. For example, two of the most frequent TCRs found within the converted Foxp3$^+$ population, with CDR3 amino acid sequences AASDYGSSGNKLI and AASANSGTYQR (Table 2.2), are found in the normal Treg cell population at frequencies of 0.8 and 0.4%, respectively. Therefore, we could expect that they would account for 0.032% (0.8 x 4%) and 0.016% (0.4 x 4%) of thymic T reg cells. However, their frequencies within the normal thymic Foxp3$^+$ population are far greater (0.4 and 0.3%, respectively).

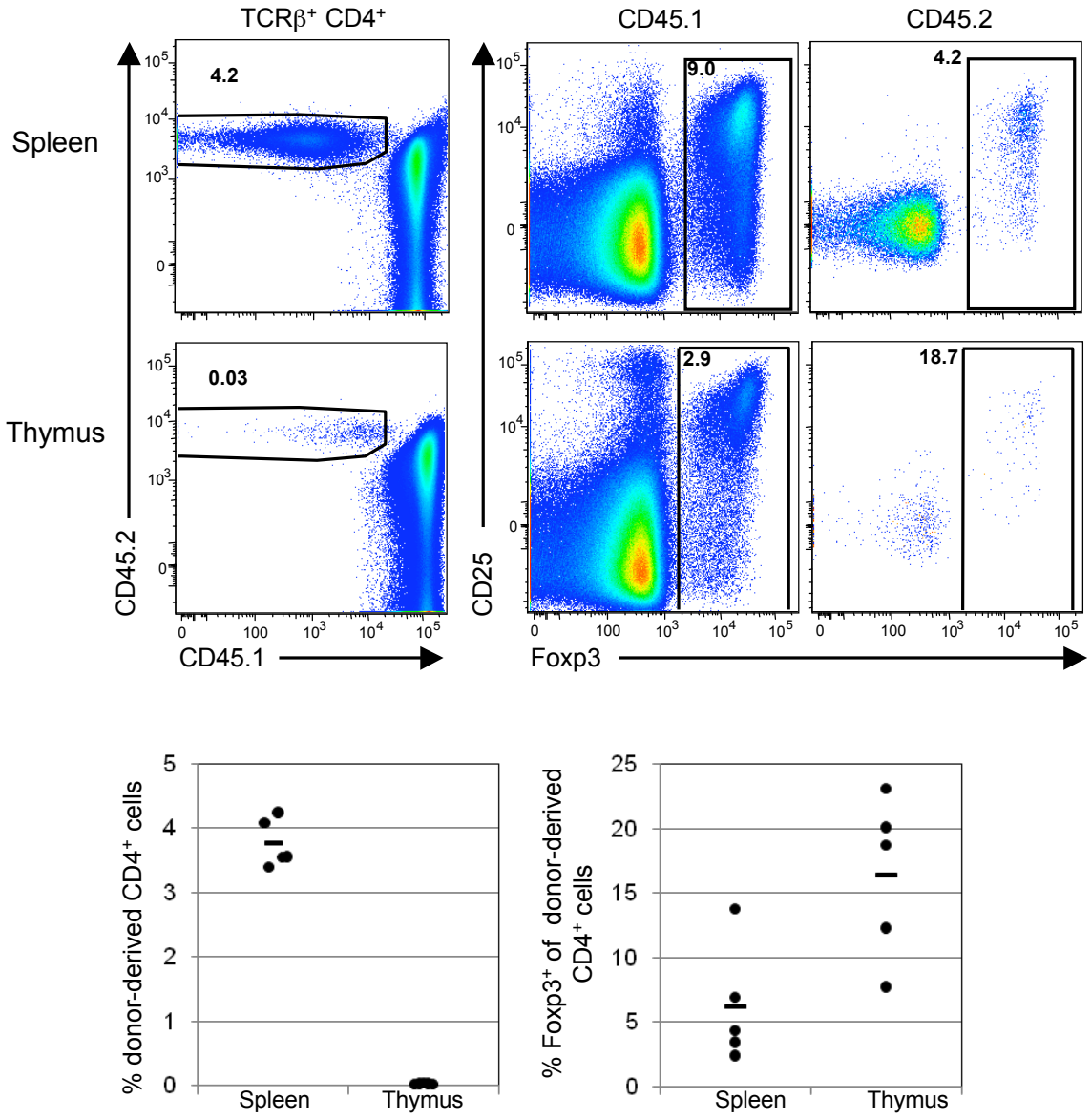


Figure 2.14 Recirculation of peripheral $CD4^+$ T cells to the thymus

alone, in the absence of thymic conditioning, is sufficient to direct peripheral conversion by introducing selected TCRα chains into peripheral monoclonal T cells from TCli αβ-TCR transgenic x *Foxp3*^{gfp} mice on a *Rag1*$^{-/-}$ background and then transferring them into *Tcrb*$^{-/-}$ hosts [33]. As the cells were isolated and treated identically, they should differ only by the additional retroviral TCRα chain at the time of transfer.

We first tested two TCRα chains (B2, B132) that were previously identified in naïve T cells, and also found primarily in the non-converted T cell subset in our lymphopenic expansion experiments (Fig. 2.15 A). These TCRs were also previously shown to be unable to facilitate in vivo expansion of T cells in *Rag1*$^{-/-}$ hosts [37]. Consistent with their low frequencies in the converted TCR data sets, we found that these naïve TCRs did not facilitate either Foxp3 induction or expansion (Fig. 2.15 B). Therefore, TCR activation and retroviral introduction of an additional TCRα chain alone are not sufficient to induce Foxp3 expression in these cells.

We then tested four TCRα chains that were identified in the highly converting Foxp3^{+} TCR data set (R19, R22, TR3, and TR5; Fig. 2.15 A, Table 2.2), and found that they also facilitated peripheral conversion in retrovirally transduced cells (Fig. 2.15 B, top). Thus, these data demonstrate that TCR specificity alone, in the absence of a unique thymic signal, is sufficient to facilitate the process of peripheral conversion.

Because these highly converting TCRs were also found in the normal Treg TCR data set, we tested the ability of other natural Treg TCRs that were not found in the conversion experiments to facilitate conversion. Interestingly, only one of the four Treg TCRs tested (R111) was able to induce Foxp3 in a sizable proportion of cells, whereas the others did not (G113, G5, G57). The inability to augment conversion was presumably not due to a

lack of TCR engagement, as these other TCRs did facilitate in vivo expansion (Fig. 2.15 B, bottom), suggesting that their cognate antigens were available in the periphery. It also appeared that certain TCRs favored expansion and conversion in different anatomic lymph nodes, which is consistent with our analysis of the normal Treg TCR repertoire (Fig. 2.2, 2.3). For example, R22 seems to promote conversion in the axillary and inguinal LNs, but less so in the mesenteric LNs (Fig. 2.15 B, top). Conversely, G57 showed expansion in the mesenteric LN but not in the axillary and inguinal LNs, (Fig. 2.15 B bottom), in accordance its distribution in the normal Treg cell population. In summary, these data suggest that TCR specificity determines whether a cell is capable of peripheral conversion, but that not all TCR specificities associated with peripheral Treg cells have this capability.

Figure 2.15 TCR specificity is sufficient to direct peripheral conversion in lymphopenic hosts. (A) Frequency distribution of TCRs tested. Data shown are the frequencies (percentages) of the TCRs in the datasets from thymocytes, normal peripheral T cells, and T cells recovered after adoptive transfer into lymphopenic hosts. (B) Retroviral manipulation of TCR specificity in peripheral T cells. Monoclonal TCli αβTCR transgenic T cells were transduced with TCRα chains, as described in Materials and methods. (top) The frequency of $Foxp3^+$ cells in the transduced ($V\alpha2^+V\beta6^+CD4^+$) T cell population was assessed by flow cytometry 2.5 – 3 wk after adoptive transfer into *Tcrb*$^{-/-}$ mice. (bottom) The absolute number of transduced ($V\alpha2^+$) cells recovered was calculated using the total cell count and the frequency of $V\alpha2^+V\beta6^+CD4^+$ T cells within the live gate, as determined by flow cytometry. Data shown are from individual mice and were obtained from five independent experiments constituting at least two independent retroviral transductions per TCR. Each symbol represents data from an individual recipient (n = 4-6), and the horizontal bars represent mean values.

A

		Thymus		Periphery														Adoptive transfer					
		Foxp3+	Foxp3−	Foxp3+					CD44lo					CD44hi					Converted			Non-converted		
ID	CDR3 sequence			Spl	Mes	Cerv	Axil	Ing	Spl	Mes	Cerv	Axil	Ing	Spl	Mes	Cerv	Axil	Ing	Spl	Mes	Cerv	Spl	Mes	Cerv
B132	AASPTASLGKLQ		0.3				0.2		0.3	0.4	0.4	0.2	0.6			0.1				0.1		0.5	1.2	0.4
B8	AASEDNNNAPR		1.1		0.1	0.1	0.3	0.1	4.2	3.0	3.0	3.1	3.0		0.1		0.3	0.3	0.2			0.7	0.5	0.9
R22	AASAGTYQR	0.3	0.1		0.1	0.4	0.2	0.6						0.1					1.3	1.3	1.2			0.1
R19	AASSGTYQR	0.3		0.2	0.5	1.7	0.2	0.2			0.1	0.1				0.1			0.8	0.5	0.5	0.1		
TR3	AASDYGSSGNKLI	0.4	0.1	0.6	0.2	0.6	1.7	1.6				0.2			0.1				5.4	5.3	10.2			
TR5	AASADNNRIF			0.1	0.2	0.1	1.5	1.0		0.1			0.1							1.3	1.4			0.2
G113	AARLNNNNAPR	0.3		0.2	0.1	0.9	1.3	0.7																
G5	AAMPNSAGNKLT			0.1		0.1	0.7	1.5										0.1				0.1		
G57	AASELYQGGRALI			0.8	2.7		0.1	0.1							0.1									
R111	AARPSGSFNKLT	0.2		0.3	1.7	2.8	0.2							0.1								0.4	0.2	

B

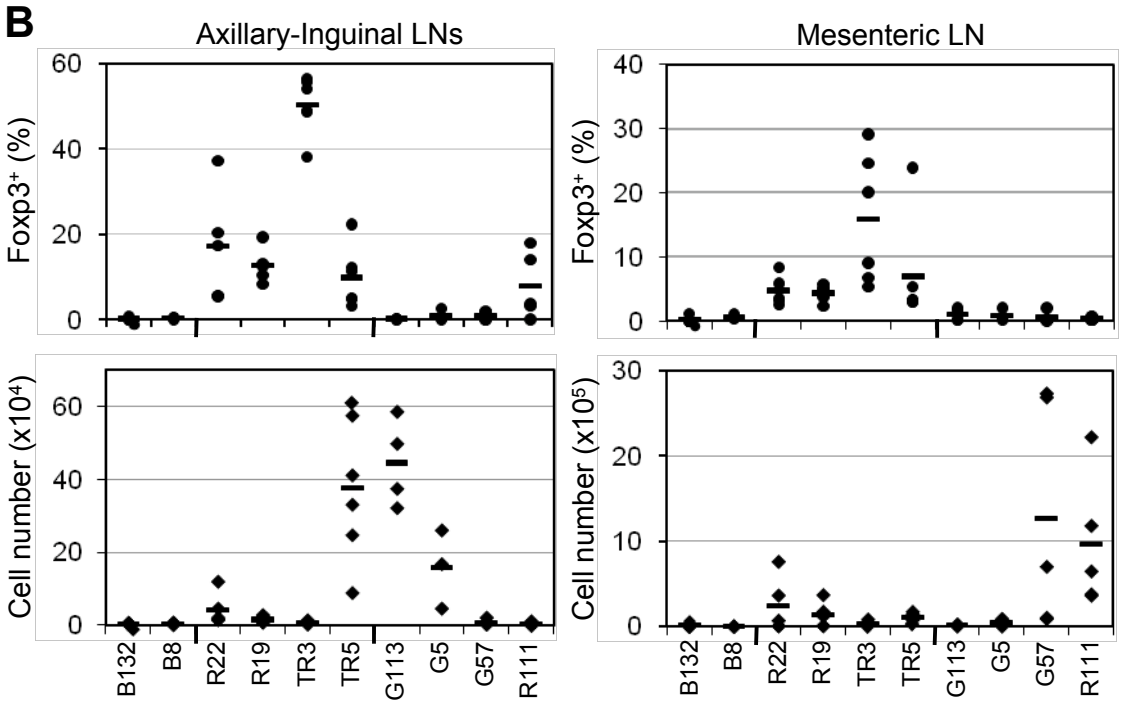


Figure 2.15 TCRs that facilitate peripheral conversion are found in the normal peripheral and thymic Treg cell TCR repertoires

Chapter 2: Discussion

Our analysis of the peripheral TRAV14 TCR repertoire strongly suggests that immune regulation is a tissue-specific activity, as the antigen-specificity of the protective Treg cell population changes considerably by anatomic location (Fig. 2.2, 2.3). These data are consistent with our previous observations of individual Treg TCR-driven proliferation in normal hosts, in which CFSE dilution was more pronounced in certain lymph nodes [33]. Thus, the observation that tissue-specific antigens greatly modify the local Treg TCR repertoire provides direct evidence for a mechanism by which the presence of an organ facilitates Treg cell-mediated tolerance to that organ [78-80].

Another intriguing observation is that the Treg and $CD44^{hi}$ TCR repertoires showed similar patterns of variability by anatomic location. This suggests that Treg and $CD44^{hi}$ populations share similar fundamental behaviors governing their interaction with tissue-specific antigens, even though the conditions for their development may be different. These data therefore support previous studies using monoclonal TCR transgenic mice [174] demonstrating that both Treg and non-Treg cells undergo clonal expansion upon antigen encounter. The expansion and maintenance of Treg cells specific for antigens normally presented in the peripheral self would favor tolerogenic Treg cell responses to those antigens, rather than novel foreign antigens. Thus, we hypothesize that shaping of the Treg cell population to the local antigenic landscape provides another mechanism for self/non-self discrimination by the immune system.

Contrary to previous studies [43], we did not find that peripheral conversion played a prominent role in the generation of the peripheral Treg cell population **(**Fig. 2.8). This difference could be explained in part by our use of Foxp3 as a marker for Treg cells,

rather than CD25 [32], to isolate non-Treg cells for adoptive transfer and analysis of conversion. Nonetheless, the use of mature Foxp3^{-}CD4^{+} thymocytes may still overestimate the role of peripheral conversion because we cannot exclude the presence of rare CD25^{-}Foxp3^{-} Treg cell precursors within this subset. We can also not be certain that these mature thymocytes are phenotypically identical to recent thymic emigrants in terms of their potential to undergo peripheral conversion. On the other hand, the use of peripheral Foxp3^{-}CD4^{+} T cells may underestimate the role of peripheral conversion because we can only assess the fraction of Foxp3^{-} cells capable of converting at steady state, and those T cells with the greatest propensity to undergo peripheral conversion are likely to be underrepresented. Thus, we believe our estimate that peripheral conversion contributes about 4-7% of the normal adult peripheral T reg cell population represents the best currently available figure.

We also studied the process of peripheral conversion during the immune response of Foxp3^{-} cells transferred into αβ-T cell deficient hosts, which may mimic the lymphopenic and Treg cell-deficient conditions during the early neonatal period. We found that peripheral conversion occurred much more readily in this environment than in a lymphoreplete host, and this made it technically feasible to perform TCR usage analysis. Interestingly, we found that peripheral conversion in polyclonal populations did not mirror previous experiments using monoclonal T cell populations, where a portion (~ 20%) of cells became Foxp3^{+} after non-inflammatory antigen encounter in both non-lymphopenic and lymphopenic models [39, 40, 173]. Rather, we observed no consistent patterns governing peripheral conversion during T cell expansion, finding that individual TCRs ranged from being highly skewed toward or against peripheral Treg cell

development (Fig. 2.11B, 2.12). Thus, the analysis of polyclonal TCR repertoires from both lymphopenic and non-lymphopenic environments do not support a model in which peripheral conversion is an automatic consequence of T cell activation, but rather one in which peripheral conversion is highly dependent on TCR specificity.

Although our data do not directly allow us to determine whether peripheral conversion occurs upon recognition of non-self antigens, it appears likely that many TCRs which facilitate conversion are reactive to self antigens. This is strictly based on the observation that several TCRs from converted Treg cells in lymphopenic hosts can also be found in the normal thymic Treg cell subset (Fig. 2.13, Table 2.2). As previously argued, we believe that thymic Treg development occurs via recognition of self [164], rather than nonself [81], antigens. Although we cannot exclude the possibility of peripheral nonself-antigen presentation in the thymus, nor the possibility that different peptides are recognized in the thymus and periphery, the most straightforward explanation is that the same self-reactivity that induces Treg cell development in the thymus also does so in the periphery. Although future experiments will be required to prove that the observed bias of converted TCRs toward the thymic Treg TCR repertoire is caused by the recognition of the same self-antigens, these data suggest that some cells that escape thymic Treg cell development express self-reactive TCRs which facilitate a second chance for Treg cell development in the periphery.

Peripherally converted Treg cells may develop directly from naïve T cells, or instead differentiate from memory/effector T cells [173]. *In vivo* data suggests that an appreciable frequency of Foxp3$^+$ cells requires one or more weeks to develop [39-41]. This time frame favors a multi-step process, but may also reflect a need for post-

conversion expansion. By contrast, *in vitro* studies have suggested that TGFβ–mediated induction of Foxp3 is considerably more efficient in naïve than memory T cells [175]. In fact, it has been reported that TGFβ is unable to induce Foxp3 in previously *in vitro* differentiated T cells [176]. Because *in vitro* TCR stimulation is used in the retroviral transduction protocol, our data directly demonstrate that, *in vivo,* peripheral conversion from previously activated T cells can still occur in the context of an appropriate TCR specificity (Fig. 2.15), hinting at possible differences between *in vitro* and *in vivo* conversion.

It is clear, however, that conversion alone is insufficient to maintain immune homeostasis, as shown by the development of colitis and other autoimmune manifestations upon transfer of Treg cell-depleted $CD4^+$ T cells [78-80, 152, 170]. However, conversion may diminish the severity of disease [41, 177]. The inability to prevent disease may be due to the limited Treg TCR repertoire generated by peripheral Treg cell development, which has been suggested to permit autoimmunity in lymphopenic mice [178]. Alternatively, it may be that peripheral conversion occurs too late to fully prevent autoimmune pathology. One interesting hypothesis is that autoimmune disease may also be exacerbated by self-reactive $Foxp3^-$ T cells expressing Treg TCRs that escape into the periphery [33], but are unable to undergo peripheral conversion due to their antigen specificity (Fig. 2.15).

These data demonstrate a critical role for TCR specificity in the decision to undergo peripheral conversion. One potential mechanism by which this occurs is that the "nature" of the TCR-ligand interaction itself could generate a unique signal that results in Foxp3 expression. This has been suggested by an αβ-TCR x cognate antigen double transgenic

model of thymic Treg cell development [36]. In this model, the high affinity ligand induced both deletion and Treg cell development, whereas a slightly different, lower affinity ligand could only induce deletion. However, a mechanism dependent on unique TCR signals would be unable to distinguish the context of T cell activation in the periphery, making it teleologically less appealing. An alternative, non-mutually exclusive, hypothesis is that the TCR specificity results in activation within an environment which facilitates peripheral Treg cell development. We favor this latter model, in which antigen presentation can occur on APCs and/or within microenvironments that determine whether a given T cell will become a regulatory or effector cell. Based on current data, we suspect that a microenvironment promoting peripheral conversion would contain TGF-β and potentially retinoic acid [40, 44, 124, 130]. Therefore, the balance of antigen presentation between two different microenvironments would determine the pathologic or tolerogenic outcome of the immune response to an antigen.

CHAPTER THREE

The Colonic Foxp3^{+} Regulatory T Cell Repertoire is Shaped by Recognition of Commensal Gut Bacteria

Chapter Three: Preface

The findings described in this chapter were a natural outgrowth of the results described in Chapter Two. In sequencing TCRs from $CD4^+$ T cells in various locations, we wondered if those found in the gut would show signs of antigen specificity and a unique TCR repertoire. In the gut, this would imply that at least a portion of the Treg cells would have developed extra-thymically, via peripheral conversion, in response to unique antigens derived from the commensal microbiota, which would not be available for selection in the thymus. With reports such as that by Sun et. al. [44] showing an increased prevalence of conversion in the GALT, and our own finding that conversion was higher in the MLN than in other LN or the spleen [150], we expected we might see more evidence of peripheral conversion in the Treg of the gut.

The large portion of these studies included in this chapter would not have been possible without the generous and enthusiastic contribution made by our collaborators Seth Bloom and his mentor Thad Stappenbeck. Seth's offer of his knowledge and skills to culture many strains of bacteria that live in the colon and cecum of the mice in our colony made possible some of the most exciting results we present in this chapter, and for this I am greatly indebted to him. He has contributed to the Materials and methods section of this chapter directly with a description of his techniques in culturing bacteria from the intestinal contents. The studies presented here are currently being prepared as a manuscript for publication.

Chapter Three: Abstract

Regulatory T cells (Treg) play an essential role in the maintenance of homeostasis in the face of constant exposure to the organisms, both beneficial and potentially harmful, which are encountered in the gut. To determine if the colon, with its unique environment and antigens derived from the commensal microbiota, harbors an equally unique Treg population we determined the repertoire of TRAV14 (Vα2) TCRα chains found on the $CD4^{+}Foxp3^{+}$ T cells of the spleen, mesenteric lymph nodes (LN), cervical LN, and colonic/cecal lamina propria (LP) in mice expressing a single TCRβ chain. We found the repertoire of the colon Treg to be unique, with no appreciable overlap with Treg from other locations in the body, nor with the naïve or $CD44^{hi}$ memory T cells in the colon. Using reporter hybridomas expressing those TCRs found most commonly on the colonic Treg, we screened for reactivity to preparations of bacteria cultured from the colon and cecum of mice in our colony. Amazingly, 3 of 12 TCRs tested were found to react to specific bacterial preps, and 4 others reacted to a preparation of autoclaved colon/cecal contents. CD4 single-positive (SP) thymocytes expressing these TCRs showed no sign of Foxp3 expression in the thymus, while those expressing Treg TCRs from other locations did. T cells expressing two of these bacterial-reactive TCRs were confirmed to show no Foxp3 expression in the thymus in mixed bone marrow chimeras. However, these T cells developed $Foxp3^{+}$ T cells in the colonic LP when hosts were cohoused with littermates of the mice expressing the fixed TCRβ chain. These studies show that the colonic $Foxp3^{+}$ Treg show unique TCR specificities, some of which develop *in situ* upon recognition of antigens derived from commensal bacteria.

Chapter Three: Introduction

The maintenance of tolerance in the gut requires that the immune system retain the ability to respond to pathogens but also be tolerant to the large number of microorganisms which inhabit the site, many of which provide essential nutrients and other benefits to their host. When this ability to maintain tolerance to the commensal microbiota fails, the result is inflammatory bowel disease (IBD), which in humans generally manifests as Crohn's disease or ulcerative colitis. These debilitating and sometimes fatal diseases affect an estimated 0.1 – 0.2% of the population [133].

One important part of the strategy employed by the immune system for accomplishing this feat are the $CD4^+Foxp3^+$ regulatory T cells (Treg). These cells are essential for the maintenance of gut homeostasis. In Foxp3-deficient *scurfy* mice, and in patients harboring a mutation in Foxp3, one of the earliest autoimmune manifestations to occur is severe enteropathy [144, 179]. Similarly, in mice engineered to express the human diptheria toxin (DT) receptor under the Foxp3 promoter, administration of DT results in elimination of the $Foxp3^+$ T cells, and severe wasting disease quickly develops [30]. A widely used model for the development of IBD is the transfer of T cells depleted of the Treg population into a lymphopenic recipient; in the absence of regulation these T cells cause intestinal inflammation, which can be prevented by the co-transfer of the $Foxp3^+$ Treg population [146].

Most Treg develop their regulatory phenotype, indicted by the expression of Foxp3, during thymic selection [33-35, 150]. However, $Foxp3^+$ regulatory cells can also develop from mature, non-regulatory $CD4^+$ T cells outside of the thymus under certain conditions, a process termed peripheral conversion [173]. This has been shown to occur

under circumstances in which cognate antigen is introduced in a non-inflammatory setting, and may be a mechanism to generate tolerance to antigens not found in the thymus. While it is estimated that peripheral conversion does not contribute greatly to the peripheral Treg repertoire [34, 35, 150], several studies have shown an increased amount of conversion in the mesenteric LN (MLN) and small intestine lamina propria, under steady state conditions and during induction of oral tolerance [44, 124, 150]. However, these studies did not address the potential for conversion in the colon.

The dendritic cells (DC) found in the gut appear to be uniquely capable of mediating peripheral conversion. In particular, the $CD103^+$ DC have been shown to carry bacterial antigens from the lamina propria to MLN [91]. DC can obtain bacterial antigens that have been transcytosed by M cells in the intestinal epithelium, by the phagocytosis of apoptotic intestinal epithelial cells which may have been invaded by bacteria, or even by direct sampling of luminal contents [90]. Therefore, it would seem likely that T cells recognizing bacterial antigens presented by these tolerogenic intestinal DC may become Treg that are then capable of mediating tolerance in the intestines. Exposure to this subset of DC also causes upregulation of gut homing molecules, due to their ability to provide retinoic acid (RA), and therefore can also cause these T cells to traffick to the LP [123, 129].

There is some evidence that Treg in the gut need to recognize bacterial antigens to efficiently preserve tolerance to the commensal bacteria. Strauch *et. al.* showed that co-transfer of $CD4^+CD62L^-$ cells (containing the regulatory population) isolated from germ free mice did not prevent disease development after transfer of $CD4^+CD62L^+$ T cells into SCID mice [147]. However, it appears from the low expression of GITR and deficiency

in Foxp3 mRNA that the germ-free $CD4^+CD62L^-$ T cells may have been relatively deficient in $Foxp3^+$ Treg. Using the transfer of $CDRB^{hi}$ cells into SCID mice, Singh *et. al.* showed that co-transferred $CDRB^{lo}$ cells from germ free mice were capable of preventing IBD only at high cell numbers, but when fewer Treg were transferred they proved to be less efficient than the comparable population from SPF mice [148]. These studies imply that without bacterial recognition, the Treg population in the colon is somewhat incomplete.

In a previous report we showed that the TCR repertoire of $Foxp3^+$ Treg varied according to their location, suggesting that the repertoire is reflective of the local antigenic milieu [150]. These TCRs also differed from those found on the activated memory T cells expressing high levels of CD44, indicating that these two populations do not overlap in their antigen specificity. We speculated that the unique antigenic environment of the colon would result in a particularly unique repertoire of TCRs being used by the colonic Treg cells, and therefore this led us to sequence the TCRα chains from $Foxp3^+$, $CD44^{hi}$, and naïve $CD44^{lo}$ $CD4^+$ T cells isolated from the colonic LP of mice expressing a fixed TCRβ chain. Indeed we found that these TCR were not shared with $Foxp3^+$ TCRs found in other locations, nor with the activated/memory $CD44^{hi}$ or naïve $CD44^{lo}$ populations.

We hypothesized that these unique specificities may be due to the availability of antigens derived from the commensal microbiota, and that these Treg may develop from $Foxp3^-$ cells via peripheral conversion. We indeed found that several of these TCRs recognized antigens derived from commensal bacteria or recognized some component of the colon/cecum contents of mice in our colony but not from germ-free mice. T cells

expressing these TCRs showed no sign of Foxp3 expression in the thymus, but two such TCRs were demonstrated to have developed Foxp3 expression after isolation from the colonic LP. Therefore, at least some Treg found commonly in the colonic LP have developed in the periphery upon recognition of an antigen derived from commensal bacteria.

Chapter Three: Materials and methods

Mice

The Foxp3^{gfp} reporter knockin, TCliβ transgenic and TCliαβ transgenic mice were kindly provided by Dr. Alexander Rudensky (then at the U. of Washington, Seattle, WA) and were bred in the specific-pathogen-free (SPF) facility at Washington University in St. Louis. *Rag1*$^{-/-}$ and *Tcra*$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). CD45.1 congenic mice were obtained from National Cancer Institute (Frederick, MD). Animals were used according to protocols approved by the Institutional Animal Care and Use Committee.

Cell purification, sorting and sequencing

Colonic lamina propria lymphocytes were isolated from the cecum and colon combined, rinsed with cold medium, cut into small pieces and incubated in RPMI containing 3% FBS, 20 mM HEPES**,** 5 mM EDTA and 0.145 mg/ml DTT for 20 minutes at 37˚C with constant stirring. Pieces then underwent three rounds of shaking in RPMI + 2 mM EDTA**,** followed by digestion of the pieces in 0.2 mg/ml Liberase CI (Roche) for 15 minutes at 37˚C with constant stirring, followed by addition of fresh enzyme and stirring for another 15 min. Digested tissue was forced through a Cellector tissue sieve (Bellco Glass, Inc.) and then underwent washing and filtering through 70 μm and 40 μm filters. Cells were spun down and resuspended in 2ml of RPMI containing 10% FBS, filtered through a 37 μm filter, counted and then stained with antibodies and either analyzed or sorted by flow cytometry as described below.

T cell suspensions from the spleen or lymph nodes to be sorted were incubated with biotinylated anti-B220 (RA3-6B2) and anti-CD8 (53-6.7) antibodies (Biolegend), followed by incubation with anti-biotin magnetic microbeads (Miltinyi Biotech) and enriched by depletion by magnetic separation using an AutoMACS (Miltinyi Biotech). Remaining cells were stained with antibodies for CD4 (RM4-S), Vβ6 (RR4-7) and CD44 (IM7) and sorted by flow cytometry using a FACSAria (Becton Dickinson). Cells to be analyzed by flow cytometry were stained with a combination of fluorescently-labeled antibodies including: Vβ6 (RR4-7), CD4 (RM4-S), CD8 (53-6.7), Vα2 (B20.1), GITR (YGITR 765), CD25 (PC61), Thy1.2 (30-H12), CD45.1 (A20), CD45.2 (I04), and CD24 (M1/69).

Cloning of TRAV14 TCRα sequences from sorted T cells was performed as previously described [33]. As before, we utilized the CDR3 amino acid sequence provided by IMGT/V-QUEST as a unique identifier for individual TCRs [163].

Statistical Analysis

The Morisita-Horn similarity index was used to assess the amount of similarity between any two sets of TCR sequences, as in previous studies [33, 37, 150]. This unit-less index ranges from 0 to 1, with 0 representing complete dissimilarity and 1 being absolutely identical [165]. The Morisita-Horn index takes into account the frequency of each TCR, and is relatively resistant to the sample size, as compared with other similarity indices such as the Jaccard. A sequence found in both data sets at greatly different frequencies would therefore contribute to dissimilarity by this index. For reference, a comparison of the first 200 randomly chosen sequences with the last 200 sequences of an

~400 sequence data set results in a Morisita-Horn index of about 0.9, which represents an experimentally derived value for the maximum expected similarity, incorporating the inherent variability due to random sampling.

Creation and screening of hybridoma cell lines expressing colonic Treg TCRs

The 58$\alpha^-\beta^-$ hybridoma cell line stably transduced with hCD4-pA-GFP-NFAT-RVT was a generous gift of Wataru Ise in the laboratory of Ken Murphy (Washington University in St. Louis) [180]. This hybridoma was then transduced with MigR1-derived retrovirus (without IRES GFP) containing TCli TCRβ chain and downstream mCD4 under an IRES. These hybridomas were sorted for expression of mCD4 and the sorted cells used as the parental cell line for introduction of TCRα chains of interest by retroviral transduction with MigR1-derived retrovirus containing the TCRα chain. After introduction of the TCRα chain, cells were allowed to expand and then sorted for high surface expression of the TCR.

Dendritic cells (DC) used in these cultures were obtained from a wild type mouse injected with 3-5 x 10^6 B16 melanoma cells engineered to produce Flt3 ligand (Flt3L) subcutaneously 7-8 days prior. Spleen and enlarged inguinal LN from the side of injection were collected and DC were enriched by use incubation with biotinylated CD11c mAb (N418) (Biolegend) followed by incubation with anti-biotin MACS microbeads (Miltinyi Biotech) and subsequent positive selection with an AUTOMacs (Miltinyi Biotech). Cells were washed, resuspended in DMEM with 10% FBS, and used as APC in screening cultures.

$1x10^4$ colonic Treg TCR-expressing reporter hybridomas cells were cultured with $1x10^4$ DC and antigen from either individual bacterial isolates, pools of bacterial isolates, medium only, autoclaved cecum/colon contents (ACP, see below), or 0.1 μg/ml αCD3. The amount of bacterial prep or ACP added was optimized experimentally; the most that could be added without compromising the health of the cells was used. Often this was about a 1:200 final dilution in the culture. In some cultures, blocking aMHC class II mAb (M5-114) was added at 10 μg/ml. After about 40-44 hours cells were collected and stained with CD4 and Vβ6, and analyzed by flow cytometery on a FACSCanto (Becton Dickinson).

Creation of bacterial and cecum/colon content preps for screening

Cecum and colon contents of two mice that were wild-type littermates of the TCli TCRβ trasnsgenic mice used for sequencing studies were aseptically harvested under anaerobic conditions, pooled in sterile pre-reduced PBS, and plated on a variety of media types in serial, half-log dilutions, which allowed isolation of unique-appearing colonies from the plates with the largest number of individually distinguishable colonies [181, 182]. The media types used for anaerobic culture were Anaerobic Reducible Blood Agar (ANB) (Remel, general anaerobic growth media), Anaerobic Reducible CNA Blood Agar (ACNA)(Remel, enriched for growth of Gram positive anaerobic organisms), Anaerobic Reducible LKV Blood Agar (LKV) (Remel, selective for growth of Gram negative obligate anaerobes), *Bacteroides fragilis* Isolation Agar (BBE) (Remel, contains gentamicin to inhibit most facultative anaerobes, bile to inhibit most anaerobic gram negative bacteria, and esculin to differentiate members of the *B. fragilis* group), and

Rogosa agar (Rog)(for cultivation of lactobacilli). The media types used for aerobic culture were Tryptic Soy Agar with 5% Sheep Blood (TSA) (Remel, general aerobic growth media), Chocolate Agar (Choc) (Remel, general aerobic growth media enriched for isolation of fastidious aerobes), Columbia CNA with 5% Sheep Blood (CCNA) (Remel, for selective isolation of gram-positive cocci and inhibition of gram-negative bacilli), and MacConkey Agar (Mac) (selective and differential for isolation of gram negative aerobes or facultative anaerobes).

Cultures were incubated for 2-7 days at 37°C in ambient room air (aerobic cultures) or 7-14 days in a Bactron-IV Anaerobic Chamber (Shel Lab) at ~37°C in an atmosphere of 5% hydrogen, 5% carbon dioxide, and 90% nitrogen (anaerobic growth). Unique-appearing single colonies were picked and sub-cultured them on fresh media, then passaged a second time to ensure purity. Sub-cultured bacteria were subsequently grown in BHI broth or Thioglycollate broth (Remel), concentrated by centrifugation, re-suspended in sterile PBS, and concentrated again by centrifugation. Cultures that did not grow in liquid media were removed from the surface of culture plates using sterile swabs and resuspended in sterile PBS. Aliquots were frozen for subsequent re-culture and for DNA isolation. The remainder was heat-killed by incubating at 75°C for 60 minutes, then frozen and preserved for subsequent T-cell analysis.

To prepare whole colon and cecal contents for screening (ACP) the contents were collected and mixed with PBS to make a slurry which was homogenized using a VWR PowerMAX homogenizer. The preparation was then autoclaved to sterilize, and filtered through sterile 70 μm nytex mesh to remove large particulates. The preparations were then frozen at –20˚C for short-term use or –80˚C for long term use.

Identification of bacterial isolates using 16S rRNA sequencing

Genomic DNA was extracted from individual isolates and the bacterial 16S rRNA gene was PCR-amplified using primers Bact-8f and Bact-1510r [84, 183]. The resulting PCR product was then sequenced using one or both of the original PCR primers. Sequences were edited and trimmed for quality using the Trev 1.9 program of the Staden Package©. When available, forward and reverse reads were assembled into a single contig using SeqMan from the DNAStar© Lasergene software package, Version 8.0.2. The 16S sequences were classified using the Ribosomal Database Project (RDP, Release 10, Update 15) Classifier and the closest cultured and un-cultured matches from GenBank were identified using RDP's SeqMatch program supplemented by BLAST analysis [184-187].

Retroviral transduction of Rag$^{-/-}$ *thymocytes and intrathymic injection*

Thymocytes from donor (CD45.2) *Foxp3^{gfp}Rag1$^{-/-}$* of 5 - 8 weeks of age were collected and underwent "spinfection" by a MigR1-derived retrovirus which expressed the TCRα chain being analyzed and the TCli TCRβ chain downstream of the P2A sequence, as described [188]. After spinfection and a rest in culture for 1 – 2 hours, cells were thoroughly washed and resuspended in PBS for intrathymic injection of sublethally irradiated (600 rad) CD45.1 congenic 6 - 10 week old hosts. Thymocytes were then analyzed by flow cytometry 14 - 16 days after injection to assess for the expression of Foxp3^{gfp} of CD45.2 (donor) CD4 SP cells.

Creation of retroviral bone marrow chimeras

Donor *Foxp3*^{gfp}*Rag1*$^{-/-}$ mice were conditioned with 5-fluorouracil before bone marrow was collected. After 2 d of culture in recombinant mouse IL-3 (10 ng/ml), recombinant human IL-6 (20 ng/ml) and recombinant mouse stem cell factor (100 ng/ml), bone marrow cells underwent 'spin infections' on two consecutive days with MigR1-derived retrovirus expressing the CT2 or CT6 TCRα chain, as well as the fixed TCli TCRβ chain downstream of the P2A sequence [188]. On the fourth day of culture, the bone marrow cells were mixed with CD45.1 *Foxp3*^{gfp} bone marrow harvested from 5-fluorouracil-treated mice at varying ratios and were injected into lethally irradiated CD45.1 hosts (1,050 rads). In some experiments, the mice were cohoused with a mature female littermate of the TCliβ transgenic mice used for sequencing studies for 7-10 days, starting at approximately two weeks after bone marrow injection. Cells from the thymus, spleen, colon LP, MLN, and pooled inguinal, axillary and cervical LNs of the mice were analyzed by flow cytometry 8-9 weeks after bone marrow injection.

Chapter Three: Results

Colonic Treg express a unique repertoire of TCRs

We previously observed that the TCR repertoire of Treg show a marked skewing of their TCR repertoire according to location, suggesting that they home to sites according to their antigen specificity much as memory T cells do [150]. The intestine is a unique site immunologically, with its access to the commensal organisms and food particles of the intestinal lumen and a need to maintain tolerance to their constantly shifting antigens. Therefore, as an extension of our previous study we chose to sequence the TCR repertoire of T cells isolated from the lamina propria (LP) of the colon and cecum (hereafter referred to as the colon). We isolated and sorted $CD4^+Foxp3^+$ (Treg), $CD4^+Foxp3^-CD44^{hi}$ (memory T cells), and $CD4^+Foxp3^-CD44^{lo}$ (naïve T cells) from the spleen, mesenteric LNs (MLN), cervical LNs (CLN) and colon in order to compare their TCR repertoires. Cells were pooled from 3 or 4 mice in each of two independent experiments, and in a third experiment cells from the spleen, MLN and colon from five mice were pooled (Table 3.1). As in previous studies [33, 37, 150], these mice express the TCRβ chain from the TCli TCR transgenic mouse (TCliβ), the $Foxp3^{gfp}$ fusion protein reporter, and are heterozygous for TCRα locus expression, ensuring that each T cell expresses a fixed TCRβ chain and a single, endogenously rearranged TCRα chain. We sequenced the TRAV14 (Va2) chains and used the CDR3 amino acid sequence of the chain as a unique identifier of that TCR.

As we hypothesized, the $Foxp3^+$ subset isolated from the colon showed a TCR repertoire that was unique, with essentially no overlap with those found in other locations

Table 3.1 TCR sequences from experiments using pooled animals

Expt 1	Colon	Spleen	Mes LN	Cerv LN	Total
Foxp3^{+}	258	409	434	467	**1568**
CD44^{hi}	120	451	490	398	**1459**
CD44^{lo}	228	337	395	397	**1357**
Total	**606**	**1197**	**1319**	**1262**	**4384**
Expt 2	**Colon**	**Spleen**	**Mes LN**	**Cerv LN**	**Total**
Foxp3^{+}	357	283	361	284	**1863**
CD44^{hi}	315	283	276	365	**1927**
CD44^{lo}	281	278	306	278	**1926**
Total	**953**	**844**	**943**	**927**	**5716**
Expt 3	**Colon**	**Spleen**	**Mes LN**	**Total**	
Foxp3^{+}	207	142	162	**511**	
CD44^{hi}	197	211	193	**601**	
CD44^{lo}	185	148	184	**517**	
Total	**589**	**501**	**539**	**1629**	

Number of sequences obtained from each population of cells isolated from the indicated tissue. Each experiment consisted of cells pooled from 3-5 mice. Cervical LN was not sequenced in Expt 3.

(Fig 3.1-3.2). While the repertoires of the Treg in each location did not overlap with the others to a large degree, the comparison with the colon was the least similar in each case, including, somewhat surprisingly, the MLN, with a Morisita-Horn similarity index of less than 0.1 in each case (Fig 3.2). As expected, the $CD44^{lo}$ naïve T cell repertoires were fairly similar across all locations, although the similarity between the colon and other locations was slightly lower, perhaps reflecting differences in the T cell trafficking patterns to the colon. The $CD44^{hi}$ memory T cell populations showed a somewhat skewed repertoire according to location, as previously reported, which held true for the colon as well, but there was a somewhat increased similarity seen between the colon and MLN in this population. In summary, our hypothesis that the colon Treg would utilize a particularly unique repertoire of TCRs was correct, perhaps due to the unique antigens available at that location.

Because these experiments used a pool of cells from several mice, we wished to compare the TCRs from individual animals in order to determine to what extent the TCR repertoire of the colonic Treg varied, and to identify the TCRs that are most commonly found on the colon Treg population. We therefore sequenced the TCRα chains from the colonic T cells in five individual animals (Table 3.2). This analysis revealed that the colonic Treg TCRs are somewhat conserved between individuals, a pattern that was also seen in the colon sequences from experiments using cells pooled from multiple mice (Fig 3.3 A and not shown). In contrast, the $CD44^{hi}$ memory T cell repertoire showed no overlap between individuals; in was, in fact, difficult to find TCRs that occurred in more than one animal. This would suggest that the memory T cell population is directed at different antigens in each animal, presumably a microbial antigen from a species that is

Figure 3.1 Foxp3^{+} Treg TCRs from the colon are unequally distributed.

The anatomical distribution of the most abundant TCR sequences within each phenotype are shown. Using the average frequencies from the pooled TCR datasets from all three experiments, the top 50 TCR sequences within a given phenotype were selected. For each TCR, the relative frequency with which it is found at each anatomical location was calculated by dividing its frequency at each location by the sum of the frequencies at all locations. The frequency is represented on the heat map by decile, with the scale indicating the colors at 100 (red), 66, 33, and 0% (yellow) for a TCR at a given location. The data are clustered such that those TCRs predominantly found in one location are shown together.

Figure 3.1 Foxp3^{+} Treg TCRs from the colon are unequally distributed.

Figure 3.2 Statistical analysis of similarity between populations from different anatomical locations. The Morisita-Horn similarity index is shown for the comparison between the colon and the mesenteric LNs (MLN), spleen (SPL) and cervical LNs (CLN) in the first set of each panel, then between the spleen and the MLN and CLN, then between the MLN and CLN. Values are for the comparison between datasets combined from all three experiments.

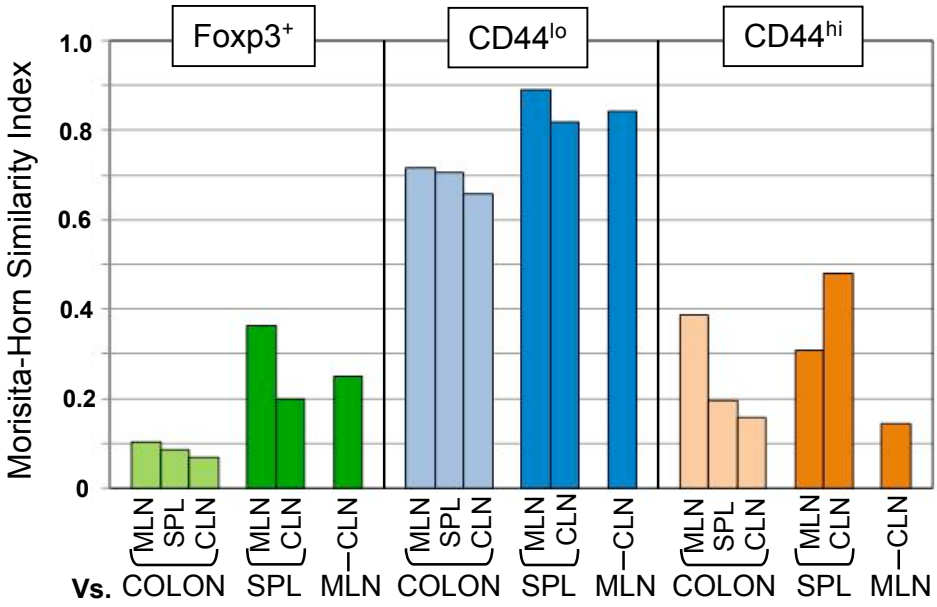


Figure 3.2 Statistical analysis of similarity between populations from different anatomical locations

Table 3.2 TCR sequencing of colon T cell from individual animals

Colon	mouse 1	mouse 2	mouse 3	mouse 4	mouse 5	Total
Foxp3^{+}	200	175	224	214	138	**951**
CD44^{lo}	209	189	201	190	176	**965**
CD44^{hi}	179	187	174	242	194	**976**
Total	**588**	**551**	**599**	**646**	**508**	**2892**

Number of sequences obtained from each population of colon/cecum lamina propria T cells from each of 5 individual mice.

Figure 3.3 Comparison of the colonic TCR repertoires of five individual mice. (A) The mean (±SEM) of the Morisita-Horn similarity indices of each pairwise comparison between the TCR repertoires of the Foxp3^{+} (green), naïve CD44^{lo} (blue), or memory CD44^{hi} (gold) populations of five individual mice (Table 3.2). (B) Comparisons between the TCR repertoires of the Foxp3^{+}, CD44^{hi}, and CD44^{lo} population within each of the five individual mice. The mean (±SEM) of the Morisita-Horn indices of these comparisons is shown.

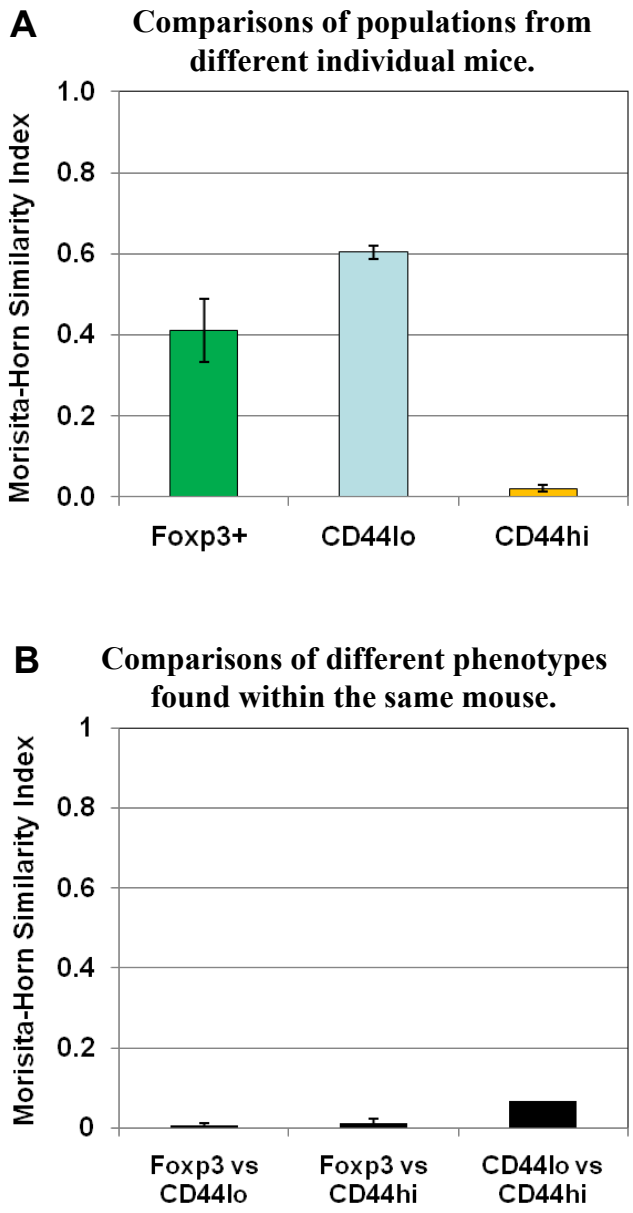


Figure 3.3 Comparison of the colonic TCR repertoires of five individual mice.

rare or transient, or a potentially pathogenic bacterium that was eliminated by the immune response. In contrast, it appears that the Treg population recognizes fairly conserved antigens; either true self antigens which are unique to the colon, and/or broadly represented bacterial antigens that are either conserved between multiple bacterial species or derive from very abundant and widespread species.

In agreement with our previous study [150], there was very little overlap in the TCR repertoires of the Foxp3^{+} Treg, CD44^{lo} naïve, and CD44^{hi} memory T cell populations. We had hypothesized there may be an increased similarity between the CD44^{hi} memory and Treg populations if indeed a sizeable number of colonic Treg arise via peripheral conversion, as this might occur alongside effector cell development to the same antigens. To the contrary there was almost no shared TCRs between these two subsets, with an average M-H value well below 0.1 (Fig 3.3 B), further supporting a model where T cell differentiation occurs in response to different stimuli in these two populations. In addition, there was no significant similarity between the Treg and naïve T cells in the colon (Fig 3.3 B).

Some common colonic Treg TCRs recognize bacterial antigens

In an attempt to determine if we could identify the antigens being recognized by colonic Treg TCRs we selected several prominent TCRs that were found at relatively high frequency within multiple mice and/or experiments, and were not found within the CD44^{lo} or CD44^{hi} datasets. This criteria ensures that we are only considering the most conserved Treg TCRs, which we believe should encompass a representative sampling of

typical Treg TCR specificities. These TCRs, and their prevalence in each dataset, are listed in Table 3.3.

We transduced a T cell hybridoma line expressing an NFAT-driven GFP reporter (as a way to assess TCR engagement) and the TCli TCRβ chain with these TCRα chains to create reporter hybridomas with the specificities of the most common colonic Treg. These were sorted by flow cytometry to obtain a population with consistent TCR expression and minimal background GFP expression. These hybridomas were then cultured with a variety of antigens and Flt3L-induced splenic DC, and assayed for NFAT-GFP expression by flow cytometry as indication of antigen recognition by the TCR.

We sought to test these hybridomas for reactivity against common commensal bacteria. In order to obtain as wide a variety as possible of the species typically found in the colon of these mice we harvested the colon and cecum contents from wild-type littermates of the TCliβ transgenic TCRα heterozygous mice being used for TCR sequencing. The commensal microbiota can vary widely between animals according to their genetics and housing conditions . Therefore, we believe the use of these littermate mice is the best approach to obtain bacterial isolates that will be relevant to the particular Treg population we have been studying.

Aerobes and anaerobes were isolated from the colon contents by growth on various nutrient agars in various conditions (see Materials and methods). Individual colonies appearing to be different species by colony morphology were subcloned and either grown in liquid media culture, or, for species that wouldn't grow to sufficient density in liquid medium, replated again at higher density and the resulting colonies scraped from the plate. These bacteria were heat-killed and then frozen for later use. In

Table 3.3 TCRs selected to screen for bacterial reactivity

Foxp3$^+$ population		COLON								MES LN			SPLEEN			CERV LN	
NAME	CDR3 AA SEQ.	ms 1	ms 2	ms 3	ms 4	ms5	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2
CT1	AASWASGYNKLT	39.0	24.0	7.6	2.3	33.3	12.8	8.7	4.8	1.6	0.3		0.5	0.4		0.4	
CT2	AASAIWNTGYQNFY	5.0	25.1	11.2	4.2	1.4	6.2	2.2	1.0		0.8			0.4			
CT7	AASATGDNRIF	0.5			2.8	3.6	11.2	1.7	4.3	0.3	0.3						
G57	AASELYQGGRALI				4.7	2.9	4.7	0.3	1.0	1.8	4.4		1.5	1.1			
CT4	AASEYSALGRLH	1.0			0.9				2.9	0.4							
CT6	AASGYSALGRLH				0.5		1.6		0.5	0.3							
CT8	AASLTGGYKVV	5.5		3.1	5.6		1.2		7.7								
CT9	AASADNRAGNKLT		16.0		18.2												
CT11	AASAPWNSNNRLT	1.5			5.6				1.9								
CT12	AASAVWANTGKLT			1.8		1.4	3.1		0.5	0.2	0.3						
CT10	AAQRRGNYKYV	2.5		4.0													
CT13	AASRERYANKMI				2.3				1.9								

The frequency (in %) with which selected colon Treg TCRs are found in individual mice (ms 1-5) and pooled mice experiment (Expt 1-3) datasets.

total, 77 isolates were obtained for screening; as the media and growth conditions used were fairly non-specific it is likely that several of these preps may consist of the same bacteria grown in more than one condition. Each of these isolates was given a name related to the type of agar on which it was grown and a randomly assigned number, and pools of 2 to 3 isolates each were created to facilitate screening. In addition, the colon and cecum contents were prepared from another TCliβ TCR$\alpha^{+/-}$ littermate control mouse, as well as a C57BL/6 germ-free mouse (see Materials and methods). These likely will contain bacteria that were not culturable with our current techniques, but that may still be recognized by our TCRs.

T cell hybridomas expressing colonic Treg TCRs were cultured with Flt3L-elicited splenic DC and pools of bacterial preps or autoclaved colonic/cecal content preps (ACP). Flow cytometry to detect NFAT-GFP showed that several of the TCRs appear to recognize antigens present in the ACP from the TCliβ littermate mouse (Fig 3.4 and 3.5), but did not show reactivity to the C57BL/6 germ-free ACP. In addition, three TCRs showed reactivity to one or more of the pools of bacterial preps (Fig 3.5). Upon screening the individual components of these pools, it was found that each of these TCRs reacted specifically to one or more individual bacterial isolates present in the pools (Fig 3.6 and Table 3.4) This reactivity was dependent upon the presence of the DCs in the culture and was abrogated by the addition of a blocking antibody to MHC class II (Fig 3.7). Therefore, 7 of the 12 prevalent colonic Treg TCRs tested showed some reactivity to bacterial-derived antigens.

The identity of several isolates from the pools to which a hybridoma was reactive was determined by 16S rRNA sequencing of the isolates, and the results are shown in

Figure 3.4 Examples of screening results of CT7- and G57-expressing hybridoma cells. Shown are representative plots demonstrating the flow cytometry assessment of hybridoma reactivity by the percentage of $Vb6^{+}Va2^{+}$ cells that express the NFAT-GFP reporter following culture with APC + the indicated antigen or no antigen control (far right) used to assess background fluorescence. ACP, autoclaved cecum/colon prep. αMHC cII, blocking antibody to MHC class II was added.

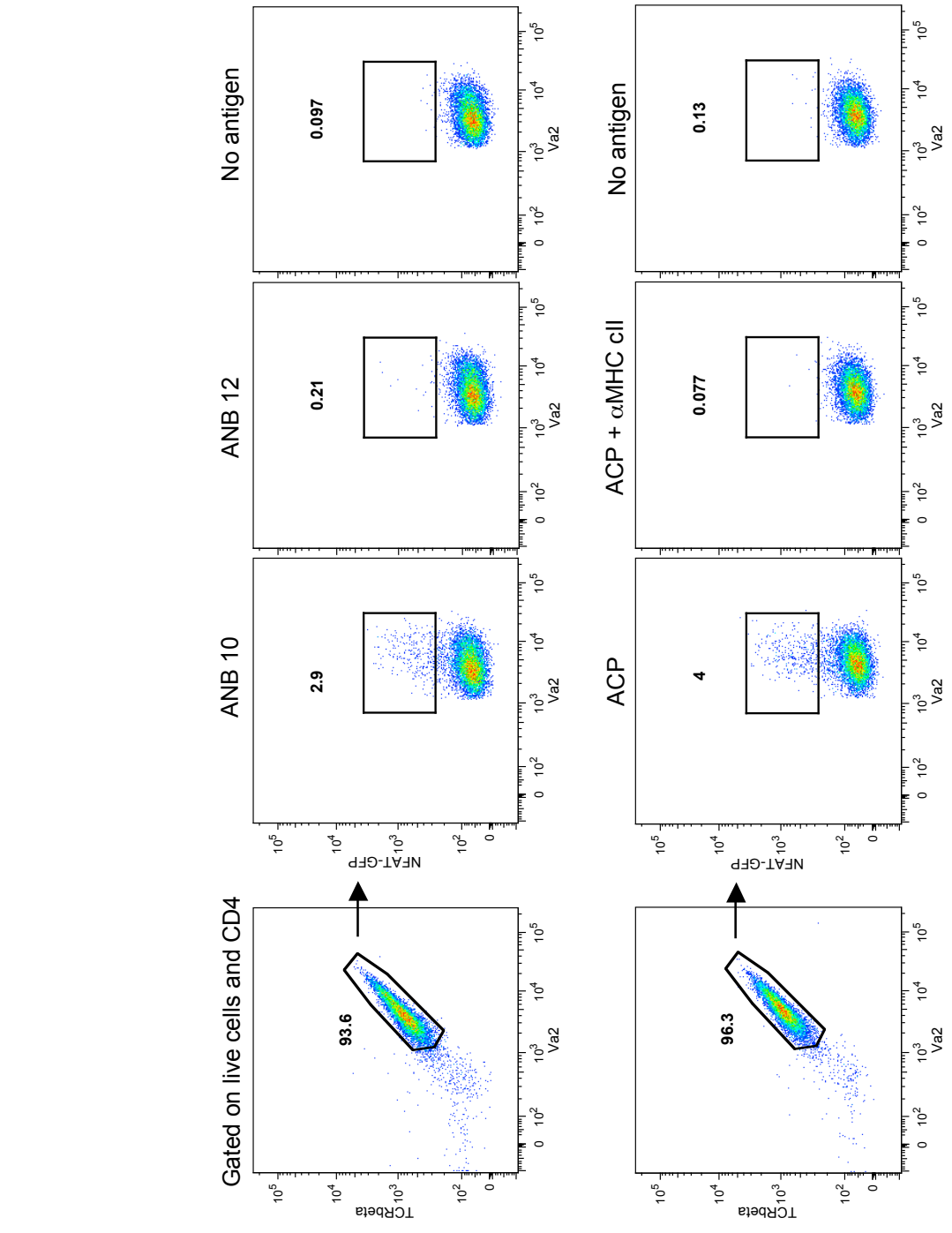


Figure 3.4 Examples of screening results using CT7- and G57- expressing hybridomas

Figure 3.5 Screening of hybridomas expressing prevalent colon TCRs against a panel of bacterial antigen pools and cecal/colonic content preparations. Hybridomas expressing the TCRs indicated and an NFAT-driven GFP reporter were screened by culture with Flt3L-elicited DC and the indicated antigen. After 40-44 hours hybridoma cells were stained for TCR expression and the percentage of cells that expressed GFP was measured by flow cytometery, as shown in Fig 3.4. The fold-increase over the percentage of cells expressing GFP in the no antigen added control culture was calculated for each sample and is shown on the bar graph. Screens of those hybridomas listed in Table 3.3 that did not show any reactivity are not depicted. ACP, autoclaved cecum/colon prep. G-F ACP, ACP from a germ-free mouse.

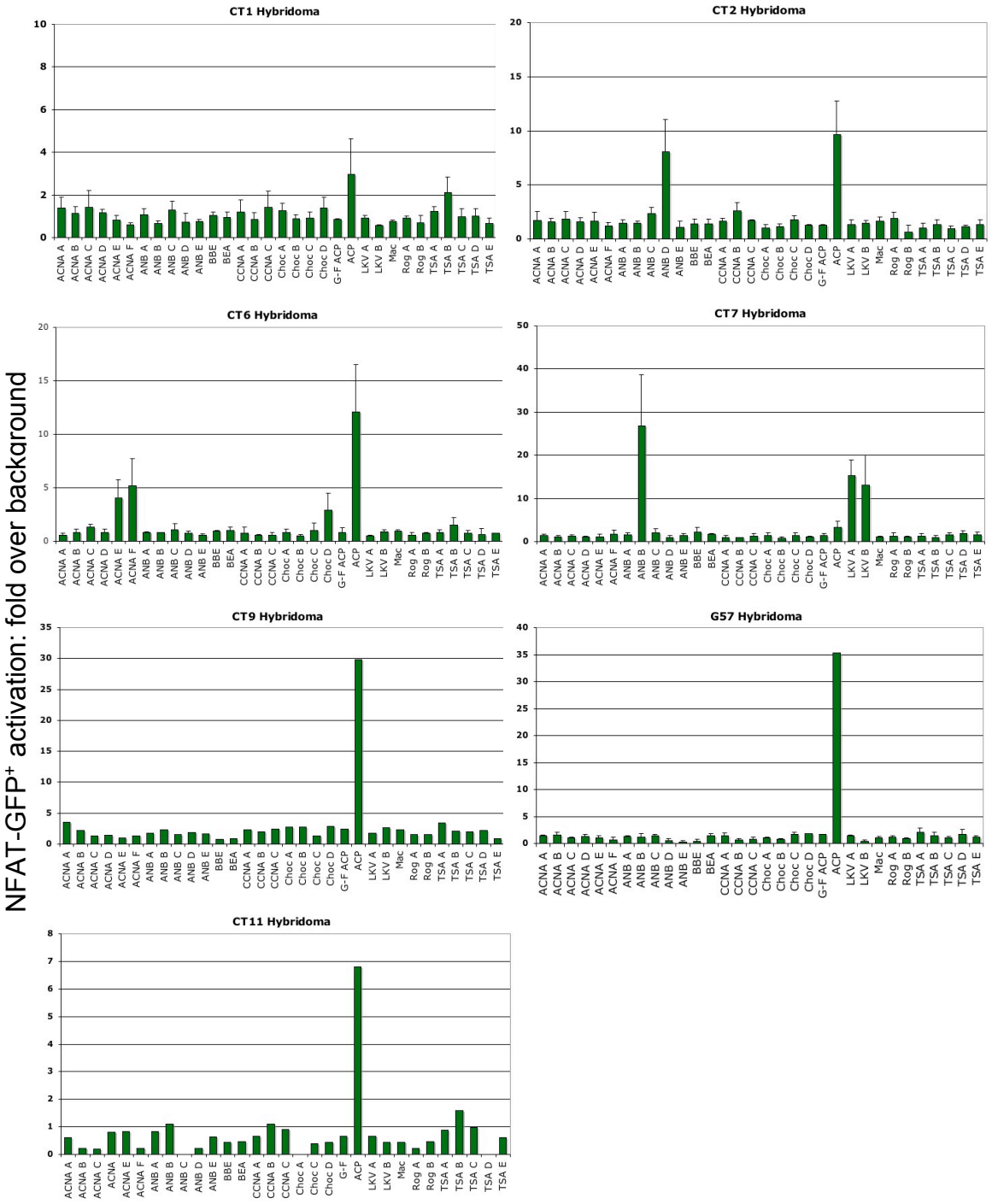


Figure 3.5 Screening of hybridomas expressing prevalent colon TCRs against a panel of bacterial antigen pools and cecal/colonic content preparations.

Figure 3.6 Reactivity to individual bacterial isolates by hybridomas expressing the CT2, CT6 or CT7 T cell receptors. The three hybridomas that showed reactivity to a pool of bacterial isolates in the assays depicted in Fig 3.5 were then screened against the individual components of that pool to determine which isolates they respond to. The fold-increase over the percentage of cells expressing GFP in the no antigen added control (shown on the right) is shown on the bar graph. Green bars show the reactivity to the pools (or no antigen control) and orange bars show the reactivity to the individual isolates which make up the pool shown on their left.

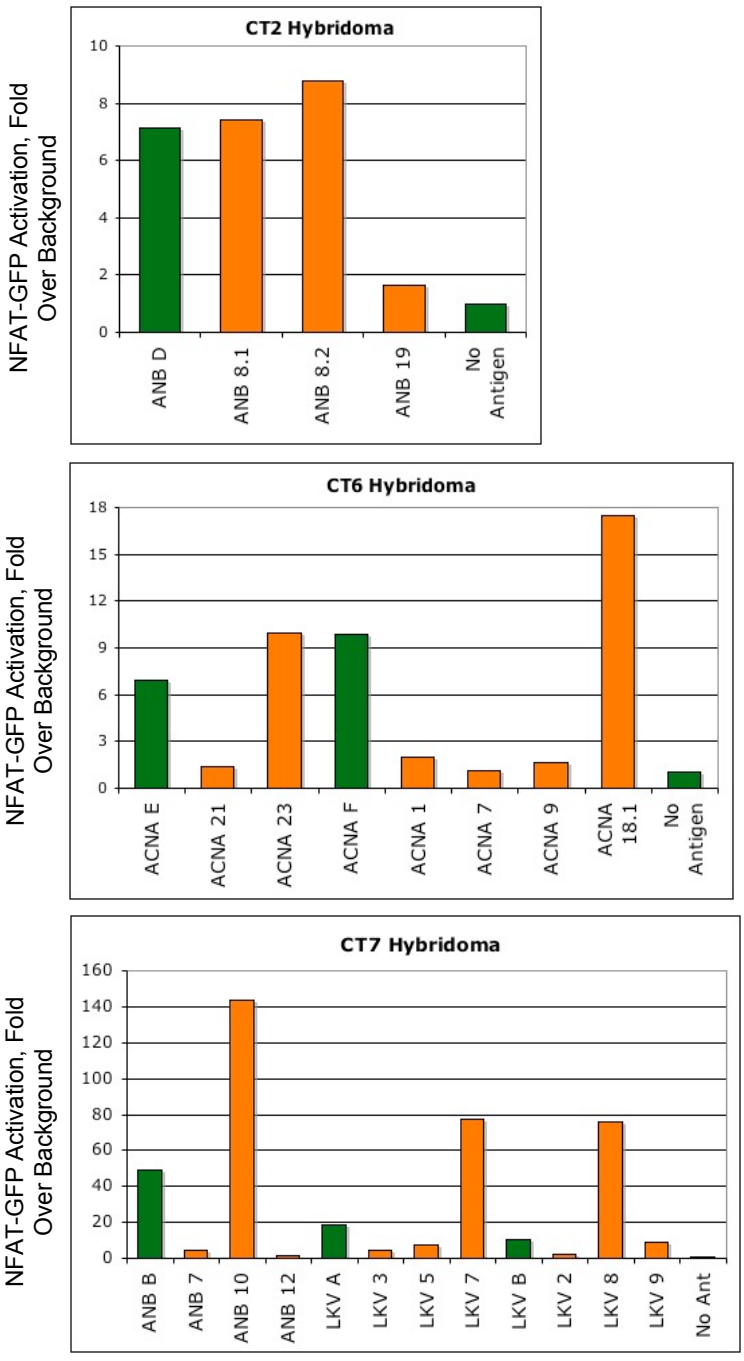


Figure 3.6 Reactivity to individual bacterial isolates by hybridomas expressing the CT2, CT6 or CT7 T cell receptors

Figure 3.7 Hybridoma reactivity to bacterial antigens is dependent upon presentation of antigen on MHC class II by APC. Shown are screens of the hybridomas indicated against the specific bacterial isolates with (green) or without (orange) addition of blocking antibody to MHC class II. Shown in blue are cultures in which no APC were added (No APC). acII, blocking antibody to MHC class II.

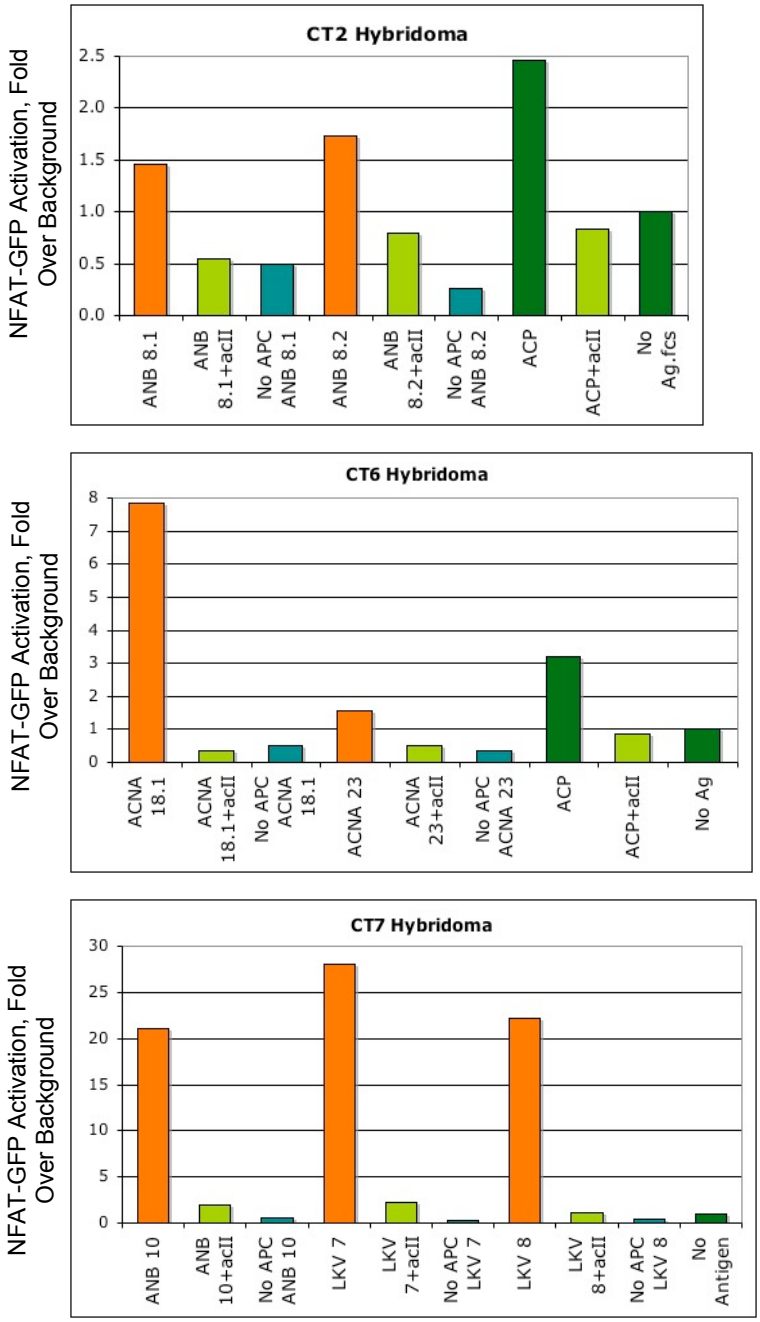


Figure 3.7 Hybridoma reactivity to bacterial antigens is dependent upon presentation of antigen on MHC class II by APC

Table 3.4 Summary of reactivity observed for each colon Treg TCR hybridoma tested.

Colon Treg TCR		Reactivity
CT1	AASWASGYNKLT	ACP
CT2	AASAIWNTGYQNFY	ANB 8.1 & 8.2, ACP
CT7	AASATGDNRIF	ANB 10, LKV 7, LKV 8
G57	AASELYQGGRALI	ACP
CT4	AASEYSALGRLH	none
CT6	AASGYSALGRLH	ACNA 18.1 & 23, ACP
CT8	AASLTGGYKVV	none
CT9	AASADNRAGNKLT	ACP
CT11	AASAPWNSNNRLT	ACP
CT12	AASAVWANTGKLT	none
CT10	AAQRRGNYKYV	none
CT13	AASRERYANKMI	none

Results of screening hybridoma expressing the indicated TCR against bacterial preps and ACP. Green=reactive to a specific bacterial prep. Gold=reactive to ACP but no specific bacteria identified. ACP=Autoclaved colonic/cecal content prep

Table 3.5. In each instance in which a hybridoma was reactive to more than one isolate, sequencing revealed that these isolates appeared to be identical, and the bacteria had likely been isolated more than once. Those isolates in the pools which did not cause hybridoma reactivity were found to be completely unrelated to the isolates that did, further confirming that each of these TCRs recognizes an antigen found only a specific species or in closely related species. In addition, the three bacteria that caused reactivity are from different phyla or orders, and are therefore not at all closely related to each other. Therefore, it seems that a diverse selection of bacteria may be recognized by colonic Treg.

T cells expressing colonic Treg TCRs do not become Foxp3$^+$ in the thymus.

Most Treg are selected to become Foxp3$^+$ regulatory cells during thymic development, presumably due to recognition of a thymically-presented self antigen. Therefore, the realization that many colonic Treg TCR recognize bacterial antigens, which presumably would not be available in the thymus, led us to wonder whether these Treg arose via peripheral conversion from mature non-regulatory cells, or if they are thymically-selected Treg which happen to cross-react with bacterial antigens. We chose several of the top colon Treg TCR to test, as well as a three Treg TCR normally found in other sites in the body, two of which we have previously shown undergo thymic Treg development (G25 and R19) [189], and as a negative control, a TCR only found on naïve T cells (B8) (Table 3.6). To test the induction of Foxp3 expression in the thymus by cells expressing these colonic Treg TCR we retrovirally introduced the TCli TCRβ chain and TCRα chain in question into thymocytes from *Foxp3^{gfp} Rag1$^{-/-}$* mice. As these mice

Table 3.5 Identification of selected bacterial isolates by 16S rRNA sequencing

Isolate name	Closest Bacterial Type Strain	Similarity by BLAST2	RDP Assignment Detail (for Confidence threshold: 80%)
***ANB 8.1**[a]	Allobaculum stercoricanis	85%	Phylum: "Firmicutes"; Class: "Erysipelotrichi"; Order: "Erysipelotrichales"; Family: Erysipelotrichaceae; Genus: Allobaculum
***ANB 8.2**[a]	Allobaculum stercoricanis	85%	Phylum: "Firmicutes"; Class: "Erysipelotrichi"; Order: "Erysipelotrichales"; Family: Erysipelotrichaceae; Genus: Allobaculum
ANB 19[b]	Propionibacterium acnes	100%	Actinomycetales; Suborder: Propionibacterineae; Family: Propionibacteriaceae; Genus: Propionibacterium
***ACNA 23**	Clostridium clostridioforme	92%	Phylum: "Firmicutes"; Class: "Clostridia"; Order: Clostridiales
ACNA 1[b]	Propionibacterium acnes	100%	Actinomycetales; Suborder: Propionibacterineae; Family: Propionibacteriaceae; Genus: Propionibacterium
ACNA 7[c]	Lactobacillus murinus	99%	Phylum: "Firmicutes"; Class: "Bacilli"; Order: "Lactobacillales"; Family: Lactobacillaceae; Genus: Lactobacillus
ACNA 9[c]	Lactobacillus murinus	99%	Phylum: "Firmicutes"; Class: "Bacilli"; Order: "Lactobacillales"; Family: Lactobacillaceae; Genus: Lactobacillus
***ANB 10**[c]	Parabacteroides distasonis	97%	Phylum: "Bacteroidetes"; Class: "Bacteroidia"; Order: "Bacteroidales"; Family: "Porphyromonadaceae"; Genus: Parabacteroides
ANB 12	Bacillus circulans	100%	Phylum: "Firmicutes"; Class: "Bacilli"; Order: Bacillales; Family: Bacillaceae; Genus: Bacillus
LKV 3[d]	Bacteroides acidifaciens	99%	Phylum: "Bacteroidetes"; Class: "Bacteroidia"; Order: "Bacteroidales"; Family: Bacteroidaceae; Genus: Bacteroides
***LKV 7**[c]	Parabacteroides distasonis	97%	Phylum: "Bacteroidetes"; Class: "Bacteroidia"; Order: "Bacteroidales"; Family: "Porphyromonadaceae"; Genus: Parabacteroides
***LKV 8**[c]	Parabacteroides distasonis	97%	Phylum: "Bacteroidetes"; Class: "Bacteroidia"; Order: "Bacteroidales"; Family: "Porphyromonadaceae"; Genus: Parabacteroides
LKV 9[d]	Bacteroides acidifaciens	99%	Phylum: "Bacteroidetes"; Class: "Bacteroidia"; Order: "Bacteroidales"; Family: Bacteroidaceae; Genus: Bacteroides

Isolates from pools which caused activation of a hybridoma were sequenced to determine their identity. * = isolate caused activation of a hybridoma. Isolates with matching letter designations were identified to be identical by sequencing. Similarity indicates similarity to the type strain indicatd. Top, isolates from pools simulatory for CT2; Middle, stimulatory for CT6; and bottom, stimulatory for CT7.

Table 3.6 TCRs used in thymic development studies

T Cell Receptor		Foxp3$^+$				CD44^{lo}				Reactivity
Name	CDR3 A.A. Sequence	COLON	MLN	SPL	CLN	COLON	MLN	SPL	CLN	
G25	AASADYSNNRLT	0.56	0.38	0.51	1.96					NA
R19	AASSGTYQR		0.36	0.20	1.42					NA
R111	AARPSGSFNKLT	0.39	2.00	0.39	3.03					NA
CT1	AASWASGYNKLT	16.04	1.44	0.47	0.20					ACP
CT2	AASAIWNTGYQNF	7.52	0.50	0.18	0.11					genus *Allobaculum*, ACP
CT4	AASEYSALGRLH	0.66	0.23							none
CT6	AASGYSALGRLH	0.28	0.12							genus *Clostridium*, ACP
CT7	AASATGDNRIF	2.96	0.21	0.04						*Parabacteroides distastonis*
G57	AASELYQGGRALI	2.30								ACP
CT9	AASADNRAGNKLT	3.80								ACP
CT11	AASAPWNSNNRLT	1.00								ACP
B8	AASEDNNNAPR					5.74	4.47	6.84	3.18	NA

Average frequency of TCR (in %) in the dataset indicated. Frequencies < 0.01% were excluded from the table. ACP = autoclaved cecum/colon prep. NA = not applicable, as TCR was not screened. Pink = Treg TCRs from non-colon sites, Green = colon Treg TCRs, Blue = naïve TCR

cannot rearrange their endogenous TCRβ chain there is an accumulation of cells in the $CD4^{-}CD8^{-}$ (DN) stage; only those cells which are successfully transduced will be able to progress through positive and negative selection. The thymocytes were then injected into the thymus of a CD45.1 congenic host in order to allow development to take place, and after 14-16 days the thymocytes from these host animals were analyzed by flow cytometry. The donor thymocytes expressing the TCR of interest were identified by CD45.2 expression, as well as Vβ6 and Vα2, and analyzed for the expression of $Foxp3^{gfp}$ (Fig 3.8 A).

We found, as expected, that the three Treg TCR normally found in non-colonic sites in the mice, R19, R111 and G25, showed induction of $Foxp3^{gfp}$ in this assay (Fig 3.8 B, red dots). In agreement with previous results, we saw that a higher percentage of G25-expressing CD4 SP thymocytes expressed Foxp3 compared to R19-expressing cells [189]. The R111 TCR showed a similar efficiency of Foxp3 induction as R19. The naïve TCR, B8, did not show any Foxp3 expression, as would be expected. These results verify this assay as capable of identifying TCRs that are normally mediate selection into the $Foxp3^{+}$ regulatory cell lineage within the thymus.

In stark contrast, all 8 colonic Treg TCRs tested in this assay showed no Foxp3 expression in the thymus (Fig 3.8 B, green dots). This included a TCR that did not show any detectable bacterial reactivity with the hybridoma screening, CT4. These results suggest that these colon Treg TCR do not encounter antigen in the thymus, but leave the thymus as naïve T cells which upregulate Foxp3 upon antigen recognition in the colon. This may be a result of interaction with a $CD103^{+}$ DC presenting antigens derived from commensal bacteria, as this subset of gut DC has been shown to efficiently mediate

Figure 3.8 Foxp3 expression by thymocytes expressing selected Treg TCRs or a naïve control TCR. Thymocytes from *Foxp3*^{gfp}*Rag1*$^{-/-}$ CD45.2+ donor mice were transduced with the indicated TCR and injected into the thymus of a sublethally irradiated CD45.1 recipient mouse. (A) (top) Gating strategy used to identify donor CD4 SP thymocytes expressing the transduced TCR 14-16 days after transfer. (bottom) Representative plots showing the level of Foxp3^{gfp} expression by recovered thymocytes expressing the R19, CT7, or B8 TCRs, as indicated. (B) Graph plotting the percentage of recovered donor CD4 SP thymocytes expressing Foxp3^{gfp} for each tested TCR. Each dot represents one animal; data from each TCR is from at least two individual experiments. Red TCRs are those normally found in non-colon Treg populations (shown in pink in Table 3.6), while those in green are colon Treg TCRs (green in Table 3.6). In blue is the control naïve T cell TCR, B8.

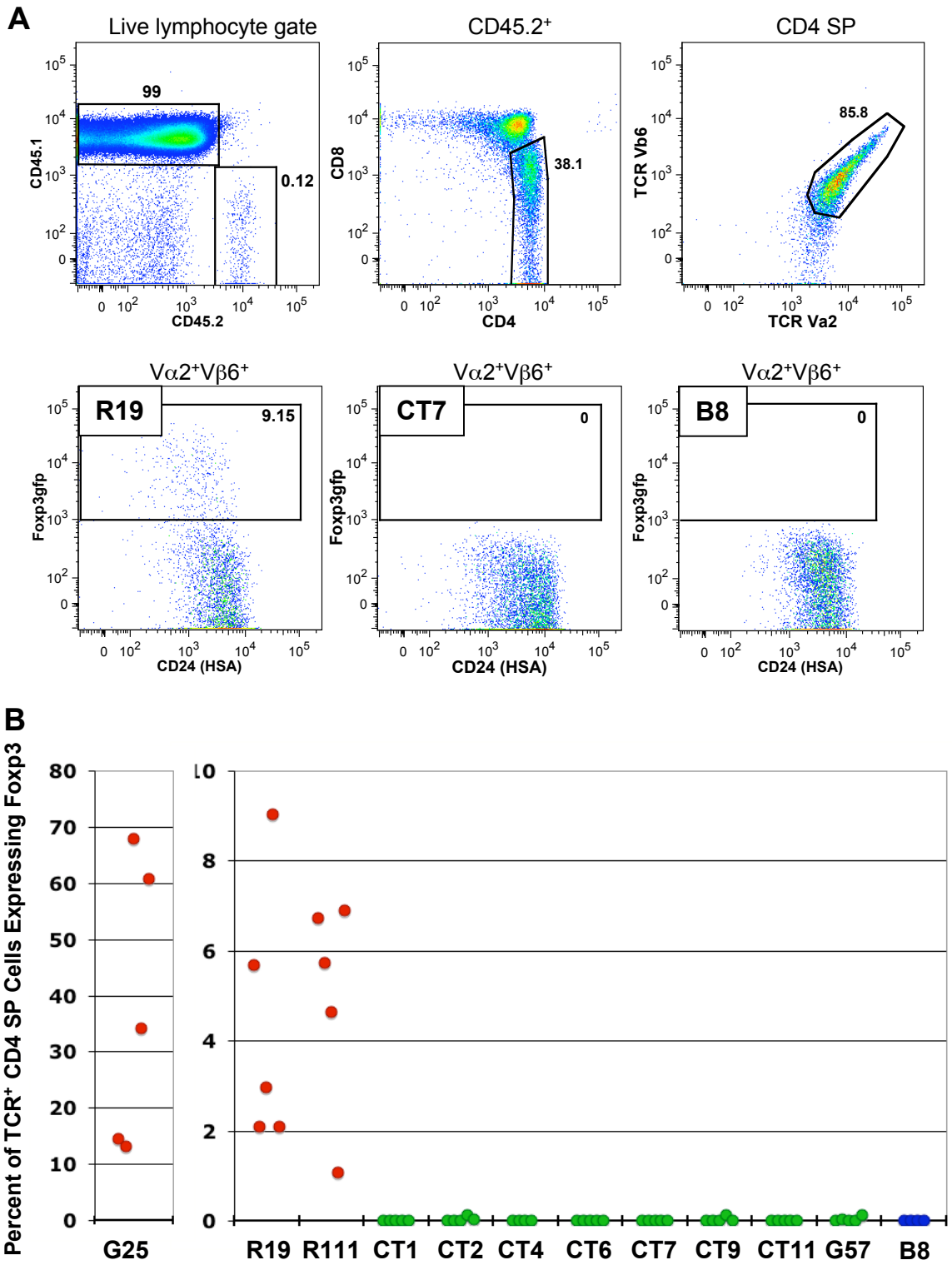


Figure 3.8 Foxp3 expression by thymocytes expressing selected Treg TCRs or naïve control TCR.

peripheral conversion, as well as upregulate gut-homing receptors on T cells [44, 124, 129]. As these TCR were all found to be quite abundant in the colon Treg TCR repertoire, it is likely that peripheral conversion plays a larger role in the generation of this population than previously suspected.

T cells expressing colonic Treg TCRs in vivo *do not express Foxp3 in the thymus, but upregulate Foxp3 upon encountering bacterial antigen in the periphery.*

To further investigate the possibility that these colon Treg are a product of peripheral conversion rather than thymic Treg selection we chose to create retroviral mixed bone marrow chimeras expressing the CT2 and CT6 TCRs. These TCRs were shown to recognize specific bacterial antigens (Fig 3.5 and Table 3.4), and did not appear to undergo thymic selection into the Treg pool (Fig 3.8 B). Retroviral bone marrow chimeras were created by mixing $Foxp3^{gfp}CD45.1^{+}$ congenic bone marrow with (CD45.2) $Foxp3^{gfp}Rag1^{-/-}$ bone marrow transduced with a retrovirus encoding the TCli TCRβ chain and either the CT2 or CT6 TCRα chain (see Materials and methods). These mice were allowed to reconstitute their T cell populations for 8 – 9 weeks, and then cells from the thymus, spleen, MLN, pooled axillary, inguinal and cervical LN (referred to as "peripheral"LN or PLN) and the colon/cecum LP were isolated and analyzed by flow cytometery.

Initial experiments confirmed that there was no detectable expression of Foxp3 in the CT2- or CT6–expressing thymocytes (orange symbols, Fig 3.9 A and 3.10). However, these initial experiments also did not show any Foxp3 expression by the CT2- or CT6-expressing cells recovered from the periphery, including the colon LP. We suspected that

Figure 3.9 Foxp3 expression by T cells expressing the CT2 TCR in mixed retroviral bone marrow chimeras. Bone marrow chimeras were made with a mixture of wild-type CD45.1$^+$ bone marrow and CT2-transduced CD45.2$^+$ bone marrow from *Foxp3^{gfp}Rag1$^{-/-}$* donors, and injected into CD45.1$^+$ lethally irradiated recipients. At 8-9 weeks of post-injection the CD45.2$^+$ T cells were recovered from the thymus, spleen, mesenteric LN, the axillary, inguinal and cervical LNs (pooled) and the colon lamina propria. The percentage of CT2-expressing CD4 SP (thymocytes) or CD4$^+$ T cells expressing Foxp3^{gfp} was assessed. (A) Results of experiments in which the chimeras were allowed to develop undisturbed (orange) or cohoused approximately two weeks after injection with a littermate mouse of the TCli TCRβ transgenic mice used for sequencing studies (Fig 3.1-3.3, Tables 3.1 and 3.2) (green). Each symbol is represents an individual animal, and data is from 3 individual experiments. (B) Additional experiment as described in A, in which the chimeras were cohoused as described. The data from this experiment is shown on a separate graph for clarity due to the development of a higher percentage of Foxp3$^+$ cells.

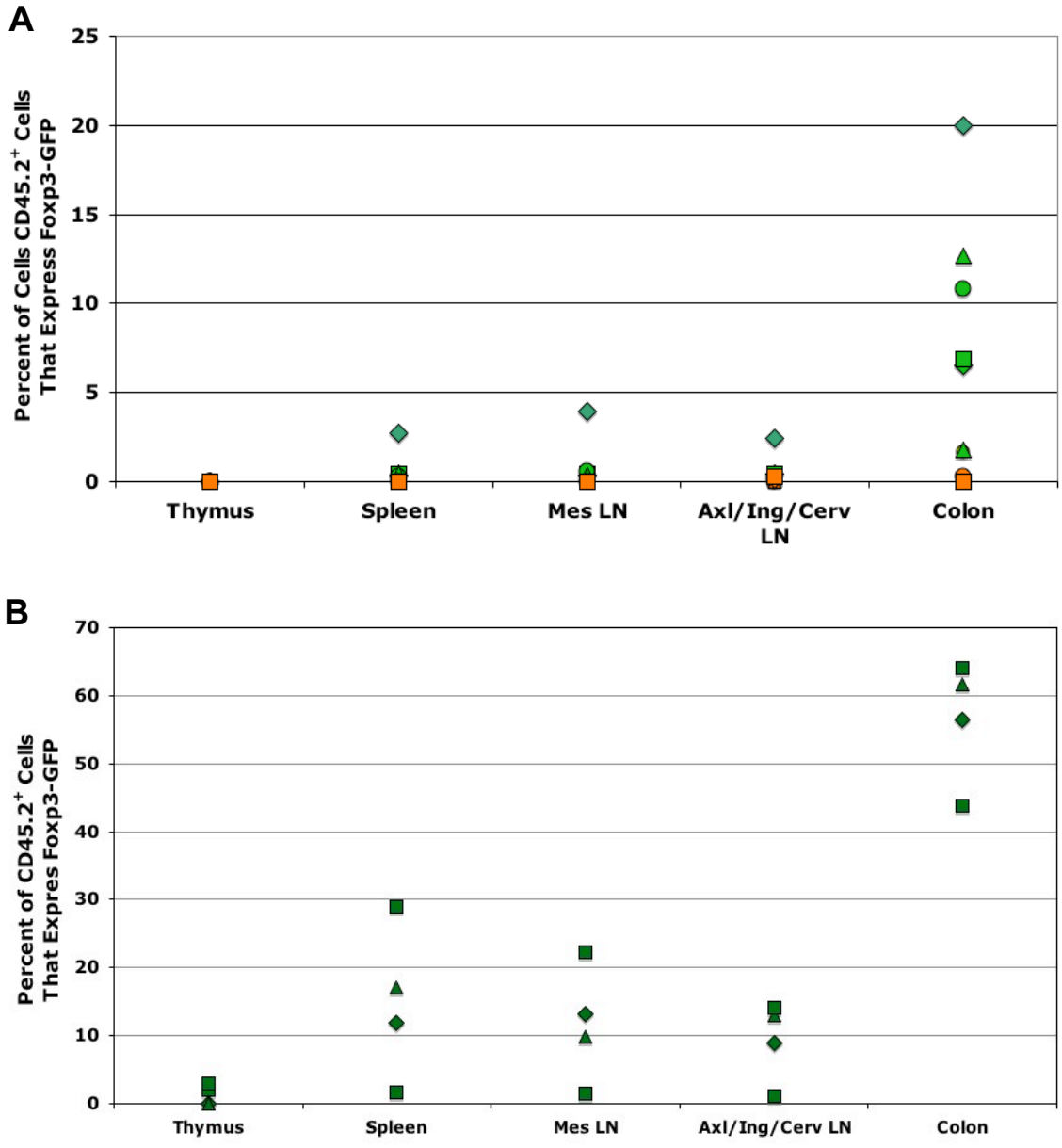


Figure 3.9 Foxp3 expression by T cells expressing the CT2 TCR in mixed retroviral bone marrow chimeras.

Figure 3.10 Foxp3 expression by T cells expressing the CT6 TCR in mixed retroviral bone marrow chimeras. Bone marrow chimeras were created with CT2-transduced CD45.2^{+} bone marrow from *Foxp3*^{gfp}*Rag1*$^{-/-}$ donors as described in Fig. 3.9. As before, the percentage of recovered CT2-expressing cells that are Foxp3^{+} is shown, and orange symbols represent mice that were not cohoused, while green symbols represent those that were. Data is from four individual experiments.

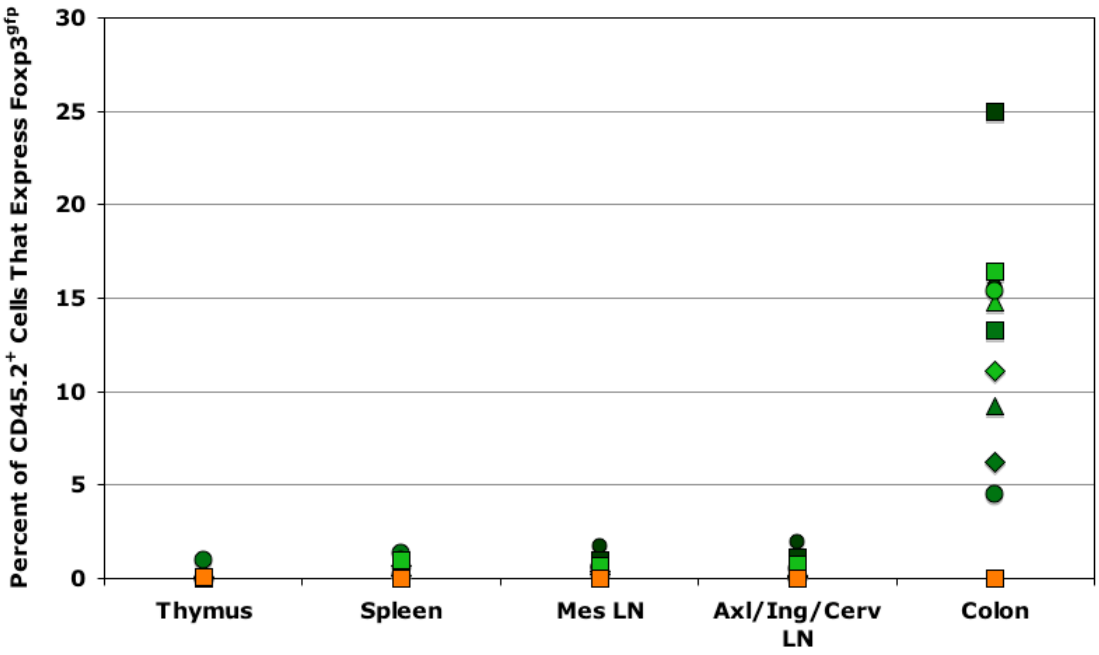


Figure 3.10 Foxp3 expression by T cells expressing the CT6 TCR in mixed retroviral bone marrow chimeras.

this might be due to a lack of the appropriate bacteria in the gut. The recipient mice in these experiments were obtained from the National Cancer Institute (NCI), and since they were not bred in our colony their colonic microbiota might have a different composition, and therefore be missing the bacteria recognized by CT2 and CT6.

Therefore, in repeat experiments we cohoused the chimeras for 7-10 days with a female TCli TCRβ chain littermate mouse, starting at approximately two weeks after bone marrow injection, to see if the bacteria could be transferred between the animals. Indeed, in the mice which had been cohoused, analysis of the peripheral T cells, particularly the colon LP T cells, showed Foxp3^{gfp} expression in the CT2- or CT6-expressing cells (Fig 3.9 A and B, 3.10, green symbols). Non-cohoused animals continued to show no sign of Foxp3 expression (orange symbols). Therefore, it seems that colon Treg expressing the CT2 or CT6 TCR are a product of peripheral conversion, which occurs as a result of bacterial-derived antigen recognition in the colon.

Chapter Three: Discussion

With these experiments we have demonstrated that the colon Treg population expresses a unique repertoire of TCRs, which does not overlap with those used by Treg in the other locations in the body that we have sequenced, nor with the $CD44^{hi}$ memory T cell population. The very distinct set of antigens recognized by these T cells may in large part be derived from the abundant commensal microbiota, as we have gone on to show that the most prominent colon Treg TCR recognize bacterial antigens. Furthermore, T cells expressing these TCRs do not show signs of expressing Foxp3 during selection in the thymus, suggesting that Foxp3 expression is a result of peripheral conversion. Indeed, for T cells expressing two of these bacterial reactive TCRs, CT2 and CT6, we have confirmed that no Foxp3 expression is seen during thymic development in a retroviral bone marrow chimera. In these mice, Foxp3 expression occurs only in the periphery, and is dependent upon commensal bacteria found in the TCli TCRβ transgenic mice used in our TCR sequencing studies. In summary, we have directly shown for the first time that some colonic Treg cells develop in response to peripheral conversion due to recognition of antigens derived from the commensal microbiota.

That peripheral conversion would be a source of colonic Treg is not particularly surprising in light of data showing abundant conversion in the small intestine and MLN in models of oral tolerance [44, 124]. Conversion in the small intestine is mediated by lamina propria $CD103^{+}$ DC which can transport antigen to the MLN to present antigen to naïve T cells . Here we show evidence that peripheral conversion also plays a role in the shaping of the Treg repertoire in the colon, as well, presumably through the same mechanism.

The lack of similarity between the colonic and MLN Treg repertoires is somewhat surprising, particularly in light of these results. One study has shown that for peripheral conversion to occur in a model of alloantigen tolerance the T cells must be able to occupy the lymph nodes; T cells from mice that did not express CD62L were not converted, presumably due to an inability to access the LN and interact with the APC mediating conversion [190]. It has also been shown that antigen presentatio in the MLN is required for oral tolerance induction [191]. It is unknown if this also holds true for T cells in the colon, as the lymphoid architecture in this region is unique. The majority of the MLN drain the small intestine rather than the colon, so while this may be a site of peripheral conversion against small intestine antigens, it may not hold true for colonic antigens. The colon lamina propria contains isolated lymphoid follicles (ILF) that might act as an additional site of conversion. Studies of germ-free mice have shown that the development of the ILFs is dependent upon the presence of the commensal microbiota, and that the bacterial load and composition affect the size and number of ILFs [192]. It is possible that the ILFs, with their proximity and responsiveness to the commensal microbiota, are actually the major site of T cell/DC interaction in the colon.

We were surprised at the number of prominent colon TCRs which showed bacterial reactivity. It would be expected that many bacterial-responsive TCRs did not show reactivity in our screen because they recognize a bacterium which was not present at a sufficient concentration in the ACP, and therefore the number of TCRs which were positive in our assay probably underestimates the true number. While several of the TCRs recognized ACP, we were able to pinpoint the reactivity to specific bacteria cultured out of mice in our colony for only three TCRs (one of which, CT7, did not show

reactivity to ACP). It is estimated that less than half of the bacterial species in the microbiota are unculturable by currently developed methods . Therefore, the four ACP-reactive TCRs which did not react to any of our bacterial isolates likely recognize antigens from organisms we were unable to culture.

The high number of TCRs reactive to ACP (6 of the 12 tested) may reflect the ability of more abundant commensal organisms to induce Treg directed against them. This may be a bacterial-driven process, such as the production of a molecule that assists in tolerance induction, or properties that maintain a tolerogenic state in APCs upon their phagocytosis. Tolerance may also just be a passive result of the lifestyle of these bacteria. For instance, they may favor a location which facilitates their uptake in a way that promotes tolerance. In effect, the ability to induce Treg tolerance may be a hallmark of a successful commensal organism, and may help explain the fairly conserved colon Treg TCR repertoire across multiple mice and experiments.

Our selection criteria of choosing colon Treg TCRs which are found almost exclusively in the colon, and were detected in multiple experiments (see Table 3.3), may have selected for bacterial-reactive TCRs. While it would be expected that some colon Treg recognize conserved self antigens, these TCRs may be found in other locations as well, and therefore not chosen by us for further study. However, the low similarity between colon and other organs studied suggest that these Treg are in the minority (Fig. 3.2) It may be that these Treg are outcompeted by the bacterial-reactive Treg in the colon, which probably receive frequent restimulation by APCs that continue to encounter these abundant bacterial antigens. DC are not the only cell type in the intestine which can present bacterial antigens, and therefore the Treg may also encounter their cognate

antigens on macrophages and on the intestinal epithelial cells (IEC), which also express MHC class II. It is possible that in the absence of bacterial-reactive Treg, more Treg recognizing conserved self antigens would inhabit the intestines and be capable of maintaining homeostasis to some degree. For example, in mice lacking a conserved noncoding sequence in the Foxp3 gene required for peripheral conversion, there is a reduction in the size of the Treg population of the GALT, but these mice do not spontaneously develop inflammation [149]. It would be interesting to see whether these mice are at increased risk for IBD development if crossed to models of spontaneous colitis development, or if the existing colon Treg repertoire that develops in these mice would be as efficient as wild type Treg in preventing disease in the classic model of transfer colitis.

Chapter Four: Discussion

Is antigen specificity necessary for regulatory T cell suppression?

The research detailed in this thesis centers around the part that antigen specificity plays in Treg function. It can be inferred from our results that, *in vivo*, Treg often do have antigen specificity. This is the simplest explanation for the segregation of TCR usage seen in the Treg populations of the spleen, lymph nodes, and even the colon. However, that does not mean that Treg must have this antigen specificity to perform their function, although most people agree that Treg need to receive TCR stimulation to mediate their suppressive functions [51, 76].

There are studies suggesting that Treg can act to suppress T cell effector function against antigens which they do not themselves recognize. For example, in a model of autoimmune gastritis, in which polyclonal T cells were able to prevent disease through "bystander suppression" [72, 73]. A follow-up paper by this group, however, reported that antigen-specific Treg were better suppressors in this system. In a model of Friend virus infection of the hematopoietic system in which a lack of regulatory activity will result in anemia, it was found that the Treg capable of preventing the anemia were not specific for Friend virus [193]. Additionally, a common model of colitis relies on the transfer of non-regulatory T cells into a lymphopenic host, and disease is prevented by the co-transfer of a population of Treg. However, it seems unlikely in this system that most of the Treg transferred are specific for gut antigens, as they generally not isolated from the GALT. Treg isolated from germ–free mice were reported to be fully capable of preventing disease in this same transfer model, although they were not as efficient as

those from conventional mice [148]. However, there is also a report that Treg from germ-free mice are unable to prevent disease in this model [147].

These studies appear to conflict with others that demonstrate antigen specificity of Treg. In one paper it was shown that *in vitro* Treg with a known antigen specificity capable of suppressing effector cells with the same specificity, but not those with a different defined specificity [75]. Additionally, studies showing that protection from organ autoimmunity relies on Treg cells that arose in an animal containing the organ in question suggest that only Treg specific for that organ can prevent disease [78, 80, 155].

These data are most easily reconciled by the theory that Treg are most effective at suppression of T cells when the antigens recognized by each are presented on the same APC (which would most commonly occur when these antigens arise from the same tissue or organism), but they can also mediate non-specific suppression less efficiently. Improved efficiency of suppression is likely to be a result of mechanisms that rely on direct interactions between the Treg and the effector cell, and/or tolerogenic effects the Treg may have on the APC. Therefore, this allows for very efficient protection of those potential targets for which Treg are specific, and explains how they are able to maintain homeostasis even while they are outnumbered *in vivo.* However, if a Treg in the vicinity of an effector cell is receiving TCR stimulation it might have a non-specific tolerogenic effect on its surrounding through the release of cytokines. The ability of Treg to control T cells with a different antigen specificity may therefore be a numbers game – if you have enough of these cells, they will abrogate the immune response through passive suppression, possibly combined with the effect of some Treg recognizing its antigen on an APC that happens, by chance, to also present antigen to the effector cell. The

combination of these will then, somewhat less efficiently, be capable of controlling responses in a "non-specific" way.

The pattern of localization seen by regulatory T cells is similar to the localization of antigen-experienced memory T cells. T cells are conditioned to stay in the area in which they recognize antigen and become activated, and regulatory T cells, as antigen-experienced cells, are no exception. Since Treg do require stimulation through their antigen receptor to suppress, whether or not their target is also recognizing the same antigen, it makes sense to keep them in the area in which they will find that stimulation. Therefore, as with memory T cells which are most effective at maintaining immunity it kept at the ready in the site where the antigen previously turned up, this pattern of localization improves the likelihood that a Treg will receive the stimulation it requires to do its job efficiently.

Does peripheral conversion play a role in the non-mucosal immune system?

Many studies in which antigen was supplied *in vivo* under non-inflammatory conditions have shown that peripheral conversion of mature T cells into $Foxp3^+$ regulatory T cells CAN occur [38, 39, 41, 162, 194, 195]. There doesn't seem to be any special characteristic of the T cells able to undergo conversion; it seems that if you present a T cell with its cognate antigen under the right conditions it may become a Treg. Our data suggests that only T cells expressing a certain subset of TCR can be converted (Fig 2.15). However, *in vivo*, this restriction likely has more to do with the antigens than the TCR or T cell properties. Only certain antigens will be presented under the proper

conditions for Treg to develop, therefore limiting the T cells capable of converting to those able to recognize this subset of antigens.

This ability of T cells to become regulatory serves as an excellent way to enforce tolerance to certain antigens, whether or not they were presented in the thymus. The body needs a system by which tolerance can be maintained to newly acquired or manufactured antigens, or even typical self antigens which may not be thymically presented. Further, even antigens which are expressed in the thymus are often expressed at low amounts, and it would be impossible for all T cells to encounter all possible antigens. This results in some T cells which strongly recognize self antigens escaping negative selection, and it has been clearly shown that there are self-reactive T cells normally in the body; in the absence of proper regulation these cells can cause autoimmunity. The flip side of this antigen limitation is that some potential Treg will also escape their induction into the Treg lineage, and enter the periphery as a typical naïve T cell.

This limited niche for Treg development was demonstrated by the recent paper from our lab showing that, for any one particular TCR specificity, there are a limited number of Treg that can be induced [189]. This number varies, most likely depending upon the availability of the particular antigen recognized. If you have a large number of T cells with a particular specificity, they cannot all receive sufficient signal to become Treg, and a lower percentage of these cells will upregulate Foxp3 in the thymus. In the TCR transgenic mouse expressing a Treg TCR, G113, there was no Foxp3 induction in the thymus. However, if the prevalence of these cells in the thymus was reduced by mixing with polyclonal T cells in a mixed bone marrow chimera, suddenly the G113 cells no longer overwhelmed the supply of their (unknown) cognate antigen, and some were

able to receive the signal to become a Treg. The TCR transgenic mouse did, however, show some Foxp3^{+} cells in the periphery even when there was no visible thymic development. These cells may be a result of a "second chance" at Foxp3 induction for cells that missed out on the opportunity in the thymus by peripheral conversion.

Our studies and others estimate that peripheral conversion does not contribute a large amount to the normal Treg repertoire. This is probably because only a few cells will require this "second chance" mechanism, which may account for the small number of Treg (4-7%) estimated to arise from conversion in the secondary lymphoid organs. However, during ontogeny the contribution may be greater, as the emerging T cells enter the somewhat lymphopenic periphery. Homeostatic expansion facilitates conversion [42, 43]. As we showed in Fig. 2.15, however, even conversion in a lymphopenic environment depends upon the TCR specificity; only those T cells receiving antigenic stimulation in the correct environment will become Treg, and therefore only the T cells recognizing self antigen will become regulatory.

It does seem, however, that peripheral conversion is not absolutely required to maintain homeostasis. A recent study identified a conserved, non-coding sequence in the Foxp3 gene which appears to be involved in peripheral conversion [149]. In spite of this defect, mice that are deficient in this region showed no spontaneous development of autoimmunity. There is likely to be a proportionally small number of cells in the periphery which have "missed" becoming Foxp3 in the thymus in a fully polyclonal animal, and therefore the thymically-derived Treg population is sufficient to maintain tolerance. Additionally, while conversion was reduced, it was not absent in these mice. However, it may be interesting to push this system and see if this lack of conversion does

affect the health of the mice under various conditions. For example, it has been reported that during infection with a pathogen, Treg can arise alongside Teff cells, and are thought to help restrain and shut down the immune response. Infection of these mice might show increased tissue damage, possibly increasing their mortality in response to certain pathogens. However, some pathogens that rely on induction of Foxp3^{+} Treg in order to establish chronic infections (such as *Leishmania major*) might be cleared by these animals [47].

Peripheral conversion and tolerance in the gut.

While it makes sense that peripheral conversion is not required on a large scale in much of the body, the mucosal immune system is in contact with an ever changing array of non-self antigens, and therefore it seems reasonable that Treg specific for harmless commensal organisms would be advantageous. As antigens derived from these organisms are unlikely to be transported to the thymus, at least in any useful amount, most Treg specific for commensal bacteria are likely to arise via conversion. Our data shows that many commonly found Treg in the colon do not arise from thymic Treg selection, and instead appear to develop in the periphery in response to exposure to bacterial antigens. While this may have been suspected, it is the first time that it has been shown that Treg recognize antigens derived from specific bacteria, and it seems that, at least in our experimental system, a large portion of the most prevalent Treg in the colon are actually bacterially-reactive. As an extension of these studies, it would be excellent to see if the Treg segregate even further within the various sections of the colon and cecum. The cecum could be processed on its own, and the colon separated into the ascending colon,

transverse colon, and rectum/anal-rectal junction. If the Treg in these portions do show differences in their TCR usage, it would be very useful to correlate the natural habitat of the bacteria to which these Treg are specific to the Treg localization, if possible.

In the previously-mentioned study of the Foxp3 CNS1-deficient mice [149], T cells show a defect in peripheral conversion, and have a lower number of $Foxp3^+$ cells in the gut-associated immune system. In spite of this, they report no development of spontaneous gut inflammation in these animals. This may seem surprising considering the evidence that conversion plays a large role in the formation of the Treg repertoire at this location. However, in these mice conversion is not completely absent, just reduced. Therefore, enough cells may created to keep homeostasis intact, at least in an unperturbed system. They also have not specifically shown that the reduction is due to a lack of conversion rather than a shorter lifespan of these converted Treg; they may, by their nature, be less stable. Therefore, at any one time the gut immune system may contain a reasonable array of bacterial-specific Treg capable of preventing disease, even if they may not survive as long. It would be very interesting to compare the repertoire of TCRs of the Treg populations in the CNS1-deficient and wild-type mice; this should allow for the determination of whether the cells expressing typical Treg TCRs do not become Foxp3+, or are simply reduced in frequency. The authors also speculate that some T cells which would normally become Treg may instead become IL-10 producing Tr1 cells; this theory could also be tested by the sequencing of these mice crossed to an IL-10 reporter mouse [25].

The real challenge of maintaining a healthy intestine becomes evident during infection with a pathogen. During such an event, there is often a breakdown in the mucus

layer and epithelial barrier, with a resulting high level of interaction between effector T cells and both the pathogenic and non-pathogenic commensal organisms. To restore homeostasis after such a pathogen is cleared and curb inflammation to allow repair of the mucosal barrier is likely a challenge requiring the highest level of suppressive activity available. In the absence of the normal number of the bacterially-responsive Treg capable of efficiently suppressing reactivity to the commensal organisms, a chronic inflammatory response may develop in the aftermath of such an event. Therefore, it would be interesting to see whether these mice develop intestinal inflammation after infection with an intestinal pathogen. Although the authors saw no difference between the CNS1 KO mice and wild-type mice in resolution of DSS-mediated colitis, it has been shown inflammation in this model is not T cell dependent [196], and therefore resolution may not require a full Treg repertoire, and the increased number of IL-10-producing Tr1 cells seen in these mice may instead compensate.

Therapeutic considerations of regulatory T cells in disease

The fact that Treg play an important role in maintaining immune homeostasis and the observations that these cells are often lacking in either numbers or suppressive capability in autoimmune diseases has led to the attempt to use these cells therapeutically [197]. Strategies are two-fold; induce and expand Treg *in vivo*, or collect cells and convert/expand them *in vitro*, for reintroduction into the patient. Many current IBD therapies such as steroid regimens, neutralization of TNFα, and blockade of IL-12p40 (as a component of both IL-12 and IL-23) may reduce innate immune activation but may also have the ability to enhance Treg function or induce Treg, possibly through the restoration

of a permissive cytokine environment [177, 198, 199]. However, these broad-based treatments can lead to unwanted side effects and susceptibility to infectious disease, so more specific ways to target Treg are being developed [197].

Efforts to collect T cells from patients and then expand or induce Treg *in vitro* have not so far been very successful [200]. There is not yet a reliable way to convert human conventional T cells into stable, functional Treg *in vitro* on any large scale. Expansion of Treg is hindered by the relatively low proportion of Treg in the blood, a lack of a good technique to efficiently purify them away from the conventional T cells, and the very large number of cells required to make a therapeutic difference. Most likely the large number required is due to the lack of Treg specificity in this approach. In mouse models of diabetes and IBD, the successful cure of ongoing inflammation by administration of Treg required the Treg to be antigen-specific [201]. Of course, in most cases it is not practical to obtain antigen-specific Treg from humans, and this is where the potential therapy has reached its largest stumbling block. It has been proposed that perhaps a vaccination of antigenic peptide may expand specific Treg *in vivo*, as a way to get around this limitation [201]

It has been discovered that certain species of commensal bacteria can help to curb inflammation in the gut; these bacteria have been termed probiotics. Many species have been described to control inflammation, and in many cases the induction of Treg may be central to this function [96]. A well-studied example is the VSL#3 cocktail of probiotics, which causes increased IL-10 production and a population of TGFβ-producing cells that prevent colitis in recipient mice upon transfer in a TGFβ-dependent manner [202]. Another is *Bifidobacteria infantis*, which led to an increase in $CD4^+CD25^+$ Treg and a

reduction in inflammation during infection with *Salmonella typhimurium*, and this protection could also be transferred with the Treg population. In some cases, bacteria that act as probiotics may produce a molecule that induce protective intestinal immune responses, such as polysaccharide A (PSA) produced by *Bacteroides fragilis* [203, 204]. Administration of purified PSA alone can protect against the induction of experimental IBD, and this is dependent upon the ability of T cells to produce IL-10, suggesting that this factor can cause the production or expansion of IL-10-producing regulatory cells of either the Tr1 or $Foxp3^+$ subsets. These probiotics have the advantage of being able to target directly an region and a subset of T cells which is directly relevant to disease in a way that is much more efficient than our current methods allow us to do *in vitro*. Additionally, it is non-invasive and very safe. These organisms and their immune effects warrant further study and hold promise in the safe effective treatment of intestinal inflammation.

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