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THE ENDOGENOUS REPERTOIRE HARBORS SELF-REACTIVE CD4^{+} T CELL CLONES THAT ADOPT A T FOLLICULAR HELPER-LIKE PHENOTYPE AT STEADY STATE

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To my mother and brothers,

whose adventurous spirits inspire boundless curiosity in me,

and

to Taral, Denali, and Everest,

whose companionships make the journey evermore colorful.

"The real voyage of discovery consists not in seeking new landscapes but in having new eyes."

-Marcel Proust

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ABSTRACT

Polymorphisms in human HLA class II alleles are associated with elevated susceptibility to many autoimmune diseases, highlighting a critical role for $CD4^+$ T helper cells in driving pathogenesis. Despite multi-layered tolerance mechanisms, the immune repertoire of healthy mice and humans harbors self-reactive $CD4^+$ conventional T cells capable of inducing autoimmunity when not properly regulated. However, little is known about the nature of these endogenous self-reactive $CD4^+$ T cells, with respect to their antigen specificity, differentiation, and functional properties at steady state and in inflammatory settings. Using a clonal approach involving deep T cell receptor (TCR) sequencing paired with the analysis of monoclonal T cell populations from TCR "retrogenic" mice, we identified numerous $CD4^+$ T cell clones that are recurrently enriched in non-lymphoid organs following sustained Treg cell ablation. These clones exhibit overt reactivity to splenic dendritic cells and are highly proliferative in the spleen and regional lymph nodes, suggestive of reactivity to widespread self-antigens, yet are not removed from the repertoire via clonal deletion. Phenotypically, these cells exhibit a T follicular helper (Tfh)-like phenotype characterized by expression of Bcl6 and PD-1, and variable expression of the canonical Tfh receptors CXCR5 and ICOS. Functionally, these cells localize as clusters within primary B cell follicles in the splenic white pulp, but do not appear to promote B cell activation. In sum, our work identifies a unique population of broadly self-reactive $CD4^+$ T cells that populate the endogenous repertoire of healthy mice.

CHAPTER I

INTRODUCTION

Innate and adaptive immunity

The concept of immunity from disease dates back as early as the 5th century BC in Greece. Historians of the time noted that individuals who had contracted and recovered from the plague somehow became "immune" or "exempt" from the disease[1]. The etiological agents of infectious diseases remained obscure until Louis Pasteur and Robert Koch identified pathogenic bacteria as the source of such diseases and established the "germ theory for disease"[2]. By the mid-1870s, it quickly became apparent that immunity to disease rests on the body's ability to defend against such pathogens.

A sophisticated network of cellular and soluble components coordinate to maintain host defense and promote homeostasis. We collectively refer to this network as the immune system. In general terms, the mammalian immune system encompasses two interconnected branches: innate immunity and adaptive immunity. The innate arm of the immune system acts as a non-specific first line of defense against pathogens. Components of the innate immune system can include physical and chemical barriers, such as the epithelial linings of our respiratory tract, gastrointestinal tracts, and skin. In addition, innate immunity also contains many cellular components that mediate immunologic functions, such as macrophages, granulocytes, dendritic cells (DCs), mast cells, and natural killer (NK) cells. Most innate immune cells sense pathogens via germline-encoded receptors that recognize universal pathogen- or danger-derived molecules[3-5], which triggers them to migrate to the site of infection and either engulf pathogens or secrete bactericidal products and pro-inflammatory factors such as cytokines, chemokines, and

interleukins[6]. The advantage of innate immunity is that responses can occur very quickly, often within a few minutes to hours[6]. However, its limitations rest on the fact that its responses are largely non-specific, and may be overwhelmed by pathogens that can subvert these generic protective mechanisms[6,7].

The adaptive arm of the immune system is classically comprised of T and B lymphocytes. As a whole, the adaptive immune response is slower to develop than the innate immune response—days as opposed to hours[6,7]. However, its advantage is two-fold: 1) it can target its response with much more precision, and 2) it can form immunological memory, which allows the system to form a faster and stronger response upon subsequent encounters with the same pathogen[7]. The remarkable degree of specificity carried out by T and B lymphocytes relies on an incredibly diverse pool of clones that each bears a uniquely rearranged receptor that can recognize a distinct target (antigen)[8]. Because each specificity exists in rare numbers within the body, T and B lymphocytes must continuously circulate throughout the blood and secondary lymphoid organs in order to survey the entire organism. Upon antigen encounter and appropriate activation within these sites, rare clones are clonally selected to proliferate to sufficient numbers and acquire effector functions tailored to the type of threat that must be neutralized[9-11]. Notably, this process enables both clonal expansion as well as the formation of memory cells that would remain in the organism even after pathogen clearance[12].

While the innate and adaptive immune systems were classically segregated into two distinct branches, it is now appreciated that the two are in fact intimately intertwined. Specifically, it is well established that DCs serve as fundamental linchpins that link the innate and adaptive immune systems[13,14]. DCs, along with macrophages and B cells, belong to a class of "professional antigen-presenting cells" (APCs), which capture and present peptide antigens on

major histocompatibility complex (MHC) molecules, and provide the critical co-stimulatory signals necessary for proper immune activation[15]. Generally, DCs only provide co-stimulatory signals upon activation (maturation)[16]. Pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) are found both on the surface and within APCs[16], which allow them to sense and recognize generic molecules derived from pathogens (pathogen-associated molecular patterns, PAMPs)[3] or those released upon cellular damage (danger-associated molecular patterns, DAMPs)[4,5]. Ligation of PRRs triggers DCs to become activated (mature)[3,16]. Thus, these innate cells regulate T and B cell activation based on whether they sense infection and/or danger. Fittingly, DCs are perfectly positioned as sentinels of infection, as they are present at all of the interfaces between the body and environment (for example, airway epithelium, skin, and mucosal surfaces). Collections of DCs are also prominently found in lymphoid organs: lymph nodes, spleen, tonsils, and thymus, where they play key roles in immune regulation. Although DCs and APCs serve as key regulators of adaptive immunity, it is important to note that this relationship is bi-directional, as adaptive immune cells can also in turn regulate DC maturation and function, either via direct surface receptor engagement or via the release of cytokines[16]. This relationship thus allows for dynamic responses that can be regulated at multiple levels.

The classic distinction between innate and adaptive immunity is further complicated by the fact that many cells actually exist on a spectrum between the two poles, rather than falling exclusively into one branch of the immune system or the other. For example, certain unconventional or innate-like lymphocytes exist apart from classical $CD4^+$ and $CD8^+$ αβT cells[17]. These cells include γδT cells, natural killer T (NKT) cells, mucosal-associated immune T (MAIT) cells, intraepithelial lymphocytes (IELs), and innate-like cells (ILCs). Generally

described as having unconventional and innate-like characteristics, the functions of many of these cells remain an active area of investigation.

Antigen recognition

In the adaptive immune system, the separation of humoral versus cellular immunity became firmly established by the 1940s when it became clear that certain types of immunity were conferred by circulating antibodies in the blood serum (for example, against diphtheria and tetanus)[18], while other forms of immune responses required cellular components (for example, delayed-type hypersensitivity)[19,20]. It is now understood that bone marrow-derived B cells mediate antibody-production, while thymus-derived T cells facilitate cell-mediated immunity[21]. Cells of the conventional CD8αβ$^+$ T cell subset (cytotoxic T lymphocytes, CTLs) can kill target cells harboring intracellular pathogens via the release of cytokines, release of cytotoxic granules perforin and granzyme, and expression of Fas ligand (FasL)[22], while cells of the conventional CD4$^+$ T cell subset (helper T cells) primarily coordinate and shape the immune response through the secretion of various cytokines[23].

Both B and T cells achieve an immensely diverse repertoire of receptors via two key processes. First, developing T and B cell progenitors undergo random somatic rearrangement of B cell receptor (BCR) or T cell receptor (TCR) gene segments, respectively—V (variable) and J (joining), and in some cases D (diversity)[8]. This process is mediated by recombinases (recombination activation gene-1 and -2; RAG-1 and RAG-2) and guided by the recombination signal sequences flanking the gene segments[24,25]. Second, at the joining sites of recombination—termed complementarity-determining region 3 (CDR3s)—individual or multiple nucleotide base pairs can be added or removed at these double-strand breaks during recombination. The

junctional diversity of CDR3 and the random rearrangement of V(D)J gene segments together form the basis for the extraordinarily diverse repertoire of BCRs and TCRs. For activated B cells undergoing a germinal center reaction, somatic hypermutation of the B cell receptor heavy and light chain variable regions also contributes to the diversity of BCRs[26].

B cell receptors (BCRs) typically recognize soluble or membrane-bound antigens by binding three-dimensional conformational epitopes[7]. In contrast, conventional αβT cells recognize only linear peptide epitopes presented on MHC molecules expressed on a cell surface[7]. $CD8^+$ T cell recognition is restricted to class I MHC molecules, which display intracellular peptides derived from cytosolic proteins[27]. In contrast, $CD4^+$ T cell recognition is restricted to class II MHC molecules, which typically present peptides derived from exogenous sources, endosomes, and lysosomes[28]. MHC class II molecules are selectively expressed on professional APCs, while MHC class I molecules are widely expressed in all nucleated cells[7]. Certain APCs are also able to take up exogenous antigens and shunt them into the cytosol to become processed in the MHC class I presentation pathway, a process referred to as cross-presentation[29]. The antigen binding structure of T cells comprises a clonally unique αβ heterodimeric TCR moiety in complex with CD3, which work in concert with CD4 or CD8 co-receptors to mediate MHC-restricted antigen recognition[30].

Immunological tolerance

The random rearrangement of V(D)J gene segments would suggest that, inevitably, there should exist random TCR and BCR sequence combinations that would recognize endogenous "self" antigens. Yet, mammals do not normally succumb to destructive autoimmunity. In this regard, a central challenge in the adaptive immune system is to establish a repertoire of mature B

and T cells that will not harm "self." It is well established that this challenge is largely resolved through the development of an immunological tolerance to self. However, the prevailing mechanisms by which an organism achieves immunological tolerance remain a complex and unresolved matter. In the next several sections, we will discuss the various tolerance mechanisms that have been described to date. We focus our earlier sections on T cell tolerance, and we will discuss B cell tolerance in ensuing sections.

Immunological tolerance is defined as a state of non-reactivity towards substances that would normally be expected to elicit an immunological response. A naturally-occurring version of this phenomenon was first observed by Ray Owen in dizygotic twin calves, which appeared to harbor red blood cells from their genetically disparate twin, yet remain healthy[31]. Anastomosis in the placenta of cattle was known to allow the exchange of blood between dizygotic twins *in utero*, creating a natural chimaera of hematopoietic stem cells[32]. It was well documented at the time that "foreign" cells should normally be rejected by the body, so it was very unusual that these chimaeras of genetically disparate cells remained within each twin throughout life. From this observation, Frank MacFarlane Burnet postulated that the neonatal period must be a critical window of time during which the immune system establishes the biological molecules that constitute "self." Any novel molecule encountered after this window would be deemed "foreign." He called this process "self/non-self discrimination"[9].

Peter Medawar and Rupert Billingham observed the same phenomenon a few years later when performing graft transplants between monozygotic and dizygotic twin calves[33]. While it was expected that monozygotic twins would tolerate genetically identical skin grafts from each other, it was rather unexpected that dizygotic twin calves also tolerated grafts from their twins. This tolerance was specific, as skin grafts from a third party were rejected. To address Burnet's

hypothesis of self/non-self discrimination and neonatal tolerance, Medawar, Billingham, and Leslie Brent attempted to experimentally create chimaeras using inbred mice[34]. By injecting neonatal and fetal mice of an inbred strain with cell suspensions from another strain, they showed that skin grafts transplanted during adulthood from the same donor strain, but not third party strains, failed to be rejected by the recipient[34]. Thus, tolerance to certain antigens seemed to be acquired through exposure of those antigens to an immature immune system. The tolerogenic window appeared to span only a few days after birth in mice, as injection of allogeneic cells after that window would elicit an immune reaction rather than tolerance. Beyond alloreactivity, tolerance could also be induced towards soluble proteins and certain pathogens (lymphocytic choriomeningitis virus) if mice were exposed *in utero*[35-37].

Neonatal thymectomy experiments in mice subsequently revealed a critical role for the thymus in establishing immune tolerance[38]. Thymectomized neonates (and later athymic nude mice[39]) appeared immunodeficient and highly susceptible to virus infections. Further, when thymectomized neonates were implanted with an allogeneic thymus, recipient mice acquired immunological tolerance to tissue grafts from the thymic donor strain but not from third party strains[40,41]. Taken together, these findings suggested that the thymus plays a critical role in the establishment of immunological tolerance through some form of self/non-self discrimination.

Recessive versus dominant tolerance

What mechanisms underlie self/non-self discrimination as it relates to immunological tolerance to self? At least three possibilities exist: 1) tolerance is established through elimination of developing T lymphocytes that react to self-antigens (a process termed clonal deletion or negative selection), 2) tolerance is established by rendering self-reactive T lymphocytes

unresponsive (anergic) to self-antigens, and 3) tolerance is established through the generation of "suppressor" cells that could restrain mature self-reactive T lymphocytes in the periphery. Because there was no method to track antigen-specific T cells at the time, investigations on this matter required the use of indirect methods prior to the 1980s.

Initial attempts to dissect mechanisms of immune tolerance centered on determining whether tolerance occurred in a recessive versus a dominant manner. To achieve this, previously tolerized mice bearing well-established skin allografts received adoptive transfer of lymphocytes isolated from untolerized syngeneic hosts. If tolerance was established through generation of suppressor cells (dominant tolerance), it would be expected that host suppressor cells would restrain alloreactive cells introduced from the newly transferred inoculum, and the established allograft would not be rejected. If tolerance was instead established through clonal deletion or anergy induction of alloreactive cells (recessive tolerance), alloreactive cells introduced from the newly transferred inoculum would be expected to breach this state of tolerance, and the established graft would then be rejected. The established graft was indeed rejected upon adoptive transfer of untolerized syngeneic lymphocytes[33,34]. Thus, it was concluded by the authors that tolerance in this setting must have been established through a recessive tolerance mechanism.

However, it is important to note that the outcome from these original studies largely depended on the strains of mice chosen as donors and recipients, and involved robust MHC-mismatched allogeneic responses. For some strains, adoptive transfer of alloreactive cells was actually not able to breach tolerance, despite high numbers of transferred cells[42]. It was since revealed that observations of dominant versus recessive tolerance were largely influenced by the nature of histocompatibility (MHC) differences between the donor and recipient mouse strains. Intriguingly, differences in class I antigens primarily led to recessive tolerance, but tolerance to

class II antigens generally depended on some kind of suppressor mechanism[42], which could be transferred to secondary hosts via adoptive transfer of cells presumed to be suppressor cells[43].

Central tolerance: negative selection

Once the technology became available, monoclonal antibodies were generated against specific TCR chains in an attempt to track antigen-specific T cells. A rare TCR β-chain (Vβ17a) was identified in mice and found to be reactive to the MHC class II I-E molecule[44]. Flow cytometry analysis revealed that mature $V\beta17a^+$ T cells were absent in all mice that bore the I-E allele but present in strains that lacked I-E. Most importantly, in I-E bearing mice, $V\beta17a^+$ T cells were actually present in immature $CD4^+CD8^+$ double-positive (DP) thymocyte populations, but absent in CD4 single-positive (SP) or CD8SP mature thymocyte populations. These results demonstrated for the first time that clonal deletion could occur, and that $V\beta17a^+$ T cells must have been deleted at the DP to SP transition following I-E encounter in the thymus. A caveat to these findings was that the $V\beta17a^+$ T cells in this study were later found to bind endogenous "superantigens" derived from mouse mammary tumor viruses[45]. Superantigens are proteins that elicit an inappropriately strong and non-specific immune response by directly cross-linking MHC class II molecules with particular TCRβ chains, irrespective of the antigen specificity of the TCR or the presented peptide antigen[46]. These unconventional ligands cause TCRs and peptide-MHCs (pMHCs) to bind differently than the canonical binding of the αβTCR to conventional pMHC complexes. Thus, although the original studies by Marrack and Kaplan demonstrated that superantigens can induce deletion of immature $CD4^+CD8^+$ DP thymocytes, such conclusions could not yet be extrapolated to conventional pMHC complexes bearing self-antigens.

The development of TCR-transgenic mice was a major advance that allowed for the study of mice bearing more conventional TCR-pMHC binding pairs. In one example, investigators compared female and male HY-transgenic (Tg) mice, which bear α- and β-TCR transgenes that encoded a MHC class I-restricted receptor specific for a peptide derived from a protein encoded by a gene on the Y chromosome[47]. A first comparison of female and male HY-Tg mice yielded results consistent with deletion of immature thymocytes in male but not female mice during the double-negative (DN) to DP transition. It was, however, realized quite early that the αβ-TCR in these mice was expressed prematurely at the DN stage rather than at the DP stage, when most endogenous αβ-TCR expression occurs[46]. HY-Tg mice re-engineered with "on-time" expression of the transgenic TCR at the DP stage showed that HY-Tg cells underwent clonal deletion at the DP to SP transition[48].

Further attempts to emulate physiological TCR expression and precursor frequencies utilized TCRβ-only transgenic mice to eliminate nonphysiologic artifacts of transgenic TCRα expression[49]. This, in combination with the use of a model self-antigen and pMHC tetramers to identify antigen-specific T cells, allows for the following advantages: 1) production of an oligoclonal repertoire with reduced clonal frequency of thymocytes specific for a model self-antigen, 2) a more physiological thymic architecture compared to mice expressing a monoclonal αβ-TCR population, and 3) in some cases, the use of mice expressing tissue-restricted antigens (TRAs) in the medulla, which mimics many naturally-occurring self antigens in the thymus. Interestingly, even in the presence of the deleting ligand, a substantial portion (25-40%) of antigen-specific T cells escaped clonal deletion[49,50]. The degree of negative selection appeared directly related to T cell avidity for the model antigen. These results revealed that low-affinity/avidity self-reactive T cell clones routinely evade negative selection and contribute to the

peripheral pool of mature T cells. Moreover, there may exist a threshold of affinity/avidity for which high-affinity self-reactive TCR clonotypes would be deleted.

The results above highlight the importance of examining thymic development of self-reactive T lymphocytes using the most physiological tools available. It is now appreciated that the nature, timing, and level of TCR and self-antigen expression, as well as the precursor frequency and ligand density, can all dramatically impact the timing and mechanisms by which tolerance is induced. One caveat of all TCR-Tg systems has to do with the level of TCR expressed on the surface of immature thymocytes, as wildtype DP thymocytes normally express very little TCR on their cell surface until they undergo positive selection[46], while TCR transgenes driven by strong promoters may express earlier and higher levels of TCR[47,49]. In addition, the examples shown by the TCRβ-only Tg systems reveal that transgenic TCRs may bind model self-antigens with superphysiological affinity/avidity[49], thereby failing to reflect what occurs to low-affinity (and perhaps more physiological) TCR clonotypes. The perfect model system with which to study central tolerance should strive to utilize: 1) appropriately-timed TCR expression at physiological levels, 2) T cells that possess affinities/avidities that fall within a physiological range for the self-antigen, 3) specificities that exist at physiological clonal frequencies, and 4) endogenous self-antigens that are expressed at natural densities with the appropriate "topology." In many cases, tetramer systems could be employed to identify an endogenous self-specific thymic population[51]. However, one should be cautious to note that tetramers may fail to detect low-avidity T cells or cells that have downregulated TCR expression. For all of the reasons outlined above, a model to track and study a completely physiological endogenous thymocyte specificity against an endogenous self-antigen remains a challenging task.

Central tolerance: clonal diversion into alternative lineages

Despite the key principles of clonal deletion, it is widely appreciated that many self-reactive TCR clonotypes (especially those bearing low-affinity TCR to self-antigens) can evade clonal deletion[49]. In fact, it has been demonstrated in recent years that in some instances, self-reactive thymocytes can be shunted into alternative lineages prior to exiting the thymus. For example, it is well established that certain self-reactive thymocyte specificities differentiate into thymic-derived regulatory T cells (tTregs)[52], which play a key role in maintaining peripheral tolerance and peripheral suppression of self-reactive conventional T (Tconv) cells[53].

Treg cells can either arise within the thymus during thymocyte development (thymic Tregs, tTregs)[54] or in the periphery via induced differentiation of conventional $CD4^+$ T cells into $Foxp3^+$ Treg cells (peripherally-induced Tregs, pTregs)[55]. While pTreg cells appear to play a significant role in the maintenance of tolerance to environmental antigens—such as those derived from commensal bacteria in the gut[55]—evidence suggests that maintenance of dominant tolerance in the periphery largely require tTreg cells bearing self-specific TCRs[56]. Mice thymectomized between 3-7 days after birth, but not after day 7, exhibit spontaneous organ-specific autoimmunity[41,57], suggesting that the 3-7 day window may be critical for the development and migration of suppressor cells from the thymus to the periphery. Efforts to better define the cell type mediating suppression of autoimmunity culminated in the identification of $CD4^+$ T cells constitutively expressing the interleukin 2 receptor (IL-2R) α-chain (CD25)[58]. These naturally arising $CD4^+CD25^+$ T cells, termed regulatory T (Treg) cells, became the best candidates for suppressor cells. However, CD25 is not a unique marker of Treg cells, as all activated T cells transiently express CD25. The field made a major leap forward when the

transcription factor Foxp3 was finally identified as a critical master regulator responsible for the development and function of the elusive Treg cells[59,60].

A series of studies demonstrated that the expression of TCRs specific for self-antigens was the crucial requirement for Foxp3 expression in developing thymocytes, and thus for tTreg cell differentiation. Multiple models of double-transgenic mice ectopically expressing a model self-antigen and its corresponding transgenic-TCR have shown that Treg cells develop in the thymus only when the cognate antigen was also expressed in the thymus[61,62]. Furthermore, thymocytes expressing a TCR-tg of high affinity for the model self-antigen gave rise to tTreg cells while those expressing a lower affinity TCR did not[63]. Similar to studies of clonal deletion, the major challenges faced by the use of TCR-transgenic mice is that the abnormal early expression of the αβ-TCR at the DN instead of DP stage, as well as high-level TCR expression, can induce early and strong TCR signaling that may not be reflective of endogenous T cell development. Yet to date, data from TCR-transgenic mouse studies remain a central basis for the prevailing notion that thymic Treg development is driven by TCR/self-pMHC interactions that fall somewhere between the binding thresholds that delineate positive selection versus negative selection (affinity/avidity model)[64].

An alternative approach employed the use of mice with limited TCR diversity to permit the sequencing of individual TCR clonotypes within the Treg and Tconv TCR repertoires[65]. The two repertoires were found to be largely distinct, with a minor (10-15%) overlap. Furthermore, T cells engineered to express TCRs identified in each subset revealed that T cells expressing Treg-derived TCRs were more likely to proliferate in lymphopenic hosts than those expressing Tconv-derived TCRs[52,65]. Together, these data reinforced the notion that Treg-derived TCRs possessed a higher level of self-reactivity than those of non-Tregs. It is important to note that lymphopenia-

induced proliferation (homeostatic proliferation) can occur for many T cells regardless of self-reactivity, due to low affinity interactions with self peptide-MHC molecules and an elevated abundance of IL-7[66,67], although it is presumed that affinity for self positively correlates with the rate of proliferation. Moreover, it is possible that proliferation is driven by exposure to commensal or environmental antigens, rather than self-ligands. While naturally-occurring tTreg cells were hypothesized to exhibit greater self-reactivity (as inferred using the various indirect methods discussed above), an endogenous thymic self-peptide ligand recognized by a naturally occurring tTreg cell clonotype remained elusive until our group identified a prostate-associated tTreg-derived TCR specific to a peptide derived from a protein found in the seminal fluid of male mice (TRPM8 channel-associated factor 3, Tcaf3)[68,69]. This antigen is not only expressed in the prostate of male mice, but also in the thymic medulla in an *Aire*-dependent fashion (discussed below). Selective deletion of the genetic sequence encoding the Tcaf3-derived peptide antigen revealed that the absence of this single peptide in the thymus was sufficient to abrogate the thymic tTreg differentiation of Tcaf3-specific thymocytes[69]. The results from these studies unequivocally demonstrated the requirement for a thymic self-antigen to promote tTreg cell differentiation.

Nonetheless, the delineation between Treg cell differentiation and negative selection remained difficult to assess. Some TCR-tg models found no increase in Treg cell numbers when antigen dose increased, but rather an increase in deletion of non-Treg cells[70]. Furthermore, other double-transgenic systems did not support the affinity model that higher affinity to a model self-antigen yields tTreg development[54,70]. It was thus unclear whether self-antigen affinity was indeed the driver of thymic Treg-cell differentiation, or whether there exist TCR-independent

factors that support the expression of Foxp3 and hence resistance to negative selection upon high-affinity self-antigen encounter.

In recent years, studies using TCRs derived from naturally arising Treg cells shed light on the nuances of thymic Treg cell development[52,65,71]. Treg-derived TCRs were obtained from repertoire analyses of two different transgenic mouse strains bearing fixed TCRβ chains. Surprisingly, TCR-transgenic mice bred to a RAG-deficient background led to virtually no Treg development in the thymus[71]. Through intrathymic transfer experiments and the generation of low-frequency bone marrow chimaeras, it soon became clear that the frequency of TCR-tg tTreg development was inversely related to the clonal frequency of TCR-tg thymocytes[68,71,72]. Additionally, the number of Treg cells developed varied considerably depending on the specificity of the TCR. Thus, there appeared to exist a limiting factor for Treg development in the thymus and a role for intraclonal competition for limited developmental factor(s) (termed a "niche effect"). Studies utilizing Nur77-eGFP reporter mice, in which the relative level of TCR signaling could be inferred from the level of eGFP expression, also supported an affinity model for tTreg development as well as the notion that tTreg progenitors compete intraclonally for developmental factors[73]. These factors remain to be directly defined, but may be related to the availability of the selecting (agonist) self-ligands in the thymus. Possible additional factors include: 1) the number or subset of APCs in the thymus, 2) the density, distribution, or nature of selecting self-antigens expressed by APCs and/or thymic epithelial cells, 3) co-stimulatory signals, or 4) soluble factors[54,74]. We discuss these elements in more detail in a later section.

Notably, certain self-reactive T cell progenitors escape clonal deletion, but adopt alternate cell fates. Specifically, interaction with self-ligands shunts these cells into unconventional lymphocyte lineages, such as invariant natural killer T (iNKT) cells[75], natural

CD8αα+ intraepithelial lymphocytes (IELs) that reside in the gut epithelium[76], and certain γδT cells[75]. iNKT cells are a minor lineage of T cells that express a semi-invariant TCR and various NK cell markers (NK1.1, DX5, and NKG2D), and recognize lipid antigens in the context of the MHC class I-like molecule, CD1d. These cells develop from DP progenitors in response to self-lipid antigens, and can be deleted upon exposure to very strong agonists, much like conventional T cell progenitors. Similarly, compelling evidence suggest that many CD8αα+ IELs (specifically TCRαβ$^+$ intestinal IELs, iIEL) arise as a result of interactions with agonist MHC class I or II ligands in the thymus and subsequent escape from thymic negative selection[76]. Finally, the γδT cell repertoire is also thought to contain self-reactive specificities, though much less is understood about the specificity of γδT cells in general.

Central tolerance: Autoimmune regulator (AIRE)

Our discussions thus far highlight the critical role of the thymus in serving as a central site for the establishment of self-tolerance, either via negative selection or via the induction of clonal diversion into tTreg or alternative lineages. We refer to tolerance established in the thymus as central tolerance. However, a challenge remained regarding how tolerance could be imparted onto thymocytes reactive to self-antigens expressed outside the thymus, such as the peripheral organs—pancreas, eyes, thyroid, secretory glands, reproductive organs, etc. This issue was illuminated by the observation that the thymus exhibits promiscuous expression of tissue-restricted antigens (TRAs)—such as insulin—specifically by medullary thymic epithelial cells (mTECs)[77-79]. Autoimmune regulator (AIRE) was subsequently discovered as a transcription factor expressed by mTECs that promotes the ectopic expression of TRAs in the thymic medulla[80]. In addition, AIRE has also been shown to play a role in the induction of genes

involved in antigen processing and presentation, and impacts DC density in the thymic medulla due to its role in promoting certain chemokine production in the thymus[81]. In humans, loss-of-function mutations of the gene result in the rare autoimmune polyglandular syndrome type-1 (APS-1; also called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, APECED) characterized by hypoparathyroidism, adrenocortical insufficiency, and mucocutaneous candidiasis[82]. In mice, loss-of-function AIRE mutations result in multi-organ autoimmunity, which varies in severity depending on the genetic background of the mouse strain[83]. These observations support the indication that AIRE plays a vital role in immune regulation.

Initial investigations of AIRE-mediated tolerance utilized TCR-transgenic systems in which TCR-tg cells were reactive to a model self-antigen expressed under an AIRE-dependent promoter (the rat insulin promoter, RIP)[84]. These studies revealed that the TCR-tg cells were clonally deleted in AIRE-expressing TCR-tg mice but remained in AIRE-deficient TCR-tg mice, suggesting that Aire mediates tolerance by promoting negative selection of thymocytes reactive to TRAs. Conclusions from these studies were further supported by a demonstration that AIRE was also required for the thymic deletion of a transgenic-TCR reactive to an endogenous self-antigen[85]. The caveats of using transgenic systems have been extensively outlined previously, so they will not be repeated here. Importantly, the original conclusions were challenged by more recent findings that Aire is critical for the thymic development of multiple naturally-occurring Treg cell specificities[68,86], and that Aire impacts the thymic repertoire of polyclonal Treg cells[68,86,87]. Further, deep sequencing approaches to compare the TCR repertoire of Treg and Tconv cells in Aire-sufficient versus Aire-deficient mice revealed that Aire directs distinct self-reactive T cell specificities into the Foxp3$^+$ Treg lineage[86]. This lineage diversion is not

complete, as some TCR clonotypes were found in both Treg and Tconv cell subsets, although the prevalence of many clones were skewed towards the Treg subset in Aire-sufficient mice and correspondingly skewed towards the Tconv cell subset in Aire-deficient mice. It was thus concluded that autoimmunity in Aire-deficient mice develop as a result of a failure to direct these self-reactive T cell specificities into the Treg lineage during thymic development, thus leaving potentially pathogenic specificities in the Tconv compartment[86]. These findings suggest that for Aire-dependent self-reactive specificities, the processes governing Treg cell differentiation and negative selection may be intricately linked.

Central tolerance: cell fate determination

In reality, the factors that dictate the fate of self-reactive thymocyte progenitors likely rely on a multifaceted combination of interrelated variables, which poses a challenge to our ability to dissect how each factor influences these fates. As mentioned previously, these fates can be influenced by distinct factors, such as self-antigen expression and presentation patterns, spatiotemporal aspects of thymocyte differentiation, as well as contextual input that accompanies the agonist TCR signal. Here, we briefly discuss the contributions of these various elements.

Lymphoid progenitors enter the thymus from the post-capillary venules located at the cortico-medullary junction, and traffic to the cortex as DN thymocytes[46]. DN thymocytes in the thymic cortex rearrange and select a TCRβ chain, then progress to the $CD4^+CD8^+$ DP stage, during which they rearrange and express a TCRα chain. If the TCRαβ complex fails to interact productively with self-peptide:MHC complexes, they undergo apoptosis (death by neglect)[88]. A successful (but weak) interaction results in positive selection and CD4 or CD8 lineage commitment, followed by migration into the thymic medulla as CD4 or CD8 single-positive (SP)

thymocytes. However, an overly strong interaction at the DP stage can also result in deletion prior to entry into the medulla[46,75]. The proportion of the pre-selection DP repertoire that can signal productively with pMHCs in the cortex has been estimated to range ~15%[89-91]. Furthermore, using an unbiased clonal approach to assess the pre-selection TCRαβ repertoire of DP thymocytes, it was shown that of the roughly 15% of TCRs that signal productively, ~7.5% were negatively selected at the DP and DP-SP transition (with rare deletion at the medullar SP stage)[89]. A majority of the negatively selected TCRs appeared to be crossreactive to multiple MHC classes and haplotypes, and select TCRs that escaped negative selection were diverted into the iIEL lineage. These findings reveal that although negative selection can target TCRs reactive to classic self-peptide:MHCs, a substantial portion of deleted thymocytes appear to be targeted towards MHC crossreactive TCR specificities.

In regards to thymocytes reactive to self-peptide:MHC complexes in the thymic medulla, while the role of affinity has already been discussed previously as it relates to negative selection and Treg cell differentiation, it is worth noting that it remains unclear what factors delineate the two developmental fates. Much of the work on quantifying clonal deletion thresholds has been carried out in MHC class I-restricted $CD8^+$ T cell progenitors[92], while thresholds driving MHC class II-restricted events have been understudied. It is possible that TCR affinities that lead to negative selection impose an upper limit on affinities that can facilitate Treg development. In support of this, experimental reduction of MHC class II expression on mTECs in TCR-tg/model-antigen double-transgenic systems have shown a shift in previously deleted TCR-tg cells towards Treg differentiation[93]. Additionally, studies using the *Nur77*-GFP transgenic mouse line—in which the expression of GFP is driven by the *Nur77* promoter (activated in response to TCR stimulation)—revealed that Treg cells perceived stronger TCR signals than Tconv cells during

thymic development and in the periphery[73]. However, studies have also reported affinities for Treg cell selection that overlap with those driving negative selection. For example, *Nur77*-GFP mice generated in mice lacking the proapoptotic molecule Bim revealed that cells rescued from clonal deletion express similar levels of GFP (i.e. self-reactivity) as Treg cells[90]. This suggests that the two may occur at similar or overlapping thresholds. Unsurprisingly, these thresholds appear to vary depending on the genetic background of the mice[94,95]. Therefore, while TCR affinity for self-ligands certainly plays an important role in driving the fate of a self-reactive thymocyte, it is likely not the only factor involved.

Two recent studies employed the use of MHC class II tetramer systems to trace the fate of endogenous $CD4^+$ T cells reactive to model self-antigens expressed under the control of various promoters that simulate either a ubiquitous or a TRA-like expression pattern[96,97]. Ubiquitous antigen expression led to efficient clonal deletion at the CD4SP stage. By contrast, TRA-like antigen expression resulted in a mild-to-moderate decrease in the number of polyclonal antigen-specific $CD4^+$ T cells, along with an emergence of $Foxp3^+$ cells, suggesting that clonal deletion and diversion into the Treg cell lineage occurred in parallel. Notably, the intensity of tetramer staining on these Treg cells was lower than that on their naive counterparts (in mice that lacked the model antigen), which suggests that high affinity TCR clonotypes may have been deleted[96]. On this basis, it was suggested that thymocyte deletion versus Treg cell-differentiation is determined by the pattern of self-antigen expression. Why would the pattern of self-antigen expression affect cell fate? Certainly, the quantity and density of antigen load would be expected to be higher in ubiquitous as opposed to TRA-like antigen expression. However, the following factors may also relate to differential antigen expression patterns: the different spatial distribution of antigen expression, the developmental stage at which these antigens are first seen

by a thymocyte, the likelihood and frequency of serial antigen encounters, the APC types that display the self-antigen of interest, and the cytokines and/or co-stimulatory molecules that accompany the TCR signal.

The density of cognate antigens presented on an APC could directly influence the magnitude of the TCR signal strength for a given self-reactive T cell. Alternatively, it has been suggested using 2-photon microscopy that negative selection requires multiple T cell-APC interactions (serial antigen encounters), while Treg differentiation is speculated to require only a single T cell-APC interaction[54,74]. Therefore, a lower availability of antigen within the thymus may be associated with a lower chance for self-reactive T cells to undergo serial antigen encounters, and thus avoid negative selection.

Central tolerance: Treg cell differentiation in the thymus

Interestingly, the observation that a $CD25^+$ but $Foxp3^{neg}$ population of CD4SP thymocytes temporally preceded the development of $CD25^+Foxp3^+$ thymic-derived Treg (tTreg) cells led to the notion that tTreg cell development could be divided into two steps[98]. The first step appears to involve a TCR-dependent upregulation of CD25 (the IL-2 receptor α-chain). In the second step, it was shown that exposure to IL-2 and IL-15 (to a lesser extent) was sufficient to induce Foxp3 expression in a TCR-independent manner. Presumably, these cytokines facilitate tTreg cell differentiation by promoting STAT5 signaling and binding to the *Foxp3* locus. Thus, the availability of IL-2 in the thymus represents an antigen-independent niche that promotes tTreg cell generation and survival. Further, highly self-reactive TCRs likely favor tTreg cell differentiation by promoting efficient TCR signaling and CD25 upregulation, thereby mediating the generation and survival of tTreg cells. Recently, an alternate two-step model has

been proposed suggesting that tTreg cells arise through two distinct developmental programs, through either $CD25^+$ progenitors or $Foxp3^{lo}$ progenitors[99]. Although the exact developmental pathways remain to be solidified, it is evident that Treg cell differentiation in the thymus likely occurs in multiple stages.

Aside from TCR and IL-2 signaling, the developmental survival of tTreg cells also depends on co-stimulatory signals. The role of co-stimulatory signals in tTreg cell development was first recognized when it was observed that B7-deficient mice lacked Treg cells[100]. Subsequently, it was shown that CD28 plays an important cell-intrinsic role in the generation of tTreg cells, as CD28-deficient mice exhibit an ~80% reduction in the frequency of thymic Treg cells[101]. This decrease in tTreg cell frequency did not coincide with a dramatic difference in the TCR repertoire of Treg cells in CD28-sufficient or -deficient mice, suggesting that CD28 signaling likely promotes either the survival or enhancement of developing Treg cell thymocytes, rather than the selection of distinct TCR specificities into the tTreg cell lineage. Cytokines such as TGF-β have also been shown to play a role in promoting the survival of thymic Treg cells[102,103] as well as other self-reactive cell types in the thymus (such as iNKT and IELs)[75], revealing a common quality among self-reactive specificities.

The antigen-presenting cells encountered by developing thymocytes may likely shape the resulting repertoire of TCR specificities selected for Treg cell development, as self-antigen reactivity and co-stimulatory signals both impact selection. Promiscuous expression of tissue-specific antigens in medullary thymic epithelial cells (mTECs) expose developing thymocytes to a broad range of peripheral tissue-restricted antigens (TRAs)[77-79]. As introduced in previous sections, ectopic expression of these TRAs in mTECs is regulated by the transcription factor AIRE[80], and has been shown to promote the thymic differentiation of select TCR specificities

reactive to these TRAs[68,86]. It is not clear whether mTECs directly promote tTreg differentiation or whether they simply provide the necessary TRAs to DCs[104]. In support of the latter, the differentiation of select tTreg-derived TCRs has been shown to depend on specific subsets of DCs in the thymus for tTreg differentiation[87]. The dependence of select tTreg specificities on DC subsets could be due to differences in the repertoire of self-ligands captured and presented by distinct DC subsets, the variability in co-stimulatory molecules expressed by distinct DC subsets, the abundance, location and distribution of DC subsets, the density of pMHC molecules presented on DCs for a given antigen, as well as the availability of cytokines in the microenvironment of distinct DC subsets.

Collectively, the fate of self-reactive T cell specificities results from an integration of many inter-related factors. Yet somehow, the fate of these cells is precisely orchestrated and not left up to chance, as distinct TCRs are recurrently and robustly deleted, directed into the tTreg lineage, or diverted into alternative lineages[52,65,86]. It should be noted however that while the TCR repertoires of Treg and non-Treg subsets are largely distinct, some TCRs still fall within both subsets (10-15%), and an overlap is always observed[65,86]. It is possible that the TCRs that drive inefficient thymic Treg cell development recognize self-antigens with low affinity, or are specific for self-antigens that are poorly expressed or presented by thymic APCs. These TCR specificities have been poorly studied, and their characterization may shed light on important aspects of thymic selection. Furthermore, because the TCRs that drive inefficient thymic Treg cell selection are presumably self-reactive, it will be important to examine the extent to which the Tconv cells expressing these TCRs are the primary drivers of autoimmune disease when immune dysregulation occurs.

Peripheral tolerance

Although a sophisticated network of central tolerance processes are imposed on self-reactive thymocytes, a fraction of self-reactive specificities nevertheless escapes clonal deletion and lineage diversion, and instead seed the peripheral T cell repertoire as potentially pathogenic specificities[49]. The fraction of the peripheral repertoire that harbors self-reactive specificities remains largely undefined, but it is widely understood that self-reactive T cells that escape central tolerance must be either disarmed or held in-check in the periphery through a multi-layered approach that involves both intrinsic and extrinsic mechanisms.

First, peripheral self-reactive T cells can simply remain ignorant of the self-antigen when anatomical barriers sequester antigen from immune surveillance (in immune privileged sites) or when self-antigen is presented at concentrations too low to stimulate T cells[105]. However, it is important to note that these cells are antigen-inexperienced and persist as naïve cells. Because they are not rendered irreversibly dysfunctional, if they become activated by external stimuli (such as infection or inflammation), ignorance can be easily overcome, and autoimmunity could potentially be induced.

Peripheral T cells that encounter and recognize self-antigens presented in the periphery can be deleted through a form of apoptosis called activation-induced cell death (AICD)[106]. AICD results from the ligation of the death domain-containing receptor Fas, whose upregulation occurs following repeated TCR stimulation. Because of this, optimal T cell activation following TCR signaling requires additional co-stimulatory signals that promote the induction of IL-2 production and the upregulation of pro-survival factors[107,108]. These signals are sent via co-stimulatory receptors that include CD28, ICOS, CD40, OX-40, and 4-1BB[107-109]. Specifically,

the CD28 ligand is provided by B7 molecules expressed by APCs upon PRR-induced activation (as described previously)[3]. In addition, co-stimulatory signals can be counter-balanced by negative signals sent through co-inhibitory receptors such as CTLA-4 and PD-1[110,111]. CTLA-4 also binds B7 molecules on APCs, but with significantly higher affinity[110]. Therefore, T cell activation is regulated by the relative expression of co-stimulatory and co-inhibitory molecules and their respective ligands. The balance between the positive and negative signals received by a T cell dictates whether the cell becomes activated or undergoes apoptosis.

Aside from AICD, TCR signaling in the absence of co-stimulatory signals could also lead to the induction of antigen-specific unresponsiveness (termed T-cell anergy), a state in which T cells are unable to respond to subsequent antigen encounter[109]. Functional, phenotypic, and molecular analyses reveal that anergic states are largely regulated and maintained by distinct molecular factors when induced *in vitro* versus *in vivo* (despite some overlapping traits), and require different strategies to restore cell function[112]. *In vivo* anergy is a desensitization state characterized by an early block in TCR signal transduction and proliferation, and a preferential block in the calcium pathway, which leads to inhibition of most cytokine production (with the notable exception of IL-10)[109,112-117]. *In vivo* anergy spontaneously reverses in the absence of the antigen. In contrast, a functional characteristic of anergic T cells induced *in vitro* is the inability to produce IL-2 or proliferate in response to antigen stimulation[113,118]. Addition of IL-2 brings these cells out of the anergic state. Irrespective to the mode of induction, anergic cells generally exist in a state of cell cycle arrest in which they are inhibited from progressing from the G1 to S phase[112,114,116,117]. Although anergic cells are largely disengaged from cell cycle reentry control mechanisms, alternative signaling pathways remain intact (such as signaling through cytokine receptors) and enable anergic cells to enter a rescued state in which they regain capability to

respond to antigen[113,118]. Therefore, a major effect of co-stimulation in anergic T cells is to produce enough cytokines and growth factors to drive the cells back into cell cycle.

In light of this, some questions remain regarding self-reactive T cells that encounter cognate self-ligands in the periphery. For example, what factors dictate whether a self-reactive T cell undergoes AICD or anergy induction when TCR signaling occurs in the absence of co-stimulation? Do these factors involve the density of self-antigens presented in the periphery, or the strength of TCR signaling experienced by the T cell? Furthermore, given the risks of autoimmunity that could be incurred by the inadvertent activation of anergic self-reactive T cells (in the right conditions), what could be the purpose of maintaining these anergic cells in the peripheral repertoire as opposed to deleting them? Could the presence of anergic cells actually serve a function or evolutionary advantage that could warrant their maintenance in the immune repertoire?

The aforementioned peripheral tolerance mechanisms involve intrinsic methods to disarm self-reactive T cells. An additional method of peripheral tolerance involves disabling and removing the self-reactive T cells from the Tconv compartment by inducing their differentiation into suppressor cells. Tconv cells activated in the presence of inhibitory cytokines such as TGF-β and IL-10 can differentiate into peripherally induced $Foxp3^+$ Treg (pTreg) cells[119]. Together, pTreg cells and tTreg cells maintain immune homeostasis via a variety of contact-dependent and contact-independent mechanisms, which include the production of inhibitory cytokines (TGF-β, IL-10, and IL-35), inhibitory receptors (CTLA-4 and LAG-3), modulation of the stimulatory properties of DCs, and IL-2 deprivation due to their high expression of IL-2Rα (CD25)[120]. Additionally, Treg cells may be more responsive to self-peptide:MHCs than their Tconv counterpart, and may compete for interactions with pMHCs presented on APCs[120].

The significant role of Foxp3$^+$ Treg cells was demonstrated when the selective depletion of Foxp3$^+$ Treg cells in mice resulted in an aggressive and fatal lymphoproliferative disease characterized by an expansion of CD4$^+$ and CD8$^+$ T cells, spontaneous activation of DCs, and infiltration of lymphocytes into non-lymphoid organs[53]. These findings revealed that, while multiple cell-intrinsic mechanisms (such as AICD and anergy induction) can promote peripheral tolerance, these methods are not sufficient. The maintenance of peripheral tolerance and immune homeostasis requires Foxp3$^+$ Treg cells to mediate immune suppression of pathogenic Tconv cells. The results from these studies also suggest that the endogenous repertoire harbors self-reactive T cell specificities that possess pathogenic potential and can become activated upon release from suppression, underscoring the importance of keeping these cells in-check.

Other types of suppressor cells have also been identified. For example, CD8$^+$ Treg cells have been described as a type of suppressor cells that can engage in direct killing of CD4$^+$ Tconv cells by perforin-mediated cytolysis or Fas-induced apoptosis[121,122]. They can also produce inhibitory cytokines such as TGF-β and IL-10 to mediate immune suppression. Type 1 regulatory (Tr1) cells are another type of CD4$^+$ suppressor cells that are Foxp3^{neg} but express CD49b and LAG-3[121,122]. These cells are induced *in vivo* following chronic antigenic stimulation in the presence of IL-10, and mediate immune suppression primarily by secreting IL-10. Tr1 cells have also been described to possess the capacity to kill myeloid APCs by releasing granzyme and perforin.

B cell tolerance

Analogous to the tolerance mechanisms imposed on cells in the T cell compartment, self-tolerance in the B cell compartment is achieved through a range of mechanisms that collectively

ensures the removal, editing, or silencing of B cells that have formed self-reactive antigen receptors during their early development in the bone-marrow. However, in contrast to T cells, B cells pose a unique challenge to the peripheral maintenance of immunological self-tolerance because B cells activated by foreign antigens can enter germinal centers (GCs), where they further modify their antigen receptor through a process called somatic hypermutation (SHM)[26]. The process of SHM inevitably leads to the emergence of activated, self-reactive GC B cells, which must be rapidly countered to avoid autoantibody production. The fate of most activated B cells heavily depends on their access to survival signals provided by T helper (Th) cells[123]. Consequently, the actions of the T helper cell compartment (and the maintenance of T cell tolerance) play a pivotal role in the regulation of activated, self-reactive B cells during GC reactions.

Within the bone marrow, it has been estimated that up to 10^{12} distinct B cell receptors (BCRs) can be produced via V(D)J recombination of immunoglobulin genes[124]. A majority of these BCRs carry significant binding affinity for soluble, cellular, or cell-surface self-antigens[124]. Within the primary B cell repertoire, B cells carrying self-reactive BCRs can be neutralized through a variety of mechanisms. First, they can be clonally deleted upon strong interaction with self-antigens in the bone marrow or in the periphery[125,126]. Second, strongly self-reactive BCR specificities in immature bone-marrow B cells can induce the reactivation of RAG protein expression, which triggers secondary V(J) rearrangement within the immunoglobulin light chain genes[127]. This process of receptor editing often leads to the loss of self-reactivity by the BCR. As a result, the BCR specificities that successfully lose self-reactivity are thereby liberated from clonal deletion and are allowed to mature and enter the peripheral repertoire. Together, these two processes eliminate about half of the original BCR specificities generated in the bone marrow[124].

In the periphery, many self-reactive B cells that escape the aforementioned processes exist either in a state of ignorance or in a functionally compromised state of anergy. B cell anergy is brought about by chronic exposure to self-antigen, and is characterized by desensitized BCR signaling and a suppressed ability to undergo plasma cell differentiation[128]. It has been estimated that the peripheral B cell repertoire contains at least 15% of BCR specificities that are demonstrably self-reactive, but are presumably ignorant or anergic[124]. Despite their compromised BCR signaling activity, anergic B cells retain both the ability to present antigen and the ability to respond to activation signals[129]. Furthermore, many BCR specificities have also been recently shown to be polyreactive[130]. Therefore, the substantial presence of these specificities underscores the need for a tight regulation of sustained tolerance in the B cell compartment.

All B cells have the potential to be activated and recruited into the GC during an immune response. However, the fate of these activated B cells depends on a number of factors. After a BCR binds an antigen, it internalizes it for proteolytic degradation and loads antigen-derived peptides on MHC class II molecules to be presented on the cell surface for recognition by $CD4^+$ T helper (Th) cells. Failure of B cells to engage with a Th cell recognizing the same antigen within 12-24 hours results in BCR-mediated apoptosis through the upregulation of the proapoptotic molecule Bim[131]. This highlights a critical role for the maintenance of self-tolerance within the $CD4^+$ T cell compartment, since self-reactive $CD4^+$ T cells that escape regulation in the peripheral repertoire may inadvertently provide stimuli that help protect self-reactive B cells from apoptosis and promote their proliferative expansion.

Activated B cells that receive proper help from their Th cell counterpart then enter the GC. The provision of Th help comes in the form of ligation with the TNF superfamily ligand CD40L and of IL-21 cytokine signals[132]. GC B cell interaction with CD40L expressed by Th

cells is required for their continual survival and differentiation into memory cells. Upon entry into GCs, B cells undergo iterative rounds of cyclic transitions between the light zone (LZ) and dark zone (DZ) of the GC as part of a positive selection process for high-affinity BCR clonotypes[133]. In the LZ, B cells bind and present antigens to cognate Th cells (called T follicular helper (Tfh) cells here). Productive interaction activates LZ B cells to enter the cell cycle and migrate into the DZ (via upregulation of CXCR4), where they undergo cell division and SHM of their immunoglobulin variable genes. Many B cells incur DNA damage and BCR loss during this process, and therefore die via apoptosis and are cleared by tingible body macrophages[134]. Those that survive SHM return to the LZ, bearing their newly mutated BCR. LZ B cells expressing BCRs with the highest affinity for the targeted antigen possess a selective advantage in accessing the antigen, and thus can preferentially interact with Tfh cells to receive help signals via CD40L and cytokine signaling. B cells receiving the proper signals in this competitive environment are then dispatched back to the LZ for further cycles of proliferation and SHM. This iterative process allows cognate B cells to retest their BCRs through a series of LZ-DZ cycles, after which, selective pressure results in the retention of a highly homogeneous population of rare founder B cell clones that dominate the GC and express a high-affinity BCR for the target antigen[133,135,136]. This entire process is referred to as affinity maturation. The ensuing differentiation of GC B cells into antibody-secreting plasma cells and memory B cells also depends on Tfh help[137].

Recently, it has been proposed that self-reactive B cells can be recruited to the GC and can undergo SHM to rapidly select for somatic mutations that increase affinity for the foreign antigen, and effectively edit the self-reactivity out from the BCR[138,139]. These self-reactive B cells may provide a distinct pool of primary specificities that can give rise to a potentially unique set of protective antibodies following SHM and selection in the GC, thus explaining a long-

standing question regarding the purpose of maintaining anergic B cells in the peripheral repertoire.

However, many of the pathogenic autoantibodies associated with autoimmune diseases in humans appear to have originated from SHM in the GC[140], suggesting a failure in the proper Tfh-mediated selection of GC B cells. Although reversion of the autoantibodies to the germline or unmutated specificity occasionally indicates that the original primary B cell clone was itself self-reactive[141], the overwhelming majority of reverted autoantibodies do not have detectable reactivity with the final autoantigen[142,143]. Therefore, most pathogenic autoantibodies originated from non-self-reactive precursor B cells that acquired their self-reactivity during GC selection. These findings demonstrate that GC B cell differentiation—though powerful—heavily depends on the activities of the relevant foreign-reactive Tfh cells for proper selection. Furthermore, because GC selection occurs in a Darwinian process, and Tfh cells exist in rare numbers in the GC, the provision of B cell help by a rare population of self-reactive Tfh cells could quickly hijack this process to form high-affinity autoantibodies and mediate selection away from the foreign antigen. In light of these observations, the existence of self-reactive Tfh cells within the endogenous repertoire at steady state merits real consideration and warrants a thorough investigation.

T follicular helper cells

Although the function of $CD4^+$ T follicular helper (Tfh) cells as B helper cells have been described for decades[144,145], the recognition of Tfh cells as an independent T helper subset was not readily appreciated until it was discovered that they depend on the expression of the transcription factor and master regulator Bcl6[146], and express the chemokine receptor CXCR5[123].

As described previously, Tfh cells are responsible for the priming and initiation of extrafollicular and GC B cell responses, and are vital for the promotion of affinity maturation as well as the maintenance of humoral memory[147-149].

The differentiation of Tfh cells is a complex and tightly controlled process that occurs in multiple stages. The first stage begins with T cell priming by MHC class II-positive dendritic cells (DCs)[147,150-152]. In addition to antigen recognition, the differentiation of Tfh cells is facilitated by exposure to cytokines IL-6 and IL-21 due to signaling through STAT3 and STAT1[153]. IL-6 induces an early wave of Bcl6 expression, but other factors are required to sustain it. As opposed to an all-or-none signaling effect, Tfh cell differentiation arises from a balance of competing cytokine-mediated signals, and can retain plasticity in their effector function. For example, IL-2 is a potent inhibitor of Tfh cell differentiation. Ligation of IL-2Rα leads to STAT5 signaling and expression of BLIMP-1 that antagonizes Bcl6 by displacing STAT3 from binding the Bcl6 locus[153-157]. Therefore, activated Tfh cells require a delicate regulation of IL-6 and IL-21 levels (as opposed to IL-2) in its immediate environment during differentiation. The role of cytokines here implies that Tfh differentiation largely occurs in microenvironments within the secondary lymphoid organs in which Tfh-promoting cytokine niches exist, such as the interfollicular zones near B cell follicles[148,158]. It also suggests a role for neighboring cells to promote an IL-2-deprived microenvironment, such as Treg cell-mediated IL-2 sinks. Failure in the maintenance of a proper balance of cytokines in the milieu surrounding a Tfh precursor could lead to its aberrant activation and differentiation into alternative effector lineages.

Activated T cells primed by DCs acquire the $CXCR5^{+}Bcl6^{+}$ phenotype within 24 to 72 hours, and require cognate T:B interactions to become fully differentiated GC Tfh cells[148,151,152].

Pre-Tfh cells upregulate expression of CXCR5, which facilitates chemotaxis toward CXCL13-rich B cell follicles. The upregulation of CCR7 by B cells at this stage facilitates their migration toward the T cell zone (rich in ligands CCL19 and CCL21), and facilitates cognate interactions with Tfh cells at the B cell-T cell junction. These cognate Tfh:B cell interactions are essential for the completion of Tfh cell differentiation[148,151,152,159,160]. The formation of stable T:B cell conjugates depends on the interactions of SLAM family proteins and SAP adaptor molecules[147,161,162]. Activated B cells express CD80, CD86, and ICOSL, which are costimulatory signals required to support the development of GC Tfh cell differentiation[107,163,164]. Compared to other effector T helper subsets, Tfh cells are more dependent on persistent TCR stimulation for differentiation and maintenance, and require CD28 and ICOS costimulation[107]. CD28 signaling is provided by CD80 and CD86 expressed on B cells, and ICOSL expression on B cells is also important for the maintenance of the Tfh cell phenotype[160,165].

In addition to its role in promoting GC B cell differentiation and affinity maturation (as described in the previous section), Tfh cells have also been implicated in maintaining GC B cell tolerance through its provision of help signals versus death signals to B cells. Specifically, upon engagement with pMHC presented on B cells, cognate Tfh cells induce the cell surface expression of two TNF superfamily ligands, CD40 and FAS ligands (CD40L and FASL)[166]. The CD40L engagement of CD40 on B cells provides at least two functions: 1) the delivery of key signals to promote B cell activation and survival, and 2) the induction of the FAS death receptor expression on B cells[132,167]. In the absence of sufficient signaling through the BCR (due to desensitization by anergy), Tfh cell-derived FASL triggers apoptosis of the anergic B cell via FAS-mediated activation of initiator caspases[167]. In contrast, sufficient BCR signaling by foreign

antigen is thought to upregulate expression of CD86 on B cells, which engages with CD28 on Tfh cells to prevent FAS-mediated cell death.

Several lines of evidence support the notion that FAS is critical for the control of self-reactivity in the GC. GC B cells express high levels of FAS, and mutations in FAS result in autoimmune lymphoproliferative syndrome (ALPS) and associated autoantibody production[168-170]. Therefore, it has been widely assumed that FAS-mediated death provides the primary mechanism through which self-reactive B cells are eliminated, and that FASL signals derive from Tfh cells. The need for a tight control of Tfh cells to prevent autoantibody production is evident in *Sanroque* (*Roquin*^{san}) mice, which produce autoantibodies and have excessive numbers of Tfh cells[166]. *Sanroque* mice harbor a defect in the ROQUIN protein, which in wildtype mice is an inhibitor of ICOS expression[171]. In the mutant *Sanroque* mice in which the negatively regulating pathway of ROQUIN is deficient, mice exhibit increased ICOS expression, spontaneous Tfh cell differentiation, and spontaneous GC responses[171]. These observations highlight a critical role for Tfh cells in the maintenance of peripheral B cell tolerance and the prevention of high-affinity autoantibody generation. Furthermore, the presence of spontaneous Tfh cell differentiation in *Sanroque* mice suggests that self-reactive specificities exist and can differentiate into Tfh cells when regulatory mechanisms are perturbed.

Significant gaps remain in our knowledge of Tfh cell-mediated selection of GC B cells. For example, although it has been implied that the expression of CD86 on B cells somehow instruct Tfh cells to promote help, the factors that dictate whether a Tfh cell provides help versus death signals to B cells remain unknown. Is B cell deletion through FAS signaling truly mediated by provision of FASL by Tfh cells, or are there other cell types involved? Further, it has been well described that Tfh cells within the GC are heterogeneous in both phenotype and function[172],

which is just beginning to be dissected. Does a division of labor exist within the GC, where functionally different populations of Tfh cells mediate survival of GC B cells as opposed to deletion of self-reactive GC B cells? What are the roles of T follicular cells that express Foxp3 (termed T follicular regulatory cells, Tfr cells), and with which cells do they interact? What factors dictate the exit of GC B cells, and is it dependent on decisions made by Tfh cells? And, finally, it is evident that the prevention of autoantibody production hinges on a tightly regulated T cell compartment. What is the prevalence of self-reactive T cells that differentiate into Tfh cells, either at steady state or during a GC reaction? If such cells exist, what could be their purpose, and how do they impact B cell tolerance and autoantibody production? Are they responsible for the prevalence of GC-derived autoantibodies associated with human autoimmune diseases, or do they play a seminal role in promoting the deletion of self-reactive B cell specificities?

Autoimmunity and immune dysregulation in humans and mice

Autoimmune diseases in humans are chronic complex inflammatory diseases that can be either systemic (e.g. systemic lupus erythematosus) or specific to a target organ or antigen (e.g. type 1 diabetes). They are typically characterized by a dysregulation of immune components—most prominently T cells and B cells—which leads to either the cell-mediated destruction of self-tissues or the production of autoantibodies, respectively. The strongest genetic associations with autoimmunity map to the HLA region, consistent with a pivotal role for T cells in driving autoimmune pathology[173]. An extensive list of autoimmune diseases has been associated with different variants of the HLA genes, with particular emphasis on MHC class II genes, specifically highlighting a role for pathological $CD4^+$ T cells in driving disease[174]. Interestingly,

other genes conferring susceptibility to autoimmunity in humans include many candidates associated with T cell-B cell collaboration[174]. Thus, there is interest in dissecting the mechanisms that lead to the dysregulation of $CD4^+$ T cells, with special regards to how this dysregulation fails at the T cell-B cell interface to drive disease.

Genome-wide associations studies (GWAS) from ten selected autoimmune conditions identified a number of genes involved in general T cell and Tfh cell biology[174]. The diseases studied included type 1 diabetes (T1D), rheumatoid arthritis (RA), juvenile idiopathic arthritis, autoimmune thyroid diseases, vitiligo, alopecia areata, systemic lupus erythematosus (SLE), multiple sclerosis (MS), primary biliary cirrhosis, and celiac disease. The study identified a susceptibility association with genes encoding costimulatory molecules such as CD28, CTLA-4, CD40, and ICOS, as well as certain corresponding ligands CD80, CD86, OX40L, and ICOSL. Cytokines such as IL-2, IL-4, IL-6, IL-21, and corresponding receptors such as IL-2Ra, IL-4R, IL-6R, and IL-21R have also been identified in GWAS studies. The chemokine receptors CXCR4 and CXCR5, and EBI-I and SLAM all relate to T cell-B cell positioning and have been implicated in disease associations as well. Lastly, a number of transcription factors associated with a GC response have been identified in GWAS data, such as BATF, IRF4, Maf, Bob1, Rel, Blimp-1. These findings demonstrate a key linkage between T cell-B cell interactions and autoimmune susceptibility. Moreover, the substantial identification Tfh-related genes in these studies further underscore the pivotal role for a tightly regulated T cell compartment, as it impinges on peripheral B cell tolerance.

In more rare instances, vaccinations protocols have also been associated with incidences of unintended autoimmunity, though large epidemiological studies are lacking to draw stronger conclusions[175]. The most notable example is the incidence of Guillain-Barre syndrome triggered

by the influenza vaccine[176]. Hypotheses to the potential causes of vaccine-induced autoimmune syndrome include molecular mimicry of the influenza antigens selected, or bystander activation of self-reactive T and B cells. Since these vaccination strategies are designed to optimize B cell antibody responses along with optimal Tfh cell help, careful strategies must be used to maintain the tightly controlled tolerance mechanisms that are imposed at the T cell-B cell interface. It also suggests that self-reactive cells exist and can be inadvertently activated in some instances.

The etiology of many autoimmune diseases is largely unresolved, but it is well known that a combination of polygenic and environmental components converge to define susceptibility or protection from disease[174]. In fact, autoimmunity in genetically susceptible individuals sometimes coincides with some type of inflammatory trigger, such as an infection. A number of mouse models that mimic human disease have shed light on the molecular mechanisms underlying certain diseases. As mentioned before, *Sanroque* mice mimic certain manifestations of disease observed in patients with systemic lupus erythematosus (SLE)[177]. It has been shown that the disease in these mice is largely caused by improperly regulated ICOS expression, resulting in spontaneous and elevated differentiation of Tfh cells that promote autoantibody production[171].

The role of mutations in the transcription factor AIRE in driving primary immune disorders (APECED, APS-1)[178] has been described in previous sections and will not be further discussed. Another primary immune disorder has been ascribed to mutations in the FOXP3 gene, which encodes the transcription factor required for the maintenance of Treg cells[59,60,179]. This disease, called immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans, leads to multi-organ autoimmunity, which presents with clinical conditions such as severe enteropathy, type 1 diabetes, and eczema[180]. The *scurfy* mouse strain harbors

mutation in the Foxp3 transcription factor, resulting in the loss of Treg cells and an X-linked lymphoproliferative disease that parallels that observed in IPEX patients[181,182]. These mice demonstrate the important role of suppressor Foxp3$^+$ Treg cells in the maintenance of immune homeostasis, as well as the importance of diverting self-reactive T cell specificities into the Treg cell lineage. In support of this, the Foxp3^{DTR} mouse strain that enables selective depletion of Foxp3$^+$ Treg cells has shown that chronic and systemic ablation of Treg cells leads to an aggressive and fatal lymphoproliferative disease[53]. It is presumed that the rapid and fatal disease in these mice is largely attributed to endogenous self-reactive T cells that become activated upon release from suppression, although the identity of these cells has yet to be defined. My thesis work centers on the use of the Foxp3^{DTR} mouse strain to reveal such endogenous self-reactive T cells that harbor pathogenic potential, which will be discussed further below.

Endogenous self-reactive T lymphocytes

The biology of naturally occurring self-reactive T conventional (Tconv) cell specificities that exist in the endogenous polyclonal repertoire is not well understood and is the subject of my thesis. A major challenge to our ability to globally survey and examine self-reactive T cells in the endogenous polyclonal repertoire hinges on the fact that the field lacks tools to accurately identify and measure self-reactivity for polyclonal cells at steady state without employing model self-antigens or tetramer systems that select for a pre-determined self-peptide specificity of interest. In this regard, the C-type lectin cell surface marker CD69 is a widely used marker of TCR signaling and T cell activation, as naïve T cells express negligible CD69, but activated T cells rapidly induce CD69 expression in an ERK-dependent fashion upon TCR signaling[183]. Constitutive expression of CD69 in naïve mice at steady state may thus indicate agonist signaling

by self-antigens. While CD69 expression is a useful marker of acute TCR signaling in some contexts, its expression is not always indicative of TCR signaling, as inflammatory stimuli (such as TLR signaling and type-I IFN signaling) can result in widespread and non-specific induction of CD69 expression[184]. Furthermore, in the thymus, its expression—along with expression of the cell surface glycoprotein CD5—are typically correlated with positive selection and thus cannot be used to distinguish between positively selected cells and cells reactive to classic self-peptide-MHC complexes[185]. Interestingly, Treg cells overall express a higher level of CD5 than Tconv cells, and cells in unprimed *Nur77*-GFP mice show that CD5 levels tend to correlate with GFP levels[90]. However, it remains unclear if CD5 expression is regulated exclusively by TCR signaling, or whether it is differentially expressed based on distinct T cell subsets.

Efforts to quantify the frequency of self-reactive T cells in the peripheral T cell pool using expression of CD69 estimate that approximately 4% of both $CD4^+$ and $CD8^+$ T cells contain self-reactive specificities[186]. This was accomplished using a CFSE-based proliferation index to back-calculate the percentage of cells in a CFSE-labeled pool of cells that proliferated and expressed elevated CD69 following Treg cell depletion. It was presumed that the proliferation of polyclonal cells upon Treg cell depletion was TCR mediated, because TCR transgenic cells co-transferred of into mice lacking the cognate antigen did not proliferate in this setting. TCR sequencing analyses of proliferating cells revealed a broad diversity of TCR clonotypes. Interestingly, proliferating cells spanned a broad range of CD5 expression, suggesting that both high- and low-affinity TCRs can proliferate upon Treg cell depletion. However, because this study was performed primarily using polyclonal populations in secondary lymphoid organs, the identity and nature of these presumed self-reactive clones could not be further elucidated.

As autoimmunity often manifests as autoimmune infiltration of lymphocytes into non-lymphoid organs, this metric is often employed in the study of self-reactive lymphocytes. Classic studies by Sakaguchi et al. revealed that autoimmune infiltration into target organs is largely mapped to the $CD4^+$ Tconv cell compartment when transferred into lymphopenic hosts, as $CD8^+$ T cells do not appear to drive infiltration in the absence of $CD4^+$ T cells[58]. Further, the co-transfer of $CD25^+$ cells (now known to be largely suppressor Treg cells) prevented this infiltration. These findings suggest that the endogenous $CD4^+$ T conventional (Tconv) repertoire harbors self-reactive specificities that are normally held in-check by Treg cells, and these Tconv cells can drive organ infiltration when released from suppression.

A second line of evidence in support of this was demonstrated when the systemic and selective depletion of $Foxp3^+$ Treg cells led to an aggressive lymphoproliferative disease characterized by splenomegaly and lymphadenopathy, expansion of $CD4^+$ and $CD8^+$ T cells, massive lymphocytic infiltrates into non-lymphoid organs, and death as early as ten days post-Treg cell-depletion[53]. In this setting, concurrent depletion of $CD4^+$ Tconv cells along with the depletion of Treg cells reduced the level of autoimmunity observed, especially in regards to dendritic cell activation[53], further supporting a central role of $CD4^+$ Tconv cells in the manifestation of disease. Following these studies, efforts to further define the nature of TCR specificities exhibited by activated T cells following Treg cell depletion led to two broad conclusions: 1) these cells do not appear to be reactive to microbial antigens derived from commensal bacteria[187], and 2) these cells do not appear to be reactive to dietary antigens derived from the gut[188]. The first conclusion was demonstrated through the systemic depletion of Treg cells in mice maintained under germ-free conditions[187]. These mice lack commensal bacteria that would normally reside in the gut, but Treg cell-depleted mice in these settings also succumbed to

fatal multi-organ autoimmunity, demonstrating that the cells responsible for disease do not depend on reactivity to microbial antigens. Interestingly, the kinetics of disease appeared delayed in germ-free mice when compared to mice maintained in specific-pathogen free (SPF) conditions, suggesting that although autoimmunity is likely driven by cells reactive to self-ligands, the microbiota may contribute to the exacerbation of disease to a certain extent. In the second study, the same findings were further extended to germ-free mice raised in an antigen-free (AF) environment[188]. In addition to the absence of commensal bacteria, these mice also lack exposure to dietary antigens as they are fed on an elementary diet. In these mice, systemic Treg cell depletion also led to the activation of a select population of naïve $CD4^+$ T cells and increased activation of host dendritic cells (DCs). Collectively, these studies support the notion that autoimmunity in these settings is largely driven by the activation of $CD4^+$ Tconv cells reactive to self-ligands rather than microbial or dietary antigens. Furthermore, these endogenous self-reactive specificities appear to harbor the capacity to pose serious threat and damage to the organism, and thus merit a thorough investigation into the nature of their self-reactivity, their phenotype and function at steady state, as well as their regulation by known immune tolerance mechanisms.

A number of questions remain unanswered regarding the nature of self-reactive T cells that are found to proliferate and infiltrate the non-lymphoid organs of Treg-depleted hosts. First, although it is known that these specificities are likely directed at self-ligands and not at ligands derived from microbial or dietary antigens, the exact nature of their antigen-specificity remains undefined. Specifically, the role of the TCR in this disease model has not been demonstrated. For example, it remains unknown whether organ-infiltration in this setting is primarily driven by TCR recognition of self-ligands expressed and presented within non-lymphoid organs or within

the draining lymph nodes. It is formally possible that the T cells directed towards target tissues of Treg-depleted hosts are largely a result of recruitment via TCR-independent mechanisms, such as inflammatory signals or non-specific activation. If TCR specificity indeed played a role, it is not known whether these cells are reactive to rare tissue-restricted antigens, sequestered antigens, or to ubiquitously expressed self-antigens.

Further, if these cells are indeed self-reactive, to what extent are these cells shaped by central and peripheral tolerance mechanisms? How do they escape negative selection in the thymus, and how are they kept in-check in the periphery? Do they largely circulate throughout the body as naïve cells ignorant of their cognate self-ligands, or do they sense their antigens but are maintained in a functionally unresponsive state of anergy? If so, what signals are required to breach the regulatory mechanisms imposed on these cells at steady state? Lastly, if self-reactive cells posed such a grave threat to the health of the organism, why are they maintained in the repertoire and allowed to circulate among the remaining pool of lymphocytes that presumably do not pose a danger to the host?

We hypothesized that the $CD4^+$ Tconv infiltrates found in the non-lymphoid organs of Treg-depleted hosts[53] would likely reveal a population of self-reactive Tconv cells that harbor pathogenic capacity and that reflect true relevance to autoimmunity. To begin to address the questions outlined above, we utilized a deep-TCR sequencing approach to identify the self-specific $CD4^+$ Tconv clonotypes that infiltrate non-lymphoid organs when released from Treg-mediated suppression[86,189]. Utilizing retrovirally expressed TCRs[69,89,190-192], we conducted a thorough examination of the development and biology of the relevant clonotypes. Our findings revealed key insights regarding the frequency, antigen-specificity, and phenotype of these clones at steady state.

CHAPTER II

MATERIALS & METHODS

Mice

C57BL/6J (B6; stock no. 000664), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (B6 CD45.1; stock no. 002014), B6.129S7-*Rag1*^{tm1Mom}/J (*Rag1*$^{-/-}$; stock no. 002216), B6.129S2-*Tcra*^{tm1Mom}/J (*Tcrα*$^{-/-}$; stock no. 002116 or 002115;), B6.129(Cg)-*Foxp3*$^{tm3(DTR/GFP)Ayr}$/J (*Foxp3*^{DTR}; stock no. 016958), B6.Cg-*Foxp3*^{tm2Tch}/J (*Foxp3*^{EGFP}*;* stock no. 006772), and B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J (*MHCII*$^{-/-}$; stock no. 003584 or 003374;) mice were obtained from The Jackson Laboratory. B6.CgTg(CD4-cre)1Cwi (*Cd4*^{Cre}; model no. 4196) mice were obtained from Taconic. TCR Vβ3 transgenic (TCRβtg) mice expressing a fixed TCRβ chain of sequence Vβ3-(TRBV26-ASSLGSSYEQY) were generated in a C57BL/6J background at the University of Chicago Transgenics Core Facility, as previously described[68]. TCRβtg and *Foxp3*^{DTR} mice were interbred to obtain TCRβtg *Foxp3*^{DTR} mice. *Cd4*^{Cre}, *Tcra*$^{-/-}$, and TCRβtg mice were interbred to obtain *Cd4*^{Cre} *Tcra*$^{-/-}$ TCRβtg mice. *Cd4*^{Cre} *Tcra*$^{-/-}$ TCRβtg and *Foxp3*^{DTR} mice were interbred to obtain *Cd4*^{Cre} *Tcra*$^{-/-}$ TCRβtg *Foxp3*^{DTR} mice. B6 CD45.1 and *Foxp3*^{DTR} mice were interbred to obtain CD45.1^{+} *Foxp3*^{DTR} mice. B6 CD45.1 and *Rag1*$^{-/-}$ mice were interbred to obtain CD45.1 *Rag1*$^{-/-}$ mice. *H2-DM*$^{-/-}$ mice in a C57BL/6J background were a generous gift from L. K. Denzin at Rutgers University. C57BL/6J mice housed under germ-free conditions (GF) were generously provided to us by E. B. Chang and B. Jabri at the University of Chicago. All mice were bred and maintained at the University of Chicago under specific-pathogen-free conditions (with the exception of germ-free mice) and in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Preparation of cell suspensions

Spleen, lymph nodes, and thymi were isolated and mechanically dissociated through 70-μm filters (Falcon) in RPMI (Gibco) supplemented with 10% FBS (Gemini Bio-Products). For the isolation of lymphocytes from non-lymphoid organs, whole male genitourinary tracts were isolated and prostate lobes were separated by microdissection. Prostate lobes, salivary glands, pancreas, and lacrimal glands were injected and digested with pre-warmed RPMI containing 0.4 mg mL^{-1} Liberase TL (Roche) and 0.2 mg mL^{-1} DNase I (Roche) for 30 min at 37°C. Digested prostate was mechanically disrupted with frosted microscope slides, while salivary glands, pancreas, and lacrimal glands were mechanically dissociated through 70-μm filters in RPMI-10% FBS. Following dissociation, each tissue sample was centrifuged at 700 × *g* for 5 min, resuspended in 5 mL of RPMI-10, overlaid on 5 mL of Histopaque 1119 (Sigma), and centrifuged at 700 × *g* for 10 min at room temperature with no brake. Viable lymphocytes were isolated from the interface, then washed and resuspended in RPMI-10% FBS.

Antibodies and flow cytometry

Typically, cell suspensions were incubated in staining buffer (phosphate-buffered saline with 2% FCS, 0.1% NaN_3, 5% normal rat serum, 5% normal mouse serum, 5% normal rabbit serum, and 10 μg mL^{-1} 2.4G2 FcR blocking antibody) for 20 min on ice (all sera from Jackson Immunoresearch). Fluorochrome-labelled monoclonal antibodies (clones denoted in parenthesis) against B220 (RA3-6B2), CCR7 (4B12), CD4 (RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD8β (YTS156.7.7), CD38 (90), CD44 (IM7), CD45.1 (A20), CD62L (MEL-14), CD69 (H1.2F3), CD73 (TY/11.8), CD80 (16-10A1), CD86 (GL-1), CXCR5 (L138D7), FR4 (12A5), GL-7 (GL7),

H2-K^b (AF6-88.5), I-A/I-E (M5/114.15.2), ICOS (C398.4A), IgD (11-26c.2a), IgM (RMM-1), NK1.1 (PK136), PD-1 (RMP1-30), TCRβ (H57-597), and Thy1.1 (OX-7) were purchased from Biolegend, eBioscience, or BD Biosciences unless otherwise noted. Intracellular staining of Bcl6 (K112-91), Egr2 (erongr2), activated Caspase-3 (C92-605), CD40L (MR1), FasL (MFL3), Ki67 (SolA15), Tbet (4B10), GATA3 (TWAJ), RORγt (Q31-378), and Foxp3 (FJK-16s) was performed using the Foxp3 Staining Buffer Set (eBioscience) at 4°C overnight. For stain sets that include antibodies against chemokine receptors, cells were stained in phosphate-buffered saline supplemented with 2% FBS at room temperature for 30 minutes. Flow cytometry data was acquired on an LSRFortessa (BD Biosciences) or LSRFortessa X-20 (BD Biosciences), or cells were FACS sorted using a FACSAria II (BD Biosciences) or FACSAria IIIu (BD Biosciences), and analyzed using FlowJo software (Tree Star). Doublets were excluded to remove dead cells when possible. Unless otherwise noted, TCR "retrogenic" cells were sorted as $CD8\alpha^-$ $CD45.1^-$ $Thy1.1^+$ and identified as $CD4^+CD8\alpha^-TCR\beta^+CD45.1^-Thy1.1^+$.

TCR sequencing and analysis

Cell populations of interest were FACS sorted into TRI Reagent (Sigma), frozen in dry ice, and stored at -80°C prior to use. RNA was isolated following a standard chloroform extraction and isopropanol precipitation protocol. Briefly, chloroform was added to each TRI reagent suspension, then vortexed and incubated for 3 min at room temperature. Samples were centrifuged at 12,000 × *g* for 15 min at 4°C, and the upper aqueous phase was extracted into a new tube, while avoiding the interface. RNA was precipitated by adding isopropanol along with 20 μg glycogen, then mixed and stored at -80°C for at least 30 min. Precipitated RNA was pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C, and the pellet was washed with 75%

ethanol, then air-dried and resuspended in molecular grade RNase-free water. Purified RNA was subjected to TCRα sequencing using the Amp2Seq service from iRepertoire, a platform based on semi-quantitative multiplex PCR coupled with Illumina sequencing. This approach allows analysis of the complete TCRα repertoire, regardless of variable-region usage. Typically, >8 x 10^5 sequence reads were obtained per sample.

For each TCRα peptide sequence, the sum of the corresponding cDNA sequence reads was divided by the total TCRα sequence reads within a given sample to obtain the frequency per sample. Recurrent clones were defined as TCRα clonotypes for which the frequency was non-zero across all five samples sequenced for a given non-lymphoid organ. Typically, the most abundant TCRα clonotypes within a site of interest were determined by ranking recurrent TCRα sequences by median frequency in decreasing order.

TCR construct design and cloning

The recombined *Trav* chain sequences of interest were obtained from cDNA sequence reads acquired by the iRepertoire sequencing platform. The *Trav* chain and the *Trac* constant region were synthesized into pUC57 plasmids (Genscript) and cloned into a conditional retroviral vector, pMGflThy1.1[89], as described below. The pMGflThy1.1 vector is designed such that expression is conditional on Cre-mediated excision of a lox-flanked STOP inserted 5' of the TCRα coding segment. Downstream of the TCRα coding segment is an IRES followed by mouse Thy1.1, such that positively infected cells could be distinguished by staining for the retrovirally encoded Thy1.1 protein and detected by flow cytometry. The *Trav* and *Trac* segments were PCR amplified using the Phusion high-fidelity polymerase (New England Biolabs) with dNTPs in 5X Phusion HF buffer (New England Biolabs). Primers were designed using the NEBuilder

Assembly Tool (New England Biolabs) and obtained from IDT. The pMGflThy1.1 vector was digested with restriction enzymes AgeI and NotI (New England Biolabs) for 12 hr at 37°C. PCR products as well as the AgeI- and NotI-digested pMGflThy1.1 vector were gel purified using a QIAquick Gel Extraction Kit (QIAGEN). Purified *Trav* and TRAC products were assembled together with the purified AgeI- and NotI-digested pMFflThy1.1 vector using the Gibson Assembly Master Mix (New England Biolabs) for 60-90 min at 50°C. Assembled product was transformed into high-efficiency 5-alpha competent *E. coli* (New England Biolabs), and plasmids carrying the correct insert were purified using an EndoFree Plasmid Maxi Kit (QIAGEN). Plasmid preparations were sequenced to verify the TCR insert. Sequence alignment and proper in-frame sequence expression was confirmed using the Snapgene software (GSL Biotech).

Retrovirus production, infection, and primary TCR retrogenic mice generation

Conditional retrovirus was produced using Plat-E cells after the *Tcra* genes were inserted into the conditional retroviral vector, pMGflThy1.1, as described above[89]. *Tcra* constructs were transfected into Plat-E packaging cells using lipofectamine (Life Technologies). Harvested retroviral supernatant was filtered through a 0.45 μm filter, frozen in a dry ice and ethanol bath, and stored at -80°C prior to use.

TCR Vβ3 transgenic *Cd4-cre Tcra*$^{-/-}$ mice (*Foxp3*^{wt} or *Foxp3*^{DTR}) were retro-orbitally injected with 150 mg kg^{-1} 5-Fluorouracil (Fresenius Kabi) 3 days prior to bone marrow harvest. Bone marrow was harvested by first cutting the epiphysis on each end of the femur bones, then by flushing the marrow out of each open end using a 30-gauge needle and syringe through a 40-μm filter. After harvest, bone marrow was cultured for 2 days in X-vivo 10 medium (Lonza) supplemented with 15% FCS, 1% penicillin and streptomycin, 100 ng mL^{-1} mouse SCF, 10 ng

mL^{-1} mouse IL-3, and 20 ng mL^{-1} mouse IL-6 (all from Biolegend). Cultures were maintained at 37°C with 5% CO_2 in a CO_2 incubator (Sanyo Electric). Stimulated cells were infected with *Tcra*-encoding retrovirus in the presence of 4 μg mL^{-1} polybrene (EMD Millipore) by centrifugation at 900 × *g* for 90 min at 37°C. After 24 hr of additional culture in X-vivo 10 medium (supplemented as described above), infected bone marrow cells were harvested and mixed with 5×10^6 freshly harvested bone marrow "filler" cells (from CD45.1 *Rag1*$^{-/-}$ mice) prior to injection. 24 hr prior to injection, recipient mice (CD45.1, *H2-DM*$^{-/-}$, or CD45.1 *Foxp3*^{DTR}) were lethally irradiated with 900 rads from an irradiator with an X-ray source. 24 hr after irradiation, recipient mice were retro-orbitally injected with the mixture of infected and filler CD45.1 *Rag1*$^{-/-}$ bone marrow to generate "primary TCR retrogenic" mice. Typically, primary TCR retrogenic mice were analyzed 6-8 weeks after reconstitution. Prior to analysis, proper expression of the transduced TCRα chains by donor cells was confirmed by staining peripheral blood leukocytes with antibody specific for CD45.2, Thy1.1, TCRβ, and CD4. In select cases where primary TCR retrogenic mice were generated in *H2-DM*$^{-/-}$ or CD45.1 *Foxp3*^{DTR} recipients, the corresponding strain were utilized as bone marrow filler cells.

Generation of low-frequency TCR retrogenic mixed bone marrow chimeras

Bone marrow cells were harvested, infected, and cultured as described above. Prior to injection, infected bone marrow cells were harvested from culture and mixed with 5×10^6 freshly harvested bone marrow "filler" cells (from CD45.1 mice), such that infected bone marrow constituted approximately 20% (or 14% or 8%) of total inoculum. 24 hr prior to injection, recipient mice (CD45.1) were sub-lethally irradiated with 500 rads from an irradiator with an X-ray source. 24 hr after irradiation, recipient mice were retro-orbitally injected with the mixture of

infected and filler CD45.1 bone marrow to generate low-frequency "TCR retrogenic" mixed bone marrow chimeric mice. Typically, chimeric mice were analyzed 6-8 weeks after reconstitution. Prior to analysis, proper expression of the transduced TCRα chains by donor cells was confirmed by staining peripheral blood leukocytes with antibody specific for CD45.2, Thy1.1, TCRβ, and CD4.

***In vitro* T cell stimulation assays**

All experiments were performed in RPMI containing 10% FBS (Atlanta Biologicals), 1% penicillin and streptomycin, 0.1% β-mercaptoethanol (Gibco), and 100 U mL^{-1} recombinant mouse interleukin-2 (IL-2) (Miltenyi Biotec), unless absence of IL-2 was specified. In addition, all cell cultures were maintained in 384-well clear round bottom ultra-low attachment spheroid microplates (Corning) at 37°C with 5% CO_2 in a CO_2 incubator (Sanyo Electric). Pooled spleen and lymph nodes were isolated from primary TCR retrogenic hosts, enriched for $CD4^+$ T cells using the mouse $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec), and stained with antibodies against CD8β, CD45.1, and Thy1.1 prior to FACS sorting. T cells expressing the TCRα chains of interest ("TCR retrogenic cells") were isolated by FACS sorting CD4-enriched lymphocytes for $CD8\beta^-CD45.1^-Thy1.1^+$ cells. Isolated TCR retrogenic cells were labeled using the CellTrace Violet (CTV) Cell Proliferation Kit (Invitrogen) following manufacturer's protocol, with slight modifications. In brief, sorted cells were resuspended in pre-warmed phosphate-buffered saline containing 0.625 μM CTV and incubated for 20 min at 37°C. The reaction was quenched by the addition of 10 mL cold RPMI containing 10% FCS (RPMI-10), then washed with RPMI-10. To obtain splenic dendritic cells (DCs), splenocytes were isolated from the spleen of various genetic mouse strains (CD45.1, GF, *H2-DM*$^{-/-}$, and *MHCII*$^{-/-}$), injected and digested with pre-warmed

RPMI containing 0.4 mg mL^{-1} Liberase TL (Roche) and 0.2 mg mL^{-1} DNase I (Roche) for 20 min at 37°C, mechanically dissociated through a 100-μm filter (Falcon), and enriched for $CD11c^+$ cells using the mouse CD11c MicroBeads UltraPure kit (Miltenyi Biotec). In select instances in which DCs from other anatomical sites were used, the same procedures were followed for cells isolated from the indicated sites. 1-2.5 × 10^4 of CTV-labeled TCR retrogenic cells were co-cultured *in vitro* with 5 × 10^4 $CD11c^+$ DCs for 5 days. When specified, anti-MHC-II blocking antibody (clone M5/114.15.2, BD Biosciences) or IgG2b, κ isotype control antibody (BD Biosciences) was added to indicated cultures at a final concentration of 5 μg mL^{-1}. As a positive control, CTV-labeled TCR retrogenic T cells were co-cultured with anti-CD3ε/anti-CD28 MACSiBead particles at a 1:1 ratio using the mouse T Cell Activation/Expansion Kit (Miltenyi Biotec) following manufacturer's protocol. For all experiments, dilution of CTV and total T cell number were assessed by flow cytometry on day 5.

Preparation of protein lysates

In select instances in which protein lysates were included in the culture of *in vitro* T cell stimulation assays, protein extracts were prepared as previously described[69]. Briefly, the prostate or salivary glands of 8-week-old male mice (B6 or *Foxp3*^{DTR} mice depleted of Treg cells for 12 days) were isolated and incubated separately in phosphate-buffered saline (PBS) for 5 min at room temperature, then centrifuged at 10,000 × *g* for 5 min at 4°C. The supernatant (i.e. secreted fraction) was removed, while the remaining pellet was resuspended in PBS and dissociated using a PowerGEN 1000 homogenizer generator (Fisher) and a hard tissue probe (Omni International). The dissociated suspension was centrifuged at 16,000 × *g* for 5 min at 4°C, and the supernatant (i.e. lysate fraction) was transferred into a new tube. The total protein content was quantified by

the Bradford assay using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories) and a spectrophotometer. The protein lysates were flash frozen in liquid nitrogen and stored at -80°C prior to use. Protein lysates were added to indicated cultures at a final concentration of 0.1 mg mL^{-1}.

***In vivo* adoptive T cell transfer**

Typically, pooled spleen and lymph nodes were isolated from primary TCR retrogenic hosts, enriched for $CD4^+$ T cells using the mouse $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec), and stained with antibodies against CD8β, CD45.1, Thy1.1 prior to FACS sorting. T cells expressing the TCRα chains of interest ("TCR retrogenic cells") were isolated by FACS sorting CD4-enriched lymphocytes for $CD8\beta^-CD45.1^-Thy1.1^+$ cells. When specified, TCR retrogenic cells were further sorted for the naïve conventional T (Tconv) cell subset by sorting on $CD62L^{hi}CD44^{lo}CD25^{lo}$ $Foxp3^{EGFP\text{-neg}}$ cells. Sorted cells were resuspended in RPMI (Gibco) and retro-orbitally injected into the specified recipients.

In experiments involving co-transfer with "filler" cells, pooled spleen and lymph nodes were isolated from 6-8 week-old mice (CD45.1 or CD45.1 $Foxp3^{DTR}$, as indicated), and cells were resuspended in 1 mL Red Blood Cell Lysing Buffer (Sigma) and incubated for 5 min at room temperature to lyse red blood cells. The reaction was quenched by the addition of 10 mL cold RPMI containing 10% FCS (RPMI-10), then washed with RPMI-10 and resuspended in RPMI prior to injection. Typically, 10×10^6 bulk filler cells were co-injected retro-orbitally with sorted TCR retrogenic T cells, in which TCR retrogenic T cells constituted ~0.5-1% of the inoculum. In instances in which sort yield was low, the amount of bulk filler cells was reduced

such that the proportion of TCR retrogenic T cells would remain as ~0.5-1% of the total inoculum.

Analysis of cytokine production

For assessment of cytokine production by intracellular staining, cells were cultured in RPMI (Gibco) containing 10% FBS (Gemini Bio-Products), 50 ng mL^{-1} PMA, and 500 ng mL^{-1} Ionomycin in U-bottom 96-well plates (Corning) for 1 hr at 37°C, followed by addition of 2 μM monensin (eBioscience) for another 4 hr at 37°C. Cells were then permeabilized using the Foxp3 Staining Buffer Set (eBioscience), and stained for intracellular cytokines at 4°C overnight. Fluorochrome-labelled monoclonal antibodies (clones denoted in parenthesis) against IL-2 (JES6-5H4), IFN-y (XMG1.2), IL-4 (11B11), and IL-10 (JES5-16E3) were purchased from BD, IL-17 (TC11-18H10.1) was purchased from Biolegend, and IL-21 (mhalx21) from eBioscience. Flow cytometry data was acquired on an LSRFortessa (BD Biosciences) or LSRFortessa X-20 (BD Biosciences)

Tissue fixation and microscopy

Spleen and lymph nodes were isolated from primary TCRrg hosts and washed in phosphate-buffered saline (PBS). Spleens were directly frozen in embedding medium for optimal cutting temperature (OCT, Sakura) using cryomold trays placed on dry ice, then stored at -80°C prior to use. Lymph nodes were fixed in BD Cytofix fixation buffer (BD) diluted four-fold in 1X PBS and incubated at 4°C overnight. Lymph nodes were then washed with PBS and cryoprotected by incubating in 30% sucrose wt/v dissolved in 1X PBS (Sigma) at 4°C overnight. The next day, lymph nodes were frozen in OCT (Sakura) using cryomold trays placed on dry ice,

then stored at -80°C prior to use. Tissues were stained with the following antibodies: B220, Bcl6 (K112-91), CD4, GL-7, and Thy1.1 (OX-7), and visualized by confocal microscopy.

In vitro assay for T cell stimulation of B cell responses

All experiments were performed in RPMI containing 10% FBS (Atlanta Biologicals), 1% penicillin and streptomycin, 0.1% β-mercaptoethanol (Gibco). In addition, all cell cultures were maintained in U-bottom 96-well plates (Corning) at 37°C with 5% CO_2 in a CO_2 incubator (Sanyo Electric). Pooled spleen and lymph nodes were isolated from primary TCR retrogenic hosts and stained with antibodies against CD4, CD45.1, and Thy1.1 prior to FACS sorting. T cells expressing the TCRα chains of interest ("TCR retrogenic cells") were isolated by FACS sorting for $CD4^+CD45.1^-Thy1.1^+$ cells. To obtain B cells, splenocytes were isolated from the spleen of CD45.1 mice and enriched for B cells using the mouse B Cell Isolation Kit (Miltenyi Biotec). 3×10^4 TCR retrogenic cells were co-cultured *in vitro* with 5×10^4 splenic B cells along with purified anti-CD3ε (145-2C11) and anti-IgM (F(ab') anti-mouse IgM Mu chain) antibodies at a final concentration of 2 μg mL^{-1} and 5 μg mL^{-1}, respectively, for 7 days. B cells cultured in the absence of T cells or in the absence of anti-CD3ε and anti-IgM antibodies as well as T cells served as negative controls. For all experiments, B cell expression of I-A/I-E (M5/114.15.2), CD80 (16-10A1), GL-7 (GL7), and intracellular IgD (11-26c.2a), IgG1 (RMG1-1), IgG2a (RMG2a-62), IgG2b (RMG2b-1) were assessed by flow cytometry on day 7. B cells were also assessed for viability using the Live/Dead fixable near-IR dead cell stain kit (Life Technologies) by flow cytometry on day 7.

Immunization with complete Freund's adjuvant

Sterile 1X phosphate-buffered saline (PBS) was emulsified in Complete Freund's Adjuvant (CFA,) at a ratio of 1:1 prior to injection. 100μL of CFA was injected subcutaneously into each flank of primary TCR retrogenic hosts using a 30-gauge needle. 13 days post-immunization, mice were analyzed either by tissue fixation and microscopy or via flow cytometry.

Systemic Treg cell ablation

Diphtheria toxin (Sigma) was reconstituted at 5 μg mL^{-1} in sterile molecular grade water following manufacturer's protocol and stored at -80°C prior to use. Diphtheria toxin aliquots were frozen and thawed once and 50 μg kg^{-1} of diphtheria toxin was injected intraperitoneally on days 0 and 1, then every other day until day 12. Mice were monitored for signs of terminal autoimmune disease and were sacrificed once moribund.

Statistical analysis

Data were analyzed using Prism software (GraphPad). For the comparison of two groups, either the Student's t-test (two-tailed) or the nonparametric Mann-Whitney test were used, depending on whether data were normally distributed. For comparison of multiple groups, one-way ANOVA was employed, coupled with Tukey's HSD post-hoc tests when appropriate. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

CHAPTER III

RESULTS

Overview

The capacity of the adaptive immune system to recognize a diverse repertoire of antigens with high specificity raises the critical importance for the establishment of stringent immune tolerance mechanisms to ensure that self-tissues are protected from autoimmune attack. The failure and success of such regulatory processes have important implications in the context of autoimmunity, transplantation, and cancer, prompting considerable interest in understanding the forces that prevent unwanted immune responses directed at self-constituents. While many autoreactive T cells are thought to be purged from the Tconv cell repertoire by clonal deletion or differentiation into innate-like lineages[44,75,76], substantial evidence suggests that this process is imperfect. For example, the systemic depletion of Treg cells results in an aggressive lymphoproliferative disease characterized by splenomegaly and lymphadenopathy, expansion of $CD4^+$ and $CD8^+$ T cells, massive lymphocytic infiltrates into non-lymphoid organs, activation of dendritic cells, and death as early as ten days post-Treg cell-depletion[53]. These observations support the notion that clonal deletion is incomplete, and that peripheral tolerance mechanisms must be in place to ensure that self-reactive specificities that escape negative selection are restrained in the periphery. Specifically, the drivers of autoimmunity in Treg-deficient settings have been primarily mapped to the $CD4^+$ conventional T (Tconv) cell subset based on three key observations. First, distinct HLA class II alleles are risk factors for many human autoimmune diseases, consistent with a key role for $CD4^+$ T cells[174]. Second, adoptive transfer of $CD4^+$ T cells—but not $CD8^+$ T cells—into lymphopenic hosts in the absence of Treg cells was sufficient

to induce multi-organ autoimmunity[58]. Third, concurrent depletion of $CD4^+$ T cells along with depletion of Treg cells reduced the level of autoimmunity observed, especially in regards to dendritic cell activation[53]. While evidence suggests that $CD4^+$ Tconv cells are targets of continuous suppression by Treg cells, the identity of these cells remains elusive. Specifically, little is known about the frequency, phenotype, and specificity of self-reactive T cells present in the endogenous repertoire.

Previous work in this field has largely relied on the study of T cells reactive to transgenically expressed or exogenously administered model antigens, or the study of endogenous T cells reactive to arbitrarily selected self peptides[47-49,96,97,193]. It remains unclear whether such specificities accurately reflect self-reactive T cells that induce immune pathology following Treg cell depletion. Additionally, several studies of self-reactive T cells employ peptide-based vaccinations to expand self-reactive T cells in the peripheral repertoire[96,97]. Although self-reactive T cells can be detected in these models, it is unclear whether these cells are pathogenic in the absence of strong adjuvants. In this study, we utilize deep-TCR sequencing approaches to identify self-reactive $CD4^+$ Tconv clones that infiltrate non-lymphoid organs when released from Treg-mediated suppression[189]. Utilizing retrovirally expressed TCRs to track self-reactive Tconv clones, we define the frequency, anatomical distribution, and phenotype of a group of self-reactive Tconv cells and the mechanisms by which these cells are regulated at steady state. The self-reactive Tconv cells detected using this approach have true relevance to autoimmunity, as they are identified directly from non-lymphoid organs following Treg cell depletion, in the absence of exogenous antigen and strong adjuvants[53].

Our studies address several fundamental questions about the nature of self-reactive Tconv cells: 1) What is the prevalence of self-reactive T cells in the peripheral repertoire? 2) What is

the nature of self-ligands recognized by these self-reactive T cells? 3) To what extent are self-reactive T cells impacted by central and peripheral tolerance mechanisms? 4) What is the pathogenic potential of self-reactive T cells when studied in isolation? 5) Are self-reactive T cells defined by specific phenotypic markers that would allow for their identification and manipulation in a polyclonal repertoire? Studies on the immunological forces that regulate these endogenous self-reactive Tconv cells are expected to significantly advance our understanding of tolerance and autoimmunity.

Recurrent Tconv cell clonotypes are detected in non-lymphoid organs of Treg-depleted mice

To identify self-reactive Tconv cell clonotypes in the endogenous repertoire, we surveyed the repertoire of $CD4^+$ Tconv cells that become enriched in non-lymphoid organs following Treg cell ablation. Male $Foxp3^{DTR\text{-}EGFP}$ hosts expressing a fixed transgenic (tg) TCRβ chain were subjected to sustained Treg cell ablation by administration of diphtheria toxin (DT)[53] (Figure 1A and 1B). Following sustained Treg cell depletion, endogenous $CD4^+$ $Foxp3^{neg}$ Tconv cells enriched in distinct non-lymphoid organs were sorted, and the TCRα chains of all Vα families were sequenced using the iRepertoire platform[189] (Figure 1A). In this study, we focused our sequencing analyses on the $CD4^+$ Tconv cell subset within the prostate, while using T cells from the salivary glands for comparative analyses.

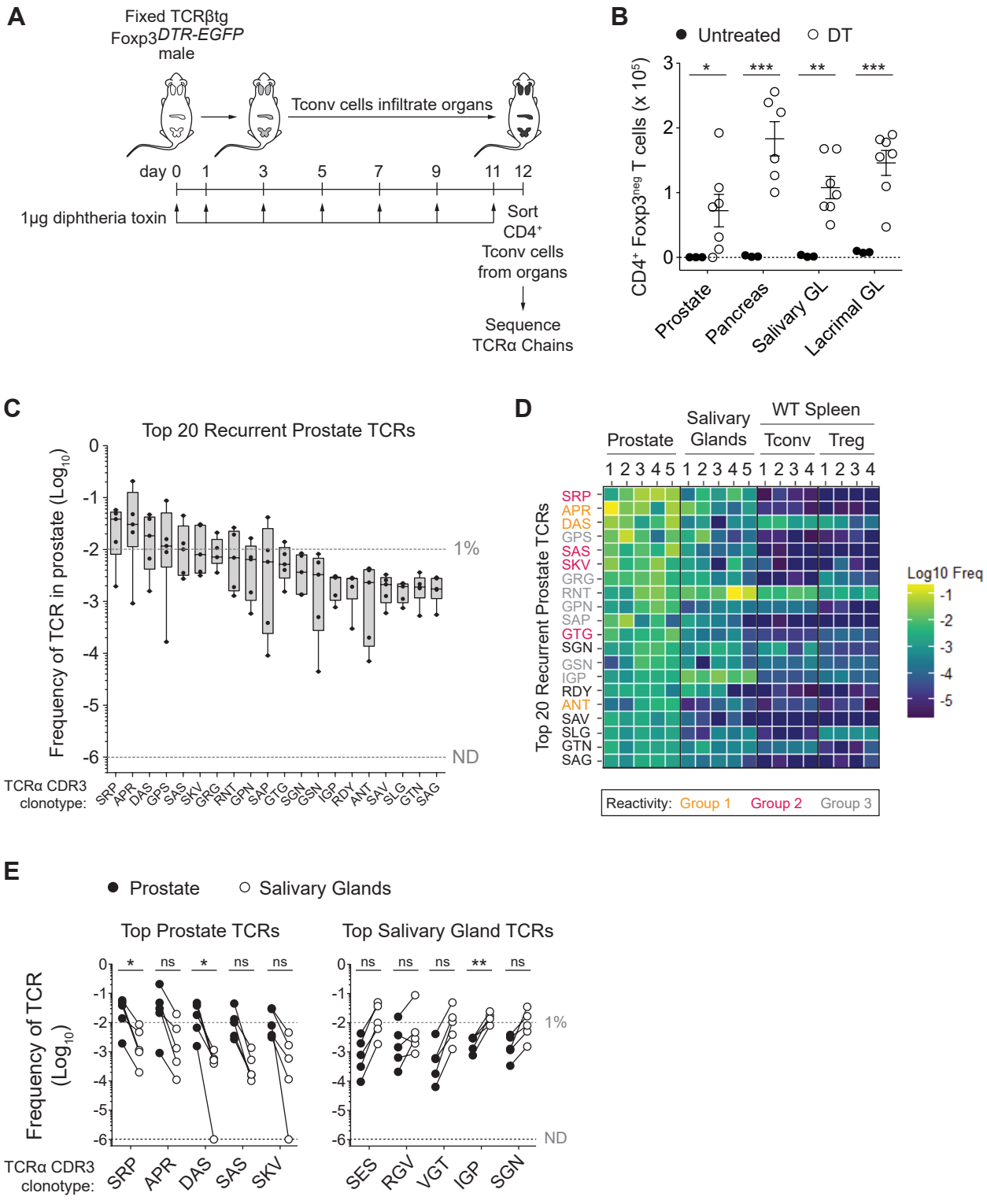

Figure 1. Recurrent Tconv cell clonotypes are detected in non-lymphoid organs of Treg-depleted mice.

Figure 1, continued.
(A) Diphtheria toxin (DT) was administered to 6-8 week-old TCRβtg Foxp3^{DTR} male mice as described in Methods. On day 12, CD4^{+} Foxp3^{neg} cells were isolated from non-lymphoid organs of Treg-depleted mice and TCRα sequences were obtained.
(B) Day 12 absolute numbers of CD4^{+} Foxp3^{neg} cells in indicated organs (prostate, pancreas, salivary glands, and lacrimal glands) of individual mice from each denoted treatment group. Closed circles, untreated; open circles, DT-treated (DT). Data are representative of three independent experiments.
(C) The log10 frequency (y-axis) of the top 20 most abundant recurrent TCRα sequences within the prostate is shown (denoted on the x-axis), ranked from left to right in order of median abundance within the prostate.
(D) The TCRα sequences from (A) are displayed alongside their corresponding log10 frequency within other organs (salivary glands) of the same Treg-depleted mice, or within pooled spleen and lymph nodes of 6-8 week-old Foxp3^{+} and Foxp3^{neg} subsets of TCRβtg Foxp3^{EGFP} male mice. Each row corresponds to individual TCRα sequences (denoted on the left), ranked from top to bottom in order of median abundance within Treg-depleted prostates. Each column corresponds to individual mice and organ, as denoted on the top of each column. Data are representative of 4-5 mice across three independent experiments.
(E) (left), The log10 frequency (y-axis; closed circles) of the top 5 most abundant recurrent TCRα sequences within the prostate is shown (denoted on the x-axis) alongside their corresponding log10 frequency within the salivary glands (open circles), as denoted. (E) (right), The same is shown for the top 5 most abundant TCRα sequences within the salivary glands. TCRα sequences are ranked from left to right in order of median abundance within the prostate (left graph) or salivary glands (right graph).

CD4^{+} Tconv cells were isolated from the prostates and salivary glands of 5 individual Treg-depleted mice. An average of ~7.2×10^4 cells per prostate and ~1.08×10^5 cells per salivary gland were obtained and subjected to TCRα sequencing. We were able to achieve complete sequence coverage for each sample, since the iRepertoire platform yields on average ~1×10^6 reads per sample. Collectively, the prostatic repertoire contained 17,154 distinct TCRα clonotypes (distinguished by CDR3α), of which 275 TCR clonotypes were recurrently detected across all 5 prostate samples sequenced. While the range of TCR frequencies within the prostate is sizeable (spanning 6 logs), the prostate appears to be largely dominated by select clonotypes,

as the frequency of the top 20 most prevalent recurrent TCR clonotypes within the prostates collectively make up ~37% of the prostatic repertoire (Figure 1C). To investigate the possibility that the enrichment of T cells in the prostate occurred as a result of TCR-independent mechanisms, we cross-referenced the frequencies of the top 20 prostatic clones to our previously published reference catalog of $CD4^+$ Tconv and Treg cells in the secondary lymphoid organs (SLOs) of TCRβtg male hosts at steady state[86]. If the prevalence of the top prostate-enriched clones is merely a passive reflection of their abundance within the peripheral repertoire, we would expect to observe a correlation between the frequency of TCRs within the prostatic repertoire and their corresponding frequency within the peripheral SLO catalog. However, our results revealed that many prostatic TCRs were not abundant in the SLOs at steady state (Figure 1D). While select TCRs were indeed found at high frequencies in the SLO reference catalog, most fell close to the limit of detection at 10^{-6} in the SLO reference catalog. Thus, we reasoned that the prostatic repertoire following Treg-ablation likely contains select tissue-enriched TCR clonotypes.

To assess whether prostatic Tconv clones might be reactive to tissue-restricted antigens or widespread antigens, we performed the same sequencing analysis for $CD4^+$ $Foxp3^{neg}$ T cells enriched in the salivary glands following Treg cell-depletion. Comparison of the prostate and salivary gland TCR repertoires revealed that prostatic TCR clonotypes are also found in the salivary glands, albeit at consistently lower frequencies (~1 log difference) (Figure 1E), and the same was true vice versa. These results suggest two possibilities: a) the TCR clonotypes are reactive to widely expressed self-antigens found in both sites, or b) the TCR clonotypes are reactive to tissue-specific antigens, but also traffic to off-target organs due to widespread inflammation.

Prostate TCRs selected for retroviral TCR expression

Clone	Vα	Jα	CDR3 Sequence	Category	Group
APR	TRAV6-7/DV9	9	ALAPRNMGYKLT	Unreactive	1
DAS	TRAV8D-1	50	ATDASSSFSKLV	Unreactive	1
ANT	TRAV7D-2	40	AAANTGNYKYV	Unreactive	1
SRP	TRAV9D-4	53	AVSRPGGGSNYKLT	Broadly self-reactive	2
SAS	TRAV9D-4	9	AVSASTMGYKLT	Broadly self-reactive	2
SKV	TRAV9D-4	53	AVSKVGGGSNYKLT	Broadly self-reactive	2
GTG	TRAV14D-3/DV8	12	ASGTGGYKVV	Broadly self-reactive	2
GPS	TRAV6-7/DV9	9	ALGPSNMGYKLT	Some self-reactivity	3
GRG	TRAV9D-4	32	AVGRGGSSGNKLI	Some self-reactivity	3
RNT	TRAV9D-4	49	AVRNTGYQNFY	Some self-reactivity	3
GPN	TRAV6-2	42	VLGPNSGGSNAKLT	Some self-reactivity	3
SAP	TRAV14-2	30	AASAPPNAYKVI	Some self-reactivity	3
GSN	TRAV9D-3	34	ALGSNTNKVV	Some self-reactivity	3
IGP	TRAV14-2	49	AAIGPGYQNFY	Some self-reactivity	3

Table 1. Selection of clonotypes for TCR retrogenic studies.
14 of the top 20 most abundant recurrent TCRα sequences within the prostate were selected for TCR retrogenic studies. The remaining 6 TCRs expressed TRAV9D-4 and were excluded from subsequent studies. The 14 selected TCRα clonotypes can be loosely segregated into three groups based on reactivity to splenic dendritic cells (see Figure 3).

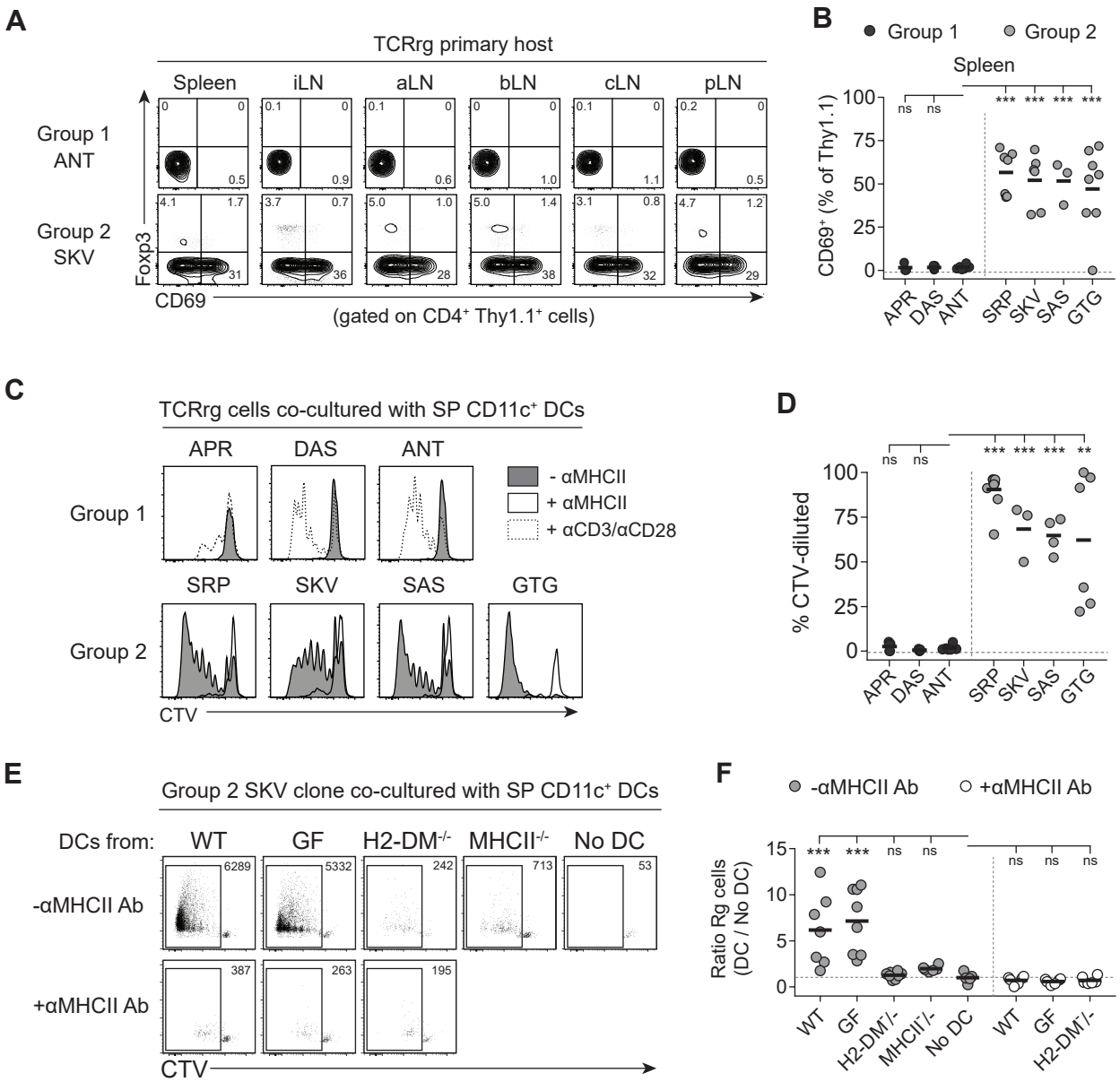

Figure 2. Several recurrent prostatic Tconv cell clonotypes exhibit widespread activation and reactivity to MHCII-restricted self-peptides.

Figure 2, continued.
From the prostatic TCRα sequences in Figure 1B, 7 TCRs were selected and retrovirally transduced into donor bone-marrow to generate TCR retrogenic (TCRrg) mice as described in Methods.
(A) 5-7 weeks after bone-marrow engraftment, secondary lymphoid organs were isolated from primary TCRrg hosts, and TCRrg ($TCR\beta^{+}CD4^{+}Thy1.1^{+}$) cells were assessed for CD69 expression via flow cytometry. TCRs were grouped based on CD69 expression: Group 1, no activation; Group 2, widespread activation. Numbers denote the percentage of cells in each quadrant. SP, spleen; iLN, inguinal; aLN, axillary; bLN, brachial; cLN, cervical; pLN, peri-aortic lymph nodes.
(B) Quantification of (A). TCRα clonotypes are denoted on the x-axis. Data are representative of three independent experiments.
(C) 5-7 weeks after bone-marrow engraftment, secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^{+}$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^{+}$) via FACS. TCRrg cells were labeled with CellTrace Violet (CTV), then 1-2.5 x 10^4 CTV-labeled TCRrg cells were co-cultured with 5 x 10^4 splenic dendritic cells (DC) isolated from B6.SJL female mice via CD11c MACS kit, along with recombinant mouse IL-2. Where indicated, anti-MHCII blocking antibody was included in the culture. As a positive control, 1-2.5 x 10^4 CTV-labeled TCRrg cells were co-cultured with anti-CD3/anti-CD28 microbeads at a 1:1 ratio. On day 5, CTV-dilution was assessed via flow cytometry.
(D) Quantification of (C). TCRα clonotypes are denoted on the x-axis. The y-axis denotes the percentage of CTV-diluted TCRrg cells. Data are representative of three independent experiments.
(E) TCRrg cells were isolated, CTV-labeled, and co-cultured with splenic $CD11c^{+}$ DCs as described in (C). DCs were isolated from various mouse strains: WT, wildtype; GF, germ-free; $H2\text{-}DM^{-/-}$, H2-DM knockout; $MHCII^{-/-}$, MHC class II knockout. Where indicated, anti-MHCII blocking antibody was included in the culture. On day 5, CTV-dilution was assessed via flow cytometry.
(F) Quantification of (E). TCRα clonotypes are denoted on the x-axis. The y-axis denotes the ratio of total TCRrg cell count within each well containing DCs divided by the cell count of TCRrg within wells containing no DCs. Data are representative of two independent experiments.

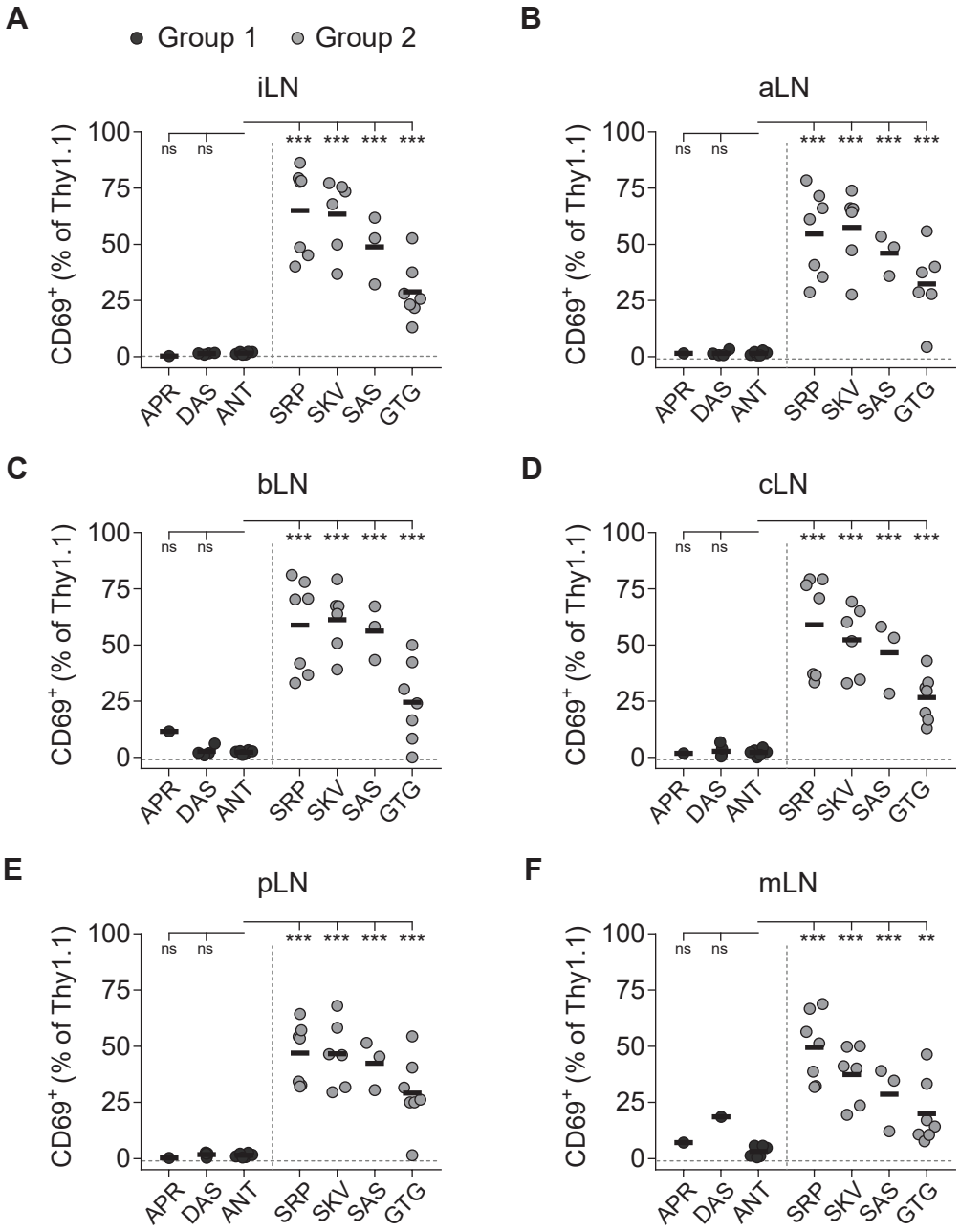

Figure 3. Several recurrent prostatic Tconv cell clonotypes exhibit widespread activation.

Figure 3, continued.
From the prostatic TCRα sequences in Figure 1B, 7 TCRs were selected and retrovirally transduced into donor bone-marrow to generate TCR retrogenic (TCRrg) mice as described in Methods.
(A-F) 5-7 weeks after bone-marrow engraftment, secondary lymphoid organs were isolated from primary TCRrg hosts, and TCRrg ($TCR\beta^{+}CD4^{+}Thy1.1^{+}$) cells were assessed for CD69 expression via flow cytometry. The percentage of TCRrg cells expressing CD69 are quantified. TCRs were grouped based on CD69 expression: Group 1, no activation; Group 2, widespread activation. SP, spleen; iLN, inguinal; aLN, axillary; bLN, brachial; cLN, cervical; pLN, peri-aortic lymph nodes. Statistical comparisons were made for each TCR using 'No DC' group as baseline.

Several recurrent prostatic Tconv cell clonotypes exhibit reactivity to widespread self-antigens

To gain insight into the antigen-specificity of prostate-enriched Tconv clones in Treg-depleted mice, we generated a series of monoclonal TCR retrogenic (TCRrg) mice expressing distinct TCRs as described in Methods[69,89,190-192]. Of the 20 most abundant recurrent TCRα sequences within the prostate, the 10 most prevalent TCRs were selected for TCR retrogenic studies (Table 1). In the remaining 10 TCRs, we selected 4 more for TCR retrogenic studies based on diverse Vα gene segment usage (Table 1). The remaining 6 TCRs expressed the same TRAV9D-4 gene and were thus excluded from subsequent studies. The collective average frequencies of the selected 14 TCRs comprise ~22% of the entire prostatic infiltrate; thus, these 14 clones represent a substantial fraction of the prostatic repertoire.

Analysis of primary TCRrg hosts expressing each of the 14 TCRs revealed that clones largely fell into three broad categories. In the first category, 3 of 14 clones (denoted as Group 1 clones) appeared largely phenotypically naive in primary TCRrg hosts, failing to upregulate expression of CD69 and CD44 in all secondary lymphoid organs surveyed (Figures 2A and 2B;

Figure 3). To screen for antigenic activity, we used an *in vitro* culture system to monitor the proliferation of TCRrg cells[69]. We found that clones expressing Group 1 TCRs did not proliferate upon co-culture with splenic DCs (Figures 2C and 2D; Figures 4A and 4B). Moreover, addition of prostatic protein extracts to the co-culture system also failed to induce T cell proliferation (Figures 4C and 4D). Group 1 TCRrg cells are functional, as addition of anti-CD3/anti-CD28 microbeads was sufficient to induce proliferation of these cells (Figures 4C and 4D). Lastly, adoptive transfer of Group 1 cells into Treg-depleted *Foxp3*^{DTR} males did not recapitulate prostate enrichment as originally observed in our sequencing analyses (Figure 17C). Thus, all of our available data suggest that Group 1 TCRs do not exhibit self-reactivity in adult mice. At the present time, it remains unclear to us why the clones in this category were recurrently detected in the prostate following Treg cell ablation.

In the second category, 4 of 14 clones (denoted as Group 2 clones) exhibited signs of overt TCR reactivity, as a large fraction of CD4^{+} TCRrg T cells in these mice expressed markers of TCR signaling (CD69) throughout all secondary lymphoid organs analyzed (Figures 2A and 2B; Figure 3). Strikingly, we found that wild-type splenic dendritic cells (DCs) alone were sufficient to induce *in vitro* proliferation of Group 2 clones in an MHC-II-dependent manner, without addition of exogenous proteins (Figures 2C and 2D; Figures 4A and 4B). Similar results were obtained when Group 2 TCRrg cells were co-cultured with DCs isolated from various lymph nodes (data not shown). These TCR clones do not appear to be reactive to foreign antigens derived from commensal bacteria, as co-culture with splenic CD11c^{+} DCs isolated from germ-free mice did not abrogate TCRrg cell proliferation (Figures 2E and 2F).

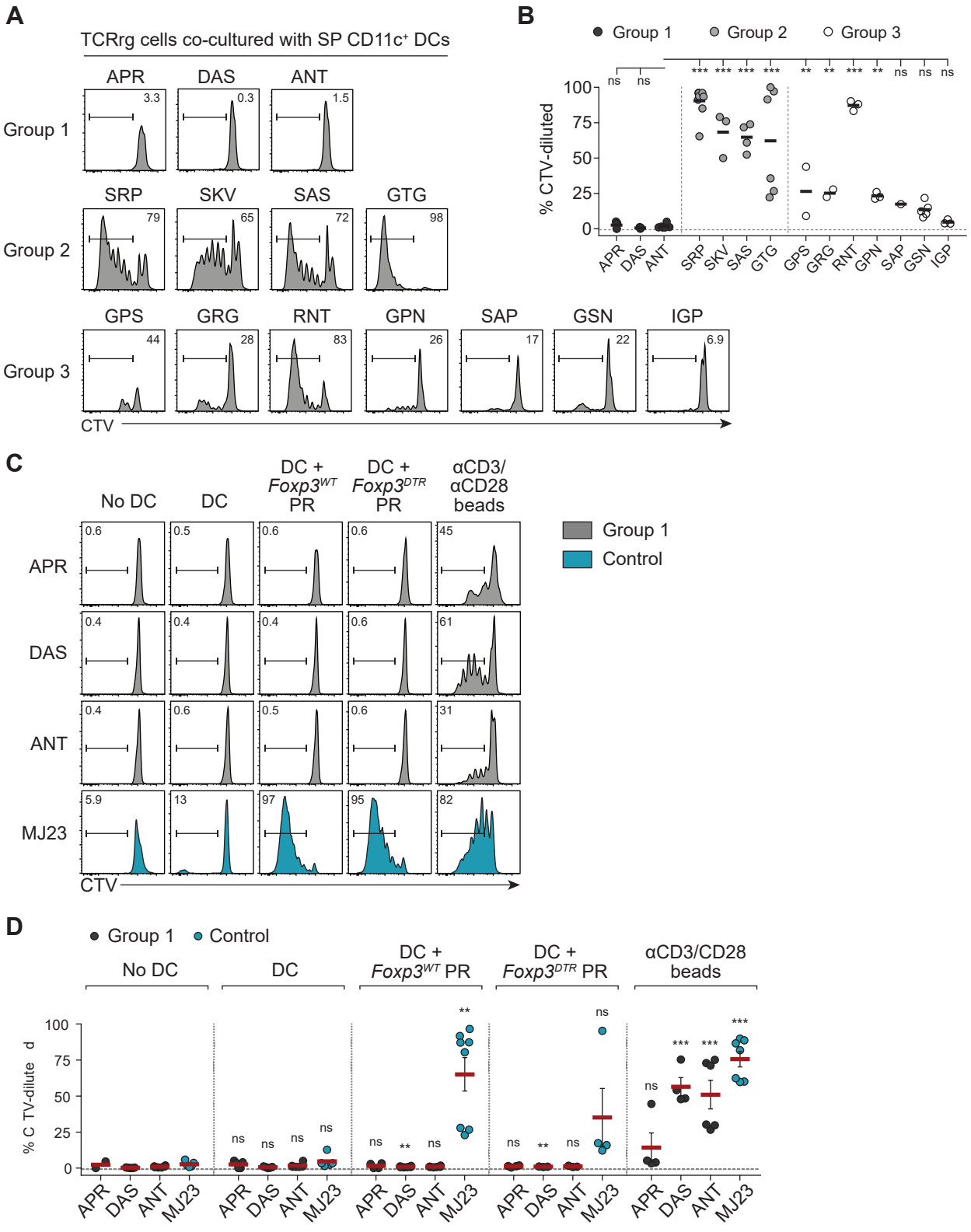

Figure 4. Prostatic Tconv cell clonotypes can be categorized based on reactivity to splenic dendritic cells and CD69 expression pattern.

Figure 4, continued.
From the prostatic TCRα sequences in Figure 1B, 14 TCRs were selected and retrovirally transduced into donor bone-marrow to generate TCR retrogenic (TCRrg) mice as described in Methods.
(A) 5-7 weeks after bone-marrow engraftment, secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^+$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. TCRrg cells were labeled with CellTrace Violet (CTV), then 1-2.5 x 10^4 CTV-labeled TCRrg cells were co-cultured with 5 x 10^4 splenic dendritic cells (DC) isolated from B6.SJL female mice via CD11c MACS kit, along with recombinant mouse IL-2. On day 5, CTV-dilution was assessed via flow cytometry. TCRs were grouped based on proliferation in response to co-culture with splenic DCs as well as CD69 expression patterns across secondary lymph nodes (not shown): Group 1, no stimulation; Group 2, overt stimulation; Group 3, minor stimulation.
(B) Quantification of (A). TCRα clonotypes are denoted on the x-axis. The y-axis denotes the percentage of CTV-diluted TCRrg cells. Data are representative of six independent experiments.
As a positive control, 1-2.5 x 10^4 CTV-labeled TCRrg cells were co-cultured with anti-CD3/anti-CD28 microbeads at a 1:1 ratio. (D) Quantification of (C). Data are representative of three independent experiments. (E) TCRrg cells
(C) 5-7 weeks after bone-marrow engraftment, secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^+$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. TCRrg cells were labeled with CellTrace Violet (CTV), then 1-2.5 x 10^4 CTV-labeled TCRrg cells were co-cultured with 5 x 10^4 splenic dendritic cells (DC) isolated from B6.SJL female mice via CD11c MACS kit, along with recombinant mouse IL-2. Where indicated, protein extracts isolated from prostatic lysates of $Foxp3^{WT}$ or Treg-depleted $Foxp3^{DTR}$ males were also included in the culture. As a positive control, 1-2.5 x 10^4 CTV-labeled TCRrg cells were co-cultured with anti-CD3/anti-CD28 microbeads at a 1:1 ratio. On day 5, CTV-dilution was assessed via flow cytometry.
(D) Quantification of (C). TCRα clonotypes are denoted on the x-axis. The y-axis denotes the percentage of CTV-diluted TCRrg cells. Data are representative of three independent experiments. Black circles, Group 1 TCRrg cells; turquoise circles, positive control MJ23tg cells.

To determine whether Group 2 clones are cross-reactive to MHC class II molecules independent of the peptide antigen, Group 2 TCRrg cells were co-cultured with splenic DCs isolated from H2-DM-deficient mice, which predominantly present MHC class II molecules bound to the invariant chain CLIP peptide[194]. These DCs still present MHC class II molecules on the cell surface, but lack surface display of diverse endogenous self-peptides. Group 2 clones failed to

proliferate following co-culture with DCs from H2-DM-deficient mice (Figures 2E and 2F), thus demonstrating that these clonotypes are specific for self-peptide antigens presented on DCs in an MHCII-dependent manner. Taken together, our observations *in vivo* and *in vitro* indicate that Group 2 TCR clonotypes are self-reactive and recognize self-antigens presented widely throughout the body. We focus the remainder of our studies on Group 2 TCR clonotypes, using Group 1 clonotypes as negative control TCRs.

It is worth noting that of the 14 TCRs we screened, there exists a third category in which 7 clones (denoted as Group 3 clones) exhibited MHCII-dependent low-level stimulation when co-cultured with splenic DCs (Figures 4A and 4B). TCRrg mice expressing Group 3 TCRs exhibited some TCR signaling in various draining lymph nodes (data not shown), but their TCR reactivity did not appear as overt as those observed for Group 2 clones. While we presume Group 3 TCRs likely also represent self-reactive specificities, our current study is focused on understanding the biology of Group 2 clonotypes.

Group 2 TCR clonotypes escape clonal deletion in the thymus and exhibit negligible thymic Treg cell development

Common thought suggests that T cells reactive to ubiquitous antigens will be purged from the repertoire by clonal deletion[96,97]. We thus investigated to what extent Group 2 clones are subjected to negative selection during thymic development. All TCR retrogenic mice generated in this study utilize a conditional retroviral construct in which the TCRα chain is expressed under the control of a *Cd4*-driven Cre-lox system[89]. This conditional system avoids the premature expression of the TCRα chain and instead induces expression of the TCRα chain at the CD4 CD8 double-positive (DP) stage of thymic development, thereby recapitulating the

natural timing of TCR expression. We assessed TCRrg thymocytes at the double-negative (DN), DP, and CD4 single-positive (CD4SP) stages for assorted hallmarks of positive selection and clonal deletion in primary TCRrg hosts (Figure 5). Positively selected clones display a $CD5^{hi}CD69^{hi}$ phenotype, while cells that undergo negative selection often display a DP^{dull} $PD\text{-}1^{hi}$ and cleaved-$Caspase\text{-}3^{hi}$ phenotype[89]. Thymic analyses of primary TCRrg hosts reveal that both Group 1 and Group 2 TCRs display markers of positive selection ($CD5^{hi}CD69^{hi}$). Furthermore, both Group 1 and Group 2 TCRs appear to exhibit some features of DP^{dull} $PD\text{-}1^{hi}$ and cleaved-$Caspase\text{-}3^{hi}$ characteristics, although these levels vary from mouse to mouse. To determine whether our findings are an artifact of the retrogenic system, we generated TCRrg mice expressing a previously described Treg-derived TCR (MJ23)[68,86]. TCRrg thymocytes expressing the MJ23 TCR appear to undergo positive selection, but do not display DP^{dull} $PD\text{-}1^{hi}$ phenotypes. Thus, our data suggest that both Group 1 and Group 2 TCRs exhibit some hallmarks associated with clonal deletion, albeit to varying degrees.

Our findings above indicate that the self-reactive nature of the TCRs expressed by Group 1 and Group 2 clones can be detected during development in the thymus, yet both Group 1 and Group 2 clones are not efficiently culled by clonal deletion, as they are readily detectable in the periphery within secondary lymphoid organs (Figure 2; Figure 3). In light of this, we aimed to assess the thymic development of representative Group 2 clones at physiological clonal frequencies. Analysis of low-frequency bone marrow chimaeras of Group 2 TCRrg mice revealed that, even when generated at frequencies below 0.1% of polyclonal thymocytes, Group 2 TCRrg cells were still readily detected in the spleen (Figure 6). Thus, while Group 2 clones exhibit some hallmarks of self-reactivity and negative selection in the thymus, they routinely escape clonal deletion and enter the peripheral repertoire as mature $CD4^{+}$ T cells.

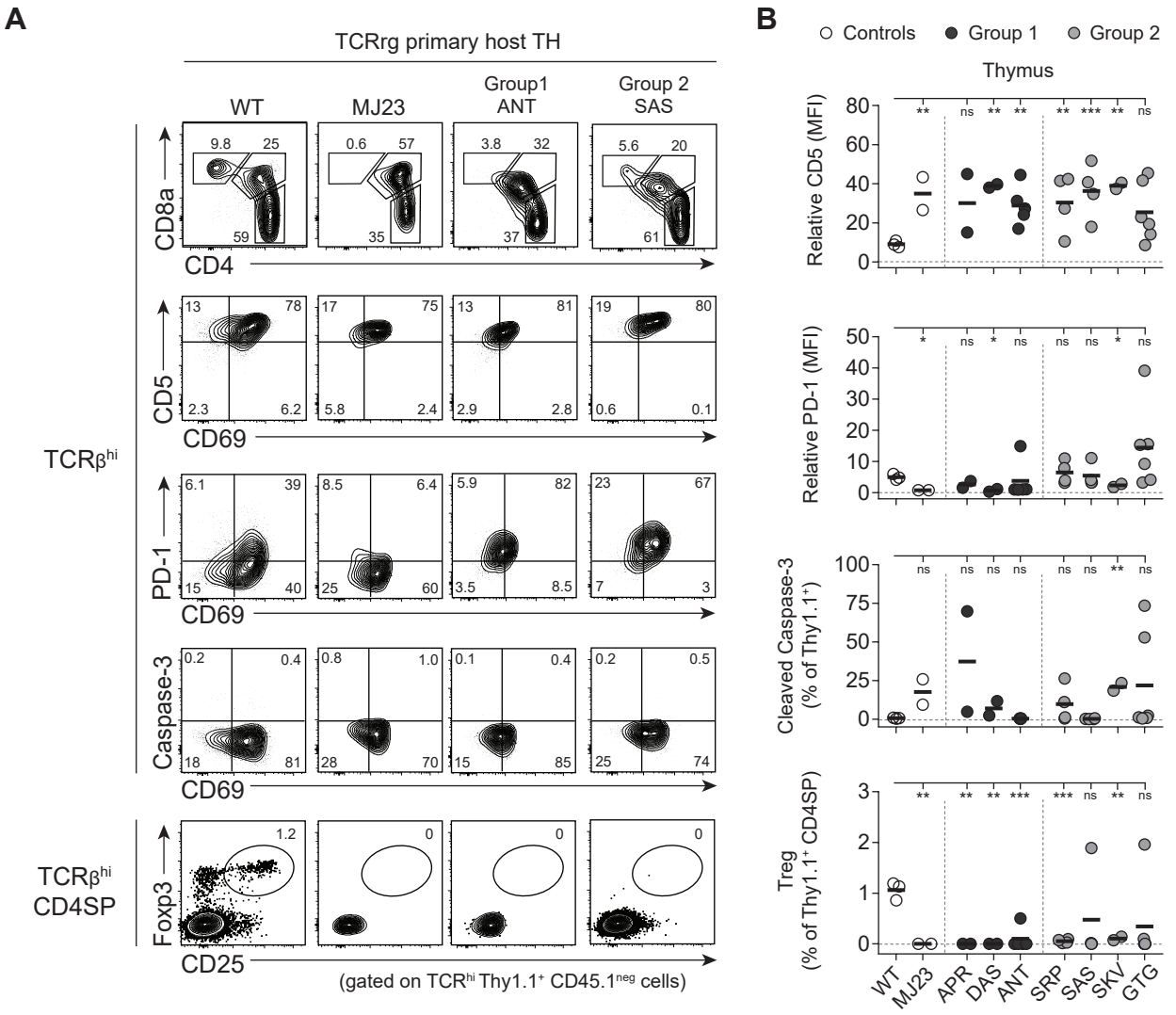

Figure 5. Group 2 TCR clonotypes escape clonal deletion in the thymus and exhibit negligible thymic Treg cell development.

(A) 5-7 weeks after primary TCRrg hosts were generated, thymi were isolated from primary TCRrg hosts, and CD73^{neg}TCRβ^{+} thymocytes were assessed for expression of CD4, CD8β, CD69, CD5, PD-1, cleaved caspase-3, CD25, and Foxp3, via flow cytometry. Numbers denote the percentage of cells falling within indicated gates or quadrants. WT, polyclonal B6.SJL cells.

(B) Quantification of (A). Data are representative of two independent experiments. CD5 and PD-1 expression are presented relative to control amounts in TCRβ^{neg}CD69^{neg} cells. MFI, mean fluorescence intensity. Open circle, WT B6.SJL host; closed dark gray circle, Group 1 TCRrg hosts; closed light gray circle, Group 2 TCRrg hosts.

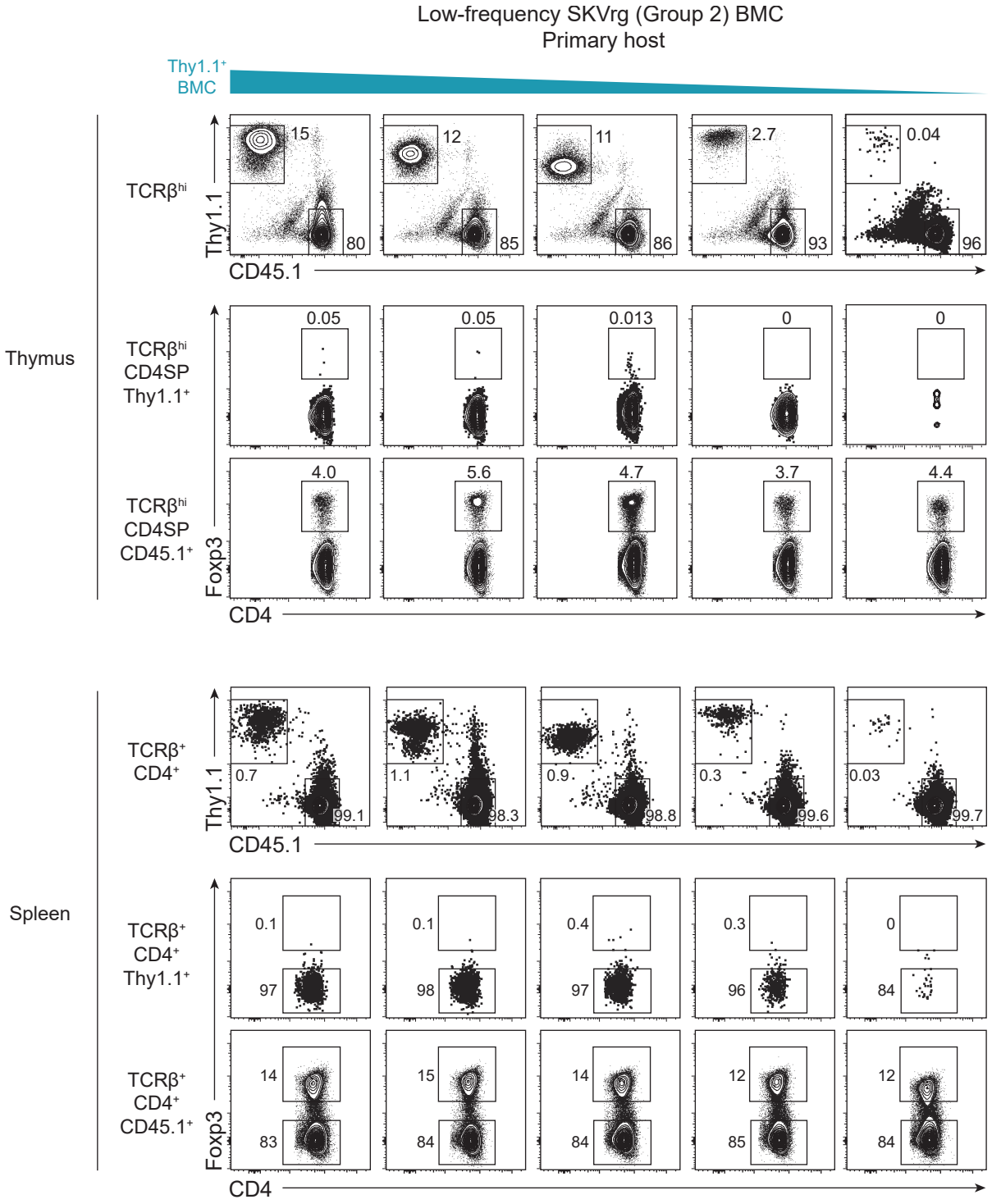

Figure 6. Group 2 TCR clonotypes at low precursor frequencies escape clonal deletion in the thymus and exhibit negligible thymic Treg cell development.

Figure 6, continued.
Low frequency bone-marrow chimaera mice were generated as described in Methods.
(Top) 5-7 weeks after primary TCRrg hosts were generated, thymi were isolated from primary TCRrg hosts, and $CD73^{neg}TCR\beta^{+}$ thymocytes were assessed for frequency of $Thy1.1^{+}$ cells as well as expression of Foxp3 within the $Thy1.1^{+}$ CD4SP TCRrg and the $CD45.1^{+}$ CD4SP polyclonal host population via flow cytometry. Numbers denote the percentage of cells falling within indicated gates or quadrants.
(Bottom) 5-7 weeks after primary TCRrg hosts were generated, the spleens were isolated from primary TCRrg hosts, and $TCR\beta^{+}CD4^{+}$ cells were assessed for frequency of $Thy1.1^{+}$ cells as well as expression of Foxp3 within the $Thy1.1^{+}$ TCRrg and the $CD45.1^{+}$ polyclonal host population via flow cytometry. Numbers denote the percentage of cells falling within indicated gates or quadrants.

In many cases, the recognition of agonist ligand in the thymus can facilitate thymic Treg cell differentiation[52,68]. The phenotype of Group 2 thymocytes suggests that these TCRs are indeed signaling with a sufficiently high TCR affinity to elicit hallmarks of negative selection in the thymus. However, when we evaluated the capacity of Group 2 TCRs to facilitate thymic Treg cell differentiation, we observed negligible tTreg cells, as measured by Foxp3 expression of Group 2 cells in the thymus (Figure 5). It is known that tTreg cell differentiation requires low precursor frequencies, since their precursors are limited by some developmental factor(s) that creates a limiting "niche"[71]. Generation of low-frequency bone-marrow chimeric Group 2 TCRrg mice did not facilitate meaningful thymic Treg cell development (Figure 6). Thus, even at low physiological precursor frequencies, they do not appear to facilitate efficient tTreg lineage diversion. It should be noted that $Foxp3^{+}$ cells have been observed on rare occasions in the periphery of Group 2 primary TCRrg hosts (Figure 2A) and in rare instances in the thymus (Figure 5B), indicating that Group 2 clones can differentiate into Treg cells in some settings. However, our available data suggest that this is likely negligible at steady state.

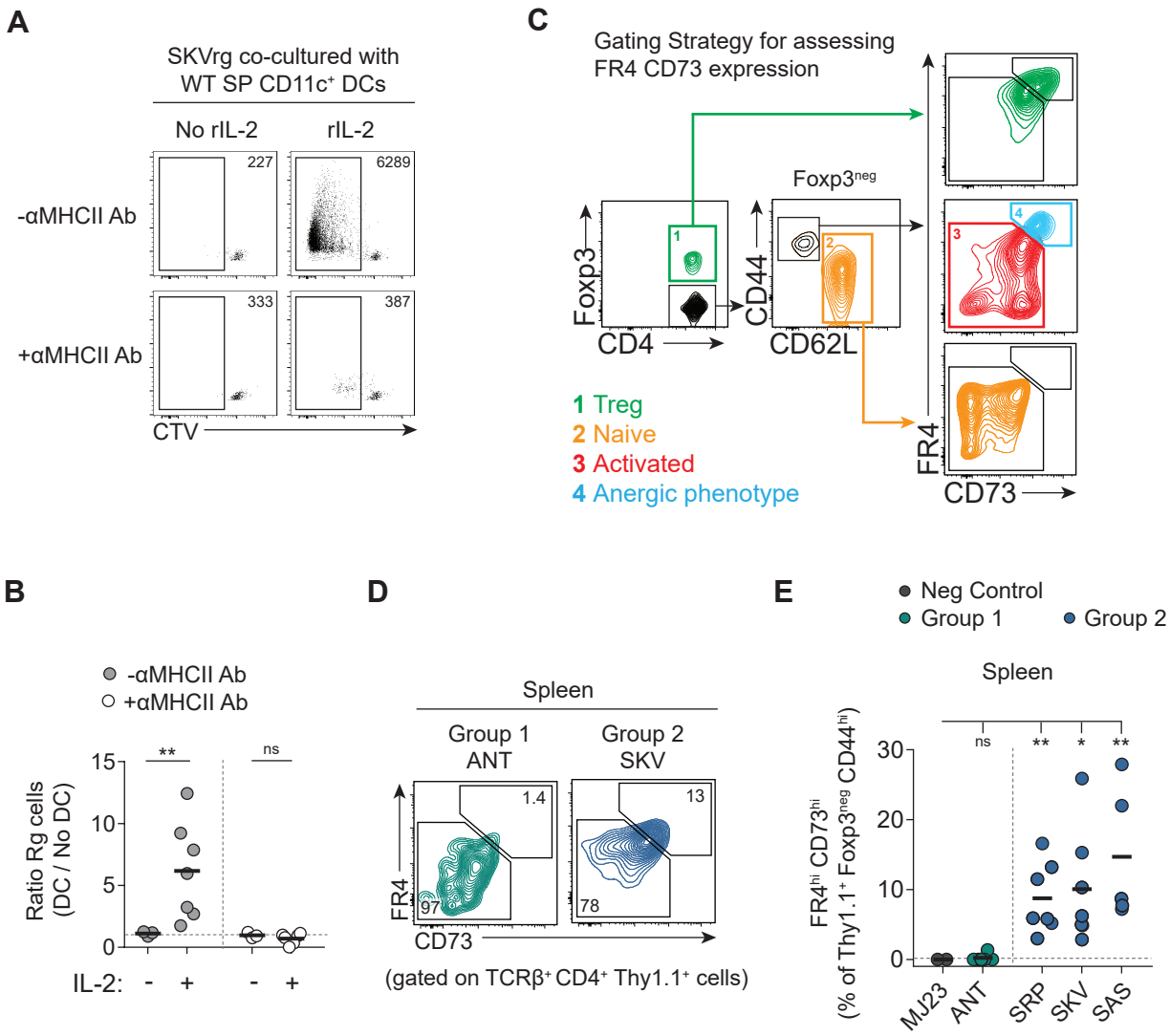

Figure 7. Group 2 clones require IL-2 to proliferate *in vitro* and adopt an FR4^{hi}CD73^{hi} phenotype in the periphery.

Figure 7, continued.
(A) 5-7 weeks after bone-marrow engraftment, secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^+$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. TCRrg cells were labeled with CellTrace Violet (CTV), then 1-2.5 x 10^4 CTV-labeled TCRrg cells were co-cultured with 5 x 10^4 splenic dendritic cells (DC) isolated from B6.SJL female mice via CD11c MACS kit, with or without recombinant mouse IL-2. Where indicated, anti-MHCII blocking antibody was included in the culture. On day 5, CTV-dilution was assessed via flow cytometry.
(B) Quantification of (A). Data are representative of two independent experiments.
(C) Gating strategy for anergic phenotype cells.
(D) Secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^+$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. ~1 x 10^5 sorted TCRrg cells were co-transferred along with 10×10^6 B6.SJL bulk pooled spleen and lymph node cells into $TCR\beta^{-/-}$ female mice, such that TCRrg cells constituted ~0.5-1% of the inoculum. 3 weeks post-transfer, secondary lymphoid organs were isolated from reconstituted $TCR\beta^{-/-}$ recipients, and TCRrg ($TCR\beta^+CD4^+Thy1.1^+$) or co-transferred polyclonal B6.SJL ($TCR\beta^+CD45.1^+Thy1.1^{neg}$) cells were assessed for expression of FR3, CD73, and Foxp3. Numbers denote the percentage of cells falling within each gate.
(E) Quantification of (D). Data are representative of four independent experiments. Green, Group 1 TCRrg cells; blue, Group 2 TCRrg cells; gray, negative control MJ23rg cells.

Group 2 clones adopt an $FR4^{hi}CD73^{hi}$ phenotype in the periphery and require IL-2 to proliferate *in vitro*

One mechanism of peripheral tolerance involves the induction of functional unresponsiveness (anergy) in self-reactive T cells that encounter their cognate antigen in the absence of co-stimulatory signals[109,113,115,116,118]. We thus evaluated whether Group 2 clones exist in an anergic state in the periphery. Several lines of evidence reveal conflicting answers to this question. First, Group 2 clones fail to proliferate *in vitro* when co-cultured with splenic DCs in the absence of recombinant IL-2 (Figures 7A and 7B), suggesting that Group 2 clones do not intrinsically produce IL-2 upon re-stimulation *ex vivo*. Addition of recombinant IL-2 was sufficient to rescue these cells and promote *in vitro* proliferation in an antigen-dependent manner

(Figures 7A and 7B; Figures 2E and 2F). Second, closer examination of Group 2 TCRrg T cells transferred into secondary hosts at low frequencies revealed that widespread T cell activation was associated with the emergence of cells characterized by an $FR4^{hi}CD73^{hi}$ phenotype (Figures 7C-E), which has previously been attributed to "anergic phenotype" cells exhibiting evidence of autoreactivity[195]. Intriguingly, surface expression of FR4 and CD73 in the periphery appears to be influenced by some limiting factor, as the emergence of the $FR4^{hi}CD73^{hi}$ phenotype seems inversely correlated with the frequency of Group 2 TCRrg cells that is present within the peripheral $CD4^+$ T cell compartment (Figure 8). The limiting factor is likely independent of the availability of cognate antigen interaction, because a large fraction of Group 2 cells (>60%) express the TCR signaling marker CD69 regardless of TCRrg cell frequency within the repertoire (Figure 8A). Thus, there likely exists at least one or more additional factor(s) that is required for the peripheral adoption of the $FR4^{hi}CD73^{hi}$ phenotype. Lastly, Group 2 clones isolated from primary TCRrg hosts did not produce the common effector cytokines IFNγ, IL-4, IL-17, IL-21, and IL-10 when re-stimulated with PMA and Ionomycin *ex vivo* (Figure 9). Together, these observations are consistent with classical descriptions of functionally unresponsive cells.

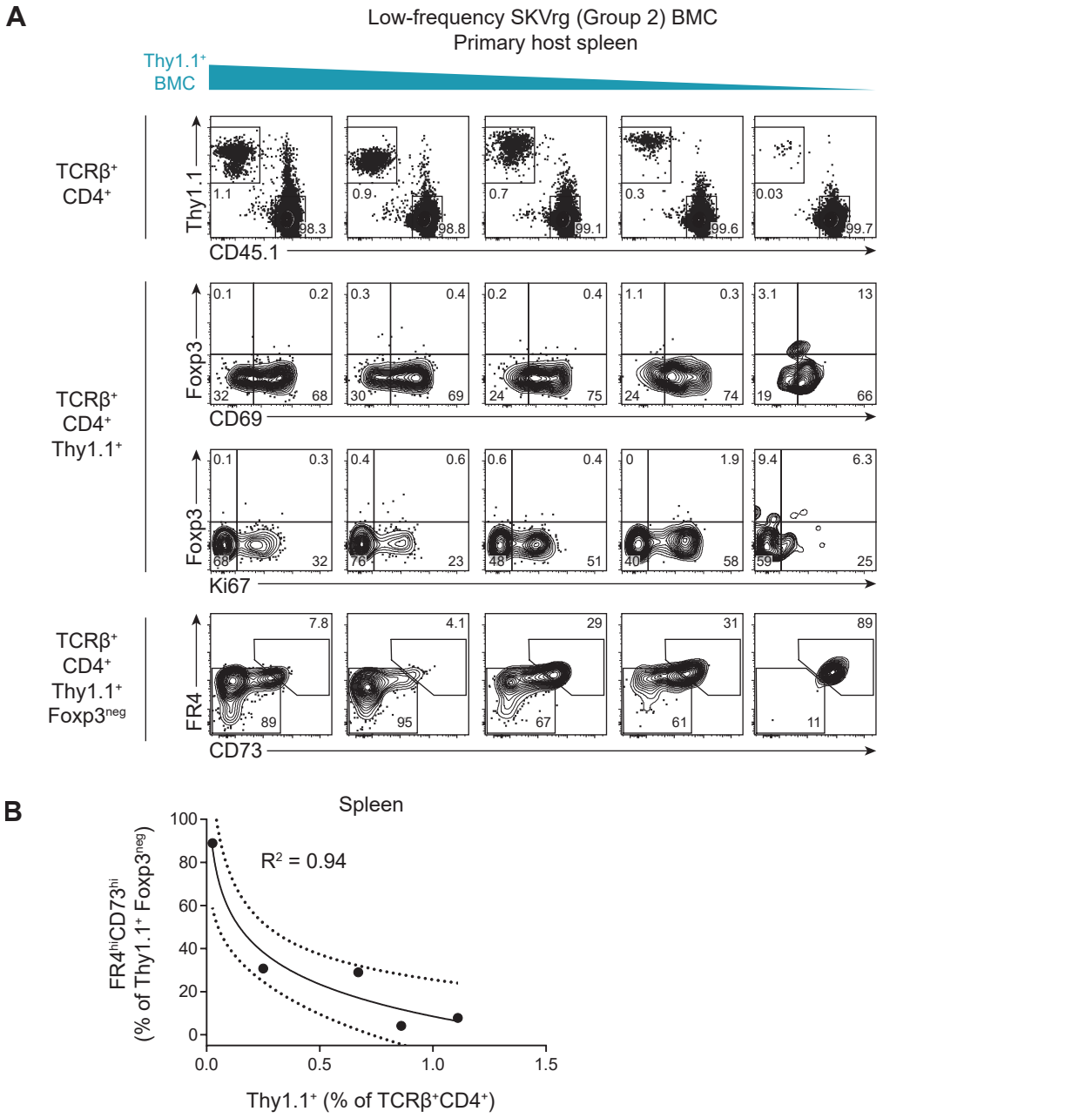

Figure 8. The emergence of the FR4^{hi}CD73^{hi} phenotype is influenced by the clonal frequency of Group 2 TCRrg cells within the peripheral CD4^{+} T cell compartment.

Low frequency bone-marrow chimaera mice were generated as described in Methods.

(A) 5-7 weeks after primary TCRrg hosts were generated, the spleens were isolated from primary TCRrg hosts, and TCRrg (TCRβ^{+}CD4^{+}Thy1.1^{+}) cells were assessed for expression of CD69, Ki67, FR4 and CD73 via flow cytometry. Numbers denote the percentage of cells falling within indicated gates or quadrants.

(B) Quantification of (A). A semilog line of best fit is drawn. Dotted lines represent 95% confidence intervals. $R^2 = 0.94$. Data are representative of one independent experiment.

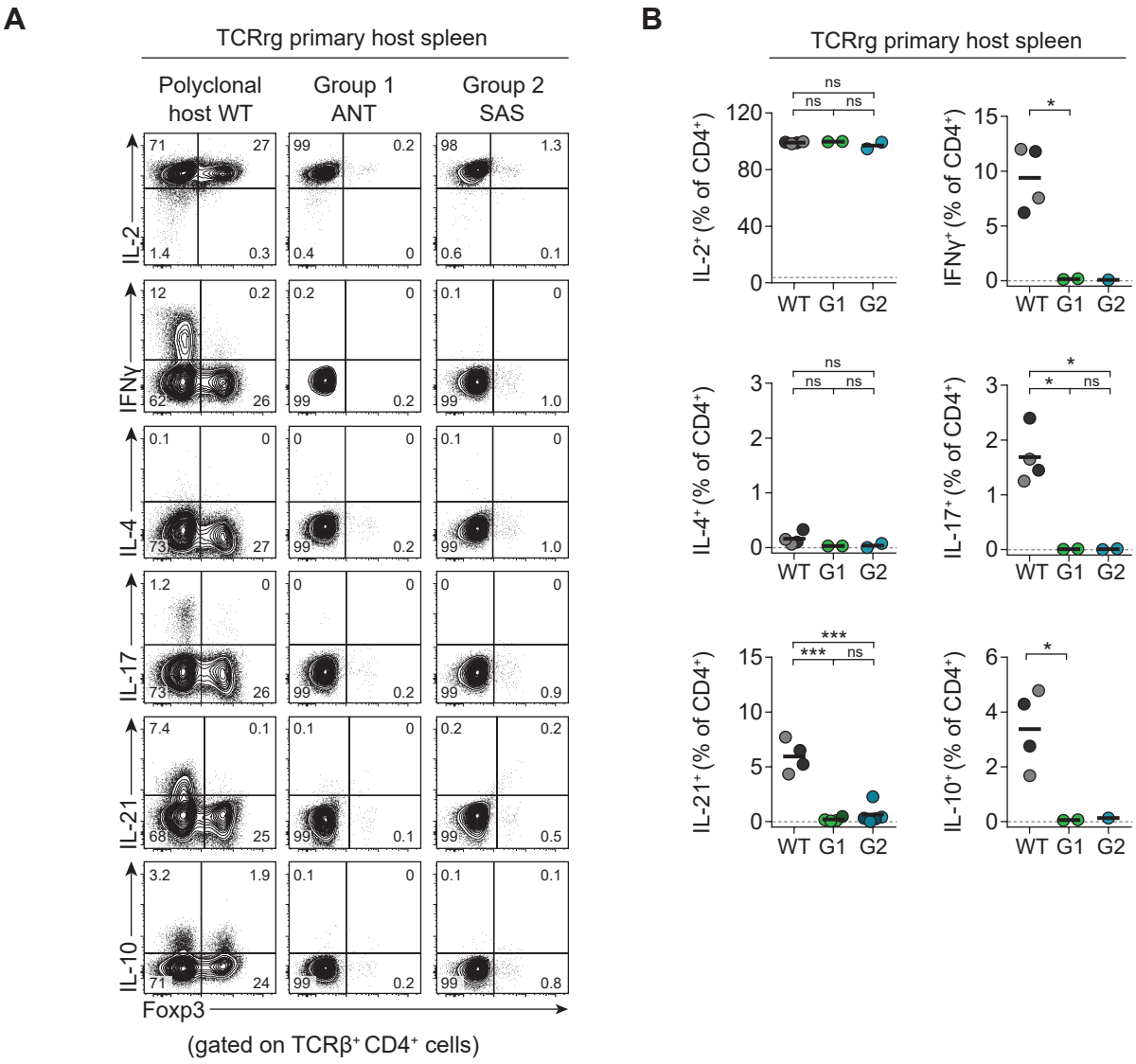

Figure 9. Group 2 clones in primary TCRrg hosts do not produce any common effector cytokines.

(A) 5-7 weeks after bone-marrow engraftment, spleens were isolated from primary TCRrg hosts, and TCRrg ($TCR\beta^+CD4^+Thy1.1^+$) cells or polyclonal wildtype cells ($TCR\beta^+CD4^+CD45.1^+$) were assessed for intracellular expression of IL-2, IFNγ, IL-4, IL-17, IL-21, IL-10 after a 5-hour stimulation with PMA/Ionomycin and monensin at 37°C via flow cytometry. Numbers denote the percentage of cells within each quadrant.

(B) Quantification of (A). Data are representative of two independent experiments. WT, polyclonal host wildtype $CD4^+TCR\beta^+$ cells; G1, Group 1 TCRrg cells; G2, Group 2 TCRrg cells.

Group 2 clonotypes adopt hallmarks of T follicular helper-like cells

However, one defining feature of Group 2 clones is that a substantial fraction of cells stain positive for the proliferation marker Ki67 (Figure 8A). This property of Group 2 clones is incongruous with the expected phenotype of *in vivo* induced anergic cells. Anergy is typically described as a nonproliferative state that results from cell cycle arrest at the G_1 phase due to lack of sufficient co-stimulatory signals to promote the G_1-to-S transition upon TCR signaling[114,116,117,196].

Given the fact that Group 2 clones are self-reactive and have the capacity to proliferate under proper signaling conditions, we next examined whether Group 2 clones adopt an effector phenotype at steady state. Group 2 clones isolated from primary TCRrg hosts at steady state did not appear to exhibit any expression of the lineage-specific transcription factors T-bet, GATA3, or RORγt that are deterministic for the differentiation of Th1, Th2, and Th17 cells, respectively (data not shown). Notably, high expression of FR4 and CD73 is a feature that has also been ascribed to T follicular helper (Tfh) cells, in addition to anergic phenotype cells[197]. In light of this, we assessed Group 2 TCRrg cells for hallmarks of Tfh cells, which include expression of Bcl6, CXCR5, ICOS, and PD-1. Strikingly, we were surprised to find that all four Group 2 TCR clonotypes express Bcl6 and PD-1 (Figures 10A-D). Expression of CXCR5 and ICOS by Group 2 TCRrg cells appears more variable, which could be a reflection of cells at varying states of activation. In addition, Group 2 TCRrg cells transferred at low frequencies into secondary hosts produce IL-21 upon re-stimulation *in vitro* (Figures 17A and 17B), demonstrating that these cells have the capacity to generate a Tfh effector function. Interestingly, the ability of Group 2 clones to produce IL-21 seems to be associated with the emergence of the $FR4^{hi}CD73^{hi}$ phenotype in these cells. Group 2 clones at high frequencies in primary TCRrg hosts are $FR4^{lo}CD73^{lo}$ and do

not produce IL-21 (Figure 8; Figure 9), while those adoptively transferred at low frequencies into secondary hosts are FR4^{hi}CD73^{hi} and readily produce IL-21 (Figure 8; Figures 17A and 17B). These findings suggest that in this context, the expression of FR4 and CD73 may be a feature more accurately associated with Tfh-like effector function than with phenotypically anergic cells. Together, our data suggest that Group 2 TCRrg cells adopt hallmarks of Tfh-like cells, but do not appear to produce IL-21 at steady state in primary TCRrg hosts.

We were curious to know at what stage Bcl6 expression occurs in the life cycle of Group 2 clones. Do Group 2 TCRrg thymocytes express Bcl6 early during thymic development, or is expression induced in the periphery? It is known that Bcl6 expression is high in all thymocytes during the pre-TCR stage of development, which then turns off at the DN3 through DP transition[198]. Consistent with this, we observed high Bcl6 expression in both polyclonal host cells and TCRrg cells at the pre-TCR stage, which was then downregulated at the TCRβ^{hi} stage of development (Figure 10F). However, Bcl6 expression was not maintained in mature Group 2 TCRrg thymocytes (Figure 10E), suggesting that Bcl6 expression is upregulated in the periphery, likely upon peripheral TCR encounter with self-antigens.

Group 2 primary TCRrg hosts are not associated with spontaneous germinal center formation

A seminal role of T follicular helper cells involves the provision of help to B cells[144,146,154,199]. Without it, activated B cells do not survive differentiation into germinal center (GC) B cells, and thereby cannot produce high-affinity antibodies. Tfh cells provide B cell help primarily through the CD40/CD40L signaling axis[200]. In return, cognate B cells provide CD80/CD86 signaling to CD28 on Tfh cells to promote Tfh cell survival[148,151,152].

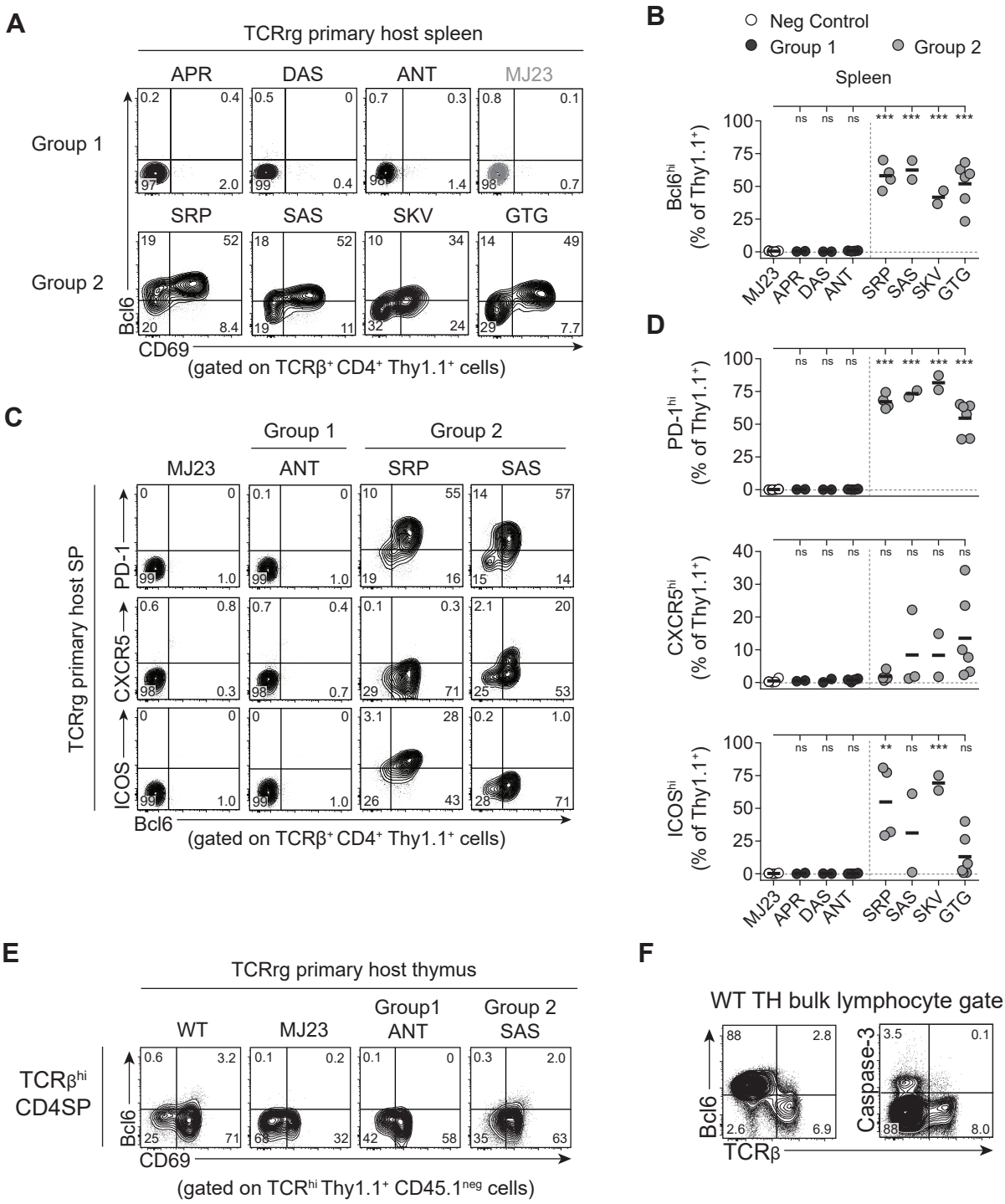

Figure 10. TCRs expressed by self-reactive Tconv cell clones display hallmarks of T follicular helper cells.

Figure 10, continued.
(A-D) 5-7 weeks after primary TCRrg hosts were generated, secondary lymphoid organs were isolated from primary TCRrg hosts, and TCRrg ($TCR\beta^+CD4^+Thy1.1^+$) cells were assessed for expression of Bcl6, PD-1, CXCR5, and ICOS via flow cytometry. Numbers denote the percentage of cells in each quadrant. SP, spleen. Data are representative of three independent experiments.
(E) 5-7 weeks after primary TCRrg hosts were generated, thymi were isolated from primary TCRrg hosts, and $CD73^{neg}TCR\beta^+$ thymocytes were assessed for expression of Bcl6 and CD69 via flow cytometry. Numbers denote the percentage of cells falling within indicated gates or quadrants. WT, polyclonal B6.SJL cells.
(F) Bulk thymocytes gated on lymphocyte gate served as positive control signals for expression of cleaved caspase-3 and Bcl6, which occur predominantly at the pre-$TCR\beta^+$ stage.

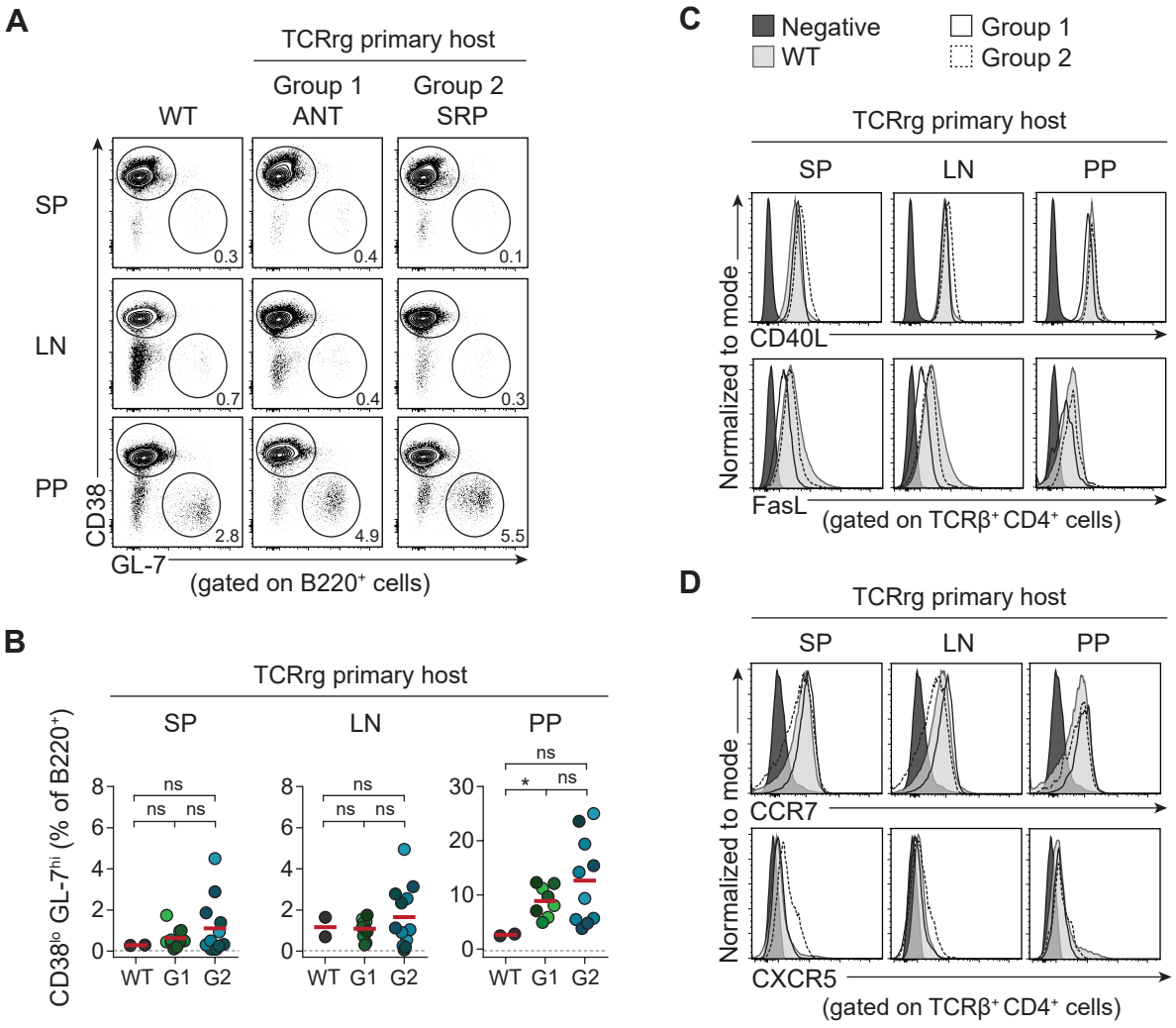

Figure 11. Group 2 TCRrg mice exhibit no spontaneous GC formation at steady state.
(A) 5-7 weeks after primary TCRrg hosts were generated, secondary lymphoid organs and Peyer's patches were isolated from primary TCRrg hosts, and B220^{+} B cells were assessed for CD38 and GL-7 expression via flow cytometry. Numbers denote the percentage of CD38^{lo}GL-7^{hi} cells. WT, polyclonal B6.SJL cells; G1, Group 1 TCRrg cells (light green = ANT, dark green = DAS, medium green = APR); G2, Group 2 TCRrg cells (light blue = SAS, dark blue = GTG). SP, spleen; LN, pooled lymph nodes (inguinal, axillary, brachial, cervical, periaortic); PP, Peyer's patches.
(B) Quantification of (A). Data are representative of three independent experiments.
(C-D) Secondary lymphoid organs and Peyer's patches were isolated from primary TCRrg hosts, and TCRrg (TCRβ^{+}CD4^{+}Thy1.1^{+}) cells were assessed for intracellular expression of CD40L and FasL (C) or surface expression of CCR7 and CXCR5 (D) via flow cytometry. Data depicted as histograms. Dark gray, negative control; light gray, polyclonal B6.SJL cells; solid line, Group 1 TCRrg cells; dotted line, Group 2 TCRrg cells. In (C), surface staining of CD40L and FasL with WT B6.SJL spleen served as negative controls. In (D), TCRβ^{neg}B220^{neg} cells served as negative controls.

Thus, stable T:B interactions ensures mutual differentiation and survival of Tfh cells and GC B cells. Based on our findings thus far, we interrogated whether Tfh-like Group 2 clones initiate spontaneous GC formation at steady state. Analysis of wildtype, Group 1, and Group 2 primary TCRrg mice revealed no differences in the prevalence of GCs (Figures 11A and 11B). Further, Group 2 TCRrg cells did not appear to express higher or lower levels of CD40L when compared to WT polyclonal or Group 1 TCRrg cells (Figure 11C).

$CD4^+Bcl6^+$ cells cluster with primary B cell follicles in the spleen of Group 2 TCRrg hosts

In the spleen and lymph nodes, T cells expressing elevated levels of the chemokine receptor CCR7 migrate deep into the T cell zone in response to a chemokine gradient rich in CCL19 and CCL21[201,202]. In contrast, T cells expressing elevated levels of the chemokine receptor CXCR5 migrate toward B cell follicles in response to its ligand CXCL13, and engage with B cells at the T cell-B cell border [148,159,203]. A fraction of Group 2 TCRrg cells exhibited downregulation of CCR7 expression and upregulation of CXCR5 expression (Figure 11D), suggesting that they may be positioned at the T cell-B cell border.

To assess positioning of Group 2 TCRrg cells, we performed immunofluorescence staining of spleens isolated from primary TCRrg hosts (Figure 12). Two observations stood out to us. First, while gross examination of the spleens isolated from Group 1 and Group 2 primary TCRrg hosts did not reveal any apparent difference in size or shape of the spleens between the two groups, immunofluorescence analysis revealed that the spleens isolated from Group 1 TCRrg mice were completely lacking in normal lymphoid morphology. These spleens lacked clear segregation of red pulp and white pulp. Although some B cells appeared to cluster together, T cells were dispersed across the spleen, with no clear formation of T cell zones.

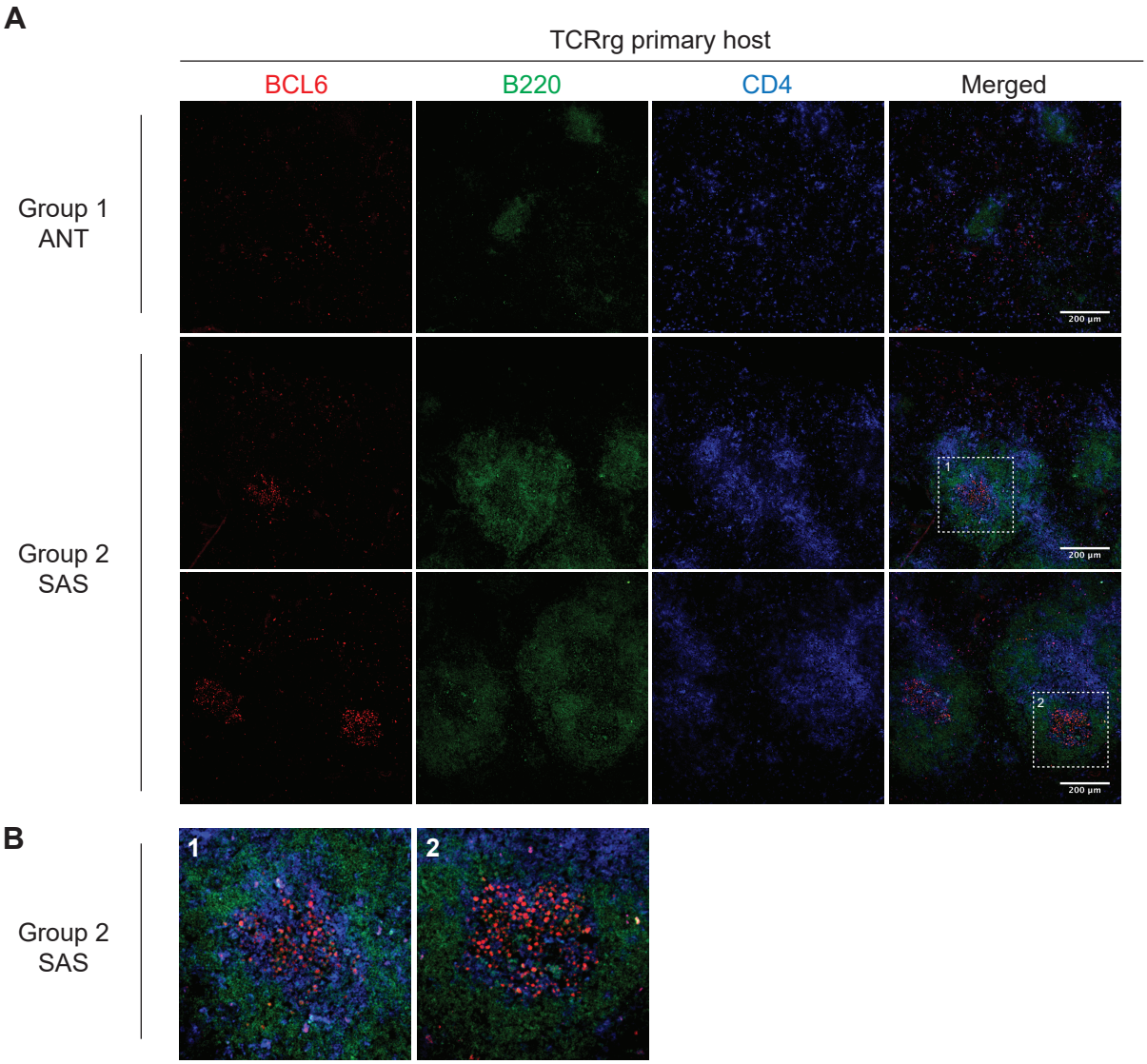

Figure 12. CD4^{+}Bcl6^{+} cells associate with primary B cell follicles in spleens of Group 2 TCRrg mice.

(A) Spleen and lymph nodes isolated from Group 1 or Group 2 primary TCRrg hosts were frozen in OCT for immunofluorescence staining as described in Methods. Tissue sections were stained for the following markers: B220, CD4, Thy1.1, Bcl6, GL-7 (no apparent signals were detected for Thy1.1 and GL-7; not shown). Localization of TCRrg cells within T cell zones, B cell zones, or at the T cell-B cell border was assessed via confocal microscopy. Representative images are shown. Red, Bcl6; green, B220; blue, CD4.

(B) Magnified view of areas denoted in (A).

It is known that total body irradiation (performed here to generate the primary TCRrg mice, see Methods) induces destruction of normal lymphatic structures[204]. Yet, spleens isolated from Group 2 TCRrg hosts (which also underwent total body irradiation) displayed the expected red pulp and white pulp, containing proper T cell zones and B cell follicles. Although these results are preliminary, this finding suggests that Group 2 clones may potentially play a central role in facilitating the proper formation of white pulps—perhaps by interacting with local dendritic cells and B cells to generate the appropriate chemokine gradients. Alternatively, it is possible that Group 1 clones somehow fail to respond appropriately to CCL19 and CCL21 chemokine gradients, and thus fail to form proper T cell zones upon lymphoid reconstitution in primary TCRrg hosts.

Secondly, our analysis also revealed that $CD4^+$ $Bcl6^{hi}$ T cells in the spleen of Group 2 primary TCRrg hosts appear to cluster densely around a single focal point within each white pulp. This space seems closely associated with primary B cell follicles. In fact, in many cases the $CD4^+$ $Bcl6^{hi}$ T cell cluster appears to be situated completely inside the B cell follicle, with the B cells forming a ring around the $CD4^+$ $Bcl6^{hi}$ T cell cluster (Figure 12B). These B cell follicles lacked expression of the germinal center marker GL-7 (data not shown); therefore, they are likely primary follicles and not germinal centers. Interestingly, each white pulp structure contains only a single cluster of $CD4^+$ $Bcl6^{hi}$ T cells within an individual B cell follicle. The $CD4^+$ $Bcl6^{hi}$ T cells do not seem to seed all B cell follicles. It should be noted that, because the Thy1.1 marker was ineffective in staining the tissue samples, we were not able to verify that the $CD4^+$ $Bcl6^{hi}$ T cell clusters in these sections corresponded directly to $Thy1.1^+$ cells. However, primary TCRrg hosts harbor a large frequency of $Thy1.1^+$ cells (frequencies can typically range anywhere from ~10% to ~60% of all $CD4^+$ T cells), and Group 2 TCRrg cells are largely $Bcl6^{hi}$ (~50-60%).

Meanwhile, the prevalence of $Bcl6^{hi}$ cells in the endogenous polyclonal wildtype compartment is much lower (only ~3% of polyclonal $CD4^+$ T cells). Thus, there is a high likelihood that the $CD4^+$ $Bcl6^{hi}$ T cell clusters seen in these sections are reflective of the localization of Group 2 TCRrg cells. In conclusion, our data suggests that Group 2 TCRrg clones are likely positioned in close proximity to B cells and may engage in continuous B cell interactions at steady state. They may also play an essential role in promoting the proper formation of lymphoid follicles within the spleen, though this remains to be confirmed.

Impact of Group 2 clones on polyclonal B cells

Our results thus far demonstrate that $CD4^+$ T cells clones that exhibit overt reactivity to widespread self-ligands are maintained in the endogenous repertoire at steady state. What might be the purpose of maintaining self-reactive Tfh-like cells in the endogenous repertoire? Aside from the functions proposed above, we envision at least two additional possibilities. First, Group 2 clones could be poised to promote the deletion of autoreactive B cells reactive to a linked self-antigen. Specifically, Group 2 clones might be poised to eliminate newly arising B cell specificities that bear self-reactive B cell receptors (BCRs) generated from somatic hypermutations (SHM) during germinal center (GC) reactions[26,166,205,206]. A second possibility is that Group 2 clones are adequately controlled by Treg cells and thus do not pose a threat to the host. This second possibility would suggest that stringent tolerance mechanisms must be in place to ensure that Group 2 clones never become inadvertently activated. This is particularly challenging in the context of an immune response to infection or vaccination strategies, in which activated Tfh cells play a vital role in promoting B cell activation and antibody-production. Unregulated self-reactive Tfh cells in this context may have the capacity to provide B cell help

and promote autoantibody production. Consequently, this possibility underscores the need to further clarify the signals and factors that are required to activate Group 2 clones *in vivo.*

Because provision of B cell help is a common function of Tfh-like cells, we first assessed the capacity of Group 2 TCRrg cells to provide B cell help when co-cultured *in vitro*[207] (Figure 13). B cells stimulated by addition of anti-IgM antibody upregulate MHC class II molecules on the cell surface. When stimulated B cells are co-cultured with polyclonal T cells or Group 1 TCRrg cells (stimulated by addition of anti-CD3ε antibody), B cells upregulate expression of the B cell activation and GC marker GL-7. However, B cells co-cultured with stimulated Group 2 TCRrg cells did not upregulate expression of GL-7 as much as in the other two groups. No significant levels of antibody isotype class-switching were observed in any of the groups studied (Figure 13B).

When B cells were assessed for viability after 7 days in culture, most B cells did not survive. However, when B cells were co-cultured with polyclonal T cells or Group 1 TCRrg cells, a small fraction (~1%) of B cells likely received help from their T cell counterpart, since they remained viable after 7 days in culture (Figure 13C). Strikingly, this was not observed when B cells were co-cultured with Group 2 TCRrg cells (Figure 13C). These findings suggest two potential explanations: 1) Group 2 TCRrg cells are incapable of providing B cell help, or 2) Group 2 TCRrg cells are actively deleting B cells that survive in culture. Previous studies by other groups have proposed that self-reactive B cells are predominantly deleted *in vivo* via Fas-mediated apoptosis upon engagement with Fas ligand (FasL)[26,166,205,206]. The FasL is presumed to be provided by T helper cells that engage with B cells upon TCR interaction with peptide-MHC presented on the B cell[26,166]. Group 2 clones did not appear to express higher or lower levels of FasL when compared to WT polyclonal or Group 1 TCRrg cells (Figure 11C).

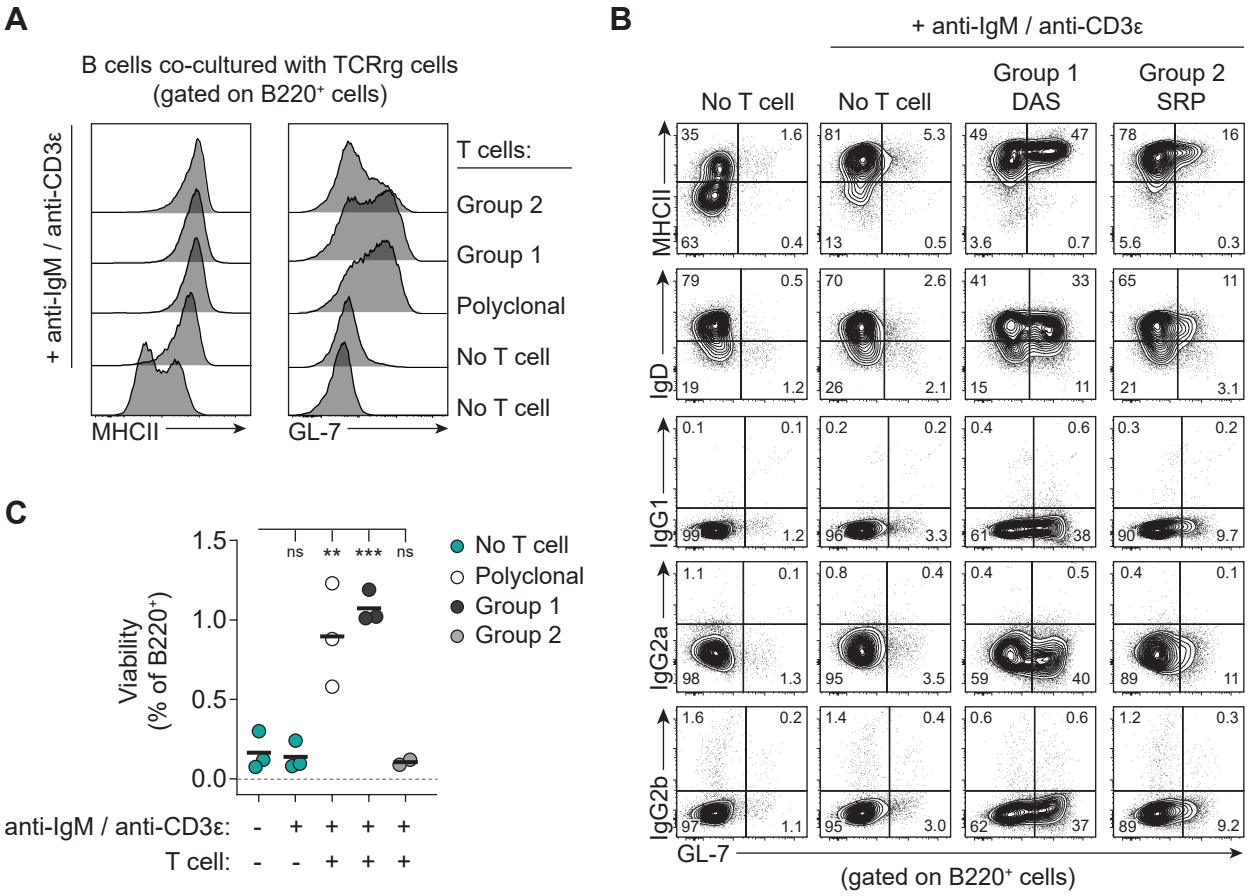

Figure 13. Group 2 clones do not readily promote B cell activation.
5-7 weeks after primary TCRrg hosts were generated, secondary lymphoid organs were isolated from primary TCRrg hosts and sorted for TCRrg cells (CD8β^{neg}CD45.1^{neg}Thy1.1^{+}) via FACS. Sorted TCRrg cells or control polyclonal wildtype CD4^{+} T cells were co-cultured with B cells isolated from B6.SJL mice, along with anti-CD3 and anti-IgM antibodies as described in Methods. 7 days later, B cells were assessed for class-switched antibody isotypes (A-B), and viability using the Live/Dead kit (C) via flow cytometry (see Methods). Numbers denote the percentage of cells falling within each quadrant.

To determine whether Fas signaling is responsible for the failure of B cells to survive when co-cultured with Group 2 TCRrg cells *in vitro*, we would predict that addition of anti-Fas or anti-FasL blocking antibody in culture should be sufficient to maintain B cell viability in this group. Furthermore, if Group 2 clones are indeed deleting B cells in culture, we would predict that Group 2 clones would exhibit a dominant effect. For example, for the ~1% of B cells that survived in culture as a result of help from co-cultured polyclonal T cells (or Group 1 TCRrg cells), addition of Group 2 TCRrg cells would induce the death of this surviving B cell fraction—or at least a reduction in the fraction of B cells that survive in culture. These results remain to be determined.

To further investigate whether activated Group 2 TCRrg cells can adopt the ability to provide B cell help, we immunized Group 2 TCRrg mice with complete Freund's adjuvant (CFA), and assessed their capacity to produce effector cytokines and promote B cell survival *in vitro*. Because the self-antigens recognized by Group 2 TCRrg cells are likely ubiquitously expressed, we reasoned that subcutaneous injection of CFA in these hosts should be sufficient to promote activation of Group 2 clones. CFA immunization indeed induced enlargement of inguinal lymph nodes draining the injection site. Strangely, Group 2 TCRrg cells failed to produce any effector cytokines upon CFA immunization (Figures 14A and 14B). In addition, Group 2 TCRrg cells did not facilitate B cell survival when co-cultured *in vitro* (Figures 14C and 14D), much like their unimmunized counterpart. Lastly, Group 2 primary TCRrg mice immunized with CFA did not exhibit elevated GC formation (data not shown).

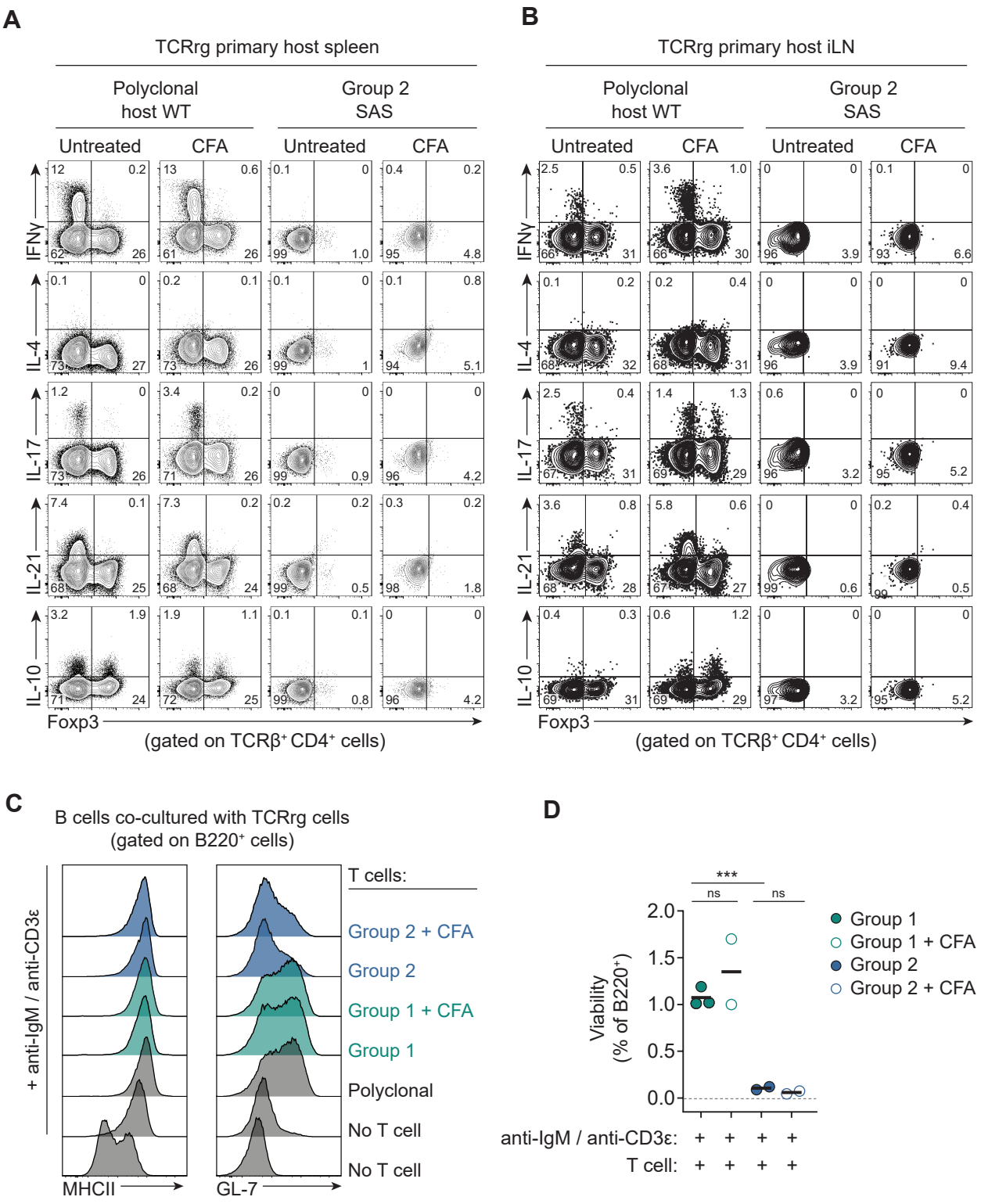

Figure 14. Group 2 clones in primary TCRrg hosts do not exhibit effector functions upon immunization with CFA.

Figure 14, continued.
5-7 weeks after primary TCRrg hosts were generated, 100μL of complete Freund's adjuvant (CFA) were injected subcutaneously into each flank of primary TCRrg hosts.
(A-B) 13 days later, the spleen (A) and draining lymph nodes (inguinal LNs) (B) were isolated from primary TCRrg hosts, and TCRrg ($TCR\beta^+CD4^+Thy1.1^+$) cells or polyclonal wildtype cells ($TCR\beta^+CD4^+CD45.1^+$) were assessed for intracellular expression of IL-2, IFNγ, IL-4, IL-17, IL-21, IL-10 after a 5-hour stimulation with PMA/Ionomycin and monensin at 37°C via flow cytometry. Numbers denote the percentage of cells within each quadrant.
(C) Secondary lymphoid organs were isolated from primary TCRrg hosts 13 days after immunization with CFA, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. Sorted TCRrg cells or control polyclonal wildtype $CD4^+$ T cells were co-cultured with B cells isolated from B6.SJL mice, along with anti-CD3 and anti-IgM antibodies as described in Methods. 7 days later, B cells were assessed for viability using the Live/Dead kit via flow cytometry (see Methods).
(D) Quantification of (C). Data are representative of one independent experiment.

Taken together, our data suggest that Group 2 clones did not readily promote B cell activation, even in the context of immunization with a strong adjuvant. Our preliminary data are consistent with the notion that Group 2 clones may not possess the capacity to promote B cell help. In addition to the functions proposed above, the following alternative possibilities may also explain why self-reactive Tfh-like cells are maintained in the endogenous repertoire: 1) Group 2 clones exist to provide an endogenous source of IL-2 (to support Treg cell homeostasis), or 2) Group 2 clones represent a population of suppressor cells (or a source of T follicular regulatory cell precursors).

The endogenous polyclonal $CD4^+$ T cell repertoire harbors $Bcl6^{hi}CD69^{hi}PD\text{-}1^{hi}$ cells

In our study, we set out to determine the frequency of self-reactive specificities that exist in the endogenous repertoire. Based on our findings that self-reactive Group 2 clones exhibit

Tfh-like characteristics (with expression of Bcl6 and PD-1 being the most pronounced in the spleen), it was of interest to us to determine the frequency, phenotype, specificity, function, and regulation of the $Bcl6^{hi}CD69^{hi}PD\text{-}1^{hi}$ polyclonal population that exists in wildtype mice at steady state.

Preliminary assessment of the $CD4^{+}$ $Foxp3^{neg}$ Tconv cell compartment in unmanipulated wildtype mice revealed that ~3% of the spleen harbored $Bcl6^{+}$ cells within the endogenous repertoire (Figure 15A). These cells displayed some characteristics of Tfh cells (such as some expression of PD-1 and CXCR5), but were largely $ICOS^{neg}$, consistent with our observations in Group 2 clones. As a comparison, the presence of canonical Tfh cells reactive to microbial antigens has been well described in the Peyer's patches at steady state[208]. These sites harbored ~23% of $Bcl6^{+}$ cells within the $CD4^{+}$ $Foxp3^{neg}$ Tconv cell compartment, and the $Bcl6^{+}$ cells here exhibited a higher resemblance to canonical Tfh cells in regards to robust expression of PD-1, CXCR5, and ICOS (Figures 15B and 15C).

Notably, of the $Bcl6^{+}$ cells in the $CD4^{+}$ Tconv cell compartment of the spleen, a majority of these cells (~65%) are $CD69^{+}$, which suggests that they are actively experiencing TCR signaling (Figure 15C, right). Therefore, Bcl6 could be a useful marker to identify self-reactive T cells in the endogenous repertoire. To confirm this, *in vitro* stimulation assay of Bcl6-expressing cells co-cultured with splenic DCs could reveal whether this population also harbors self-reactive specificities that can recognize ubiquitously expressed self-antigens in an MHC class II-restricted manner.

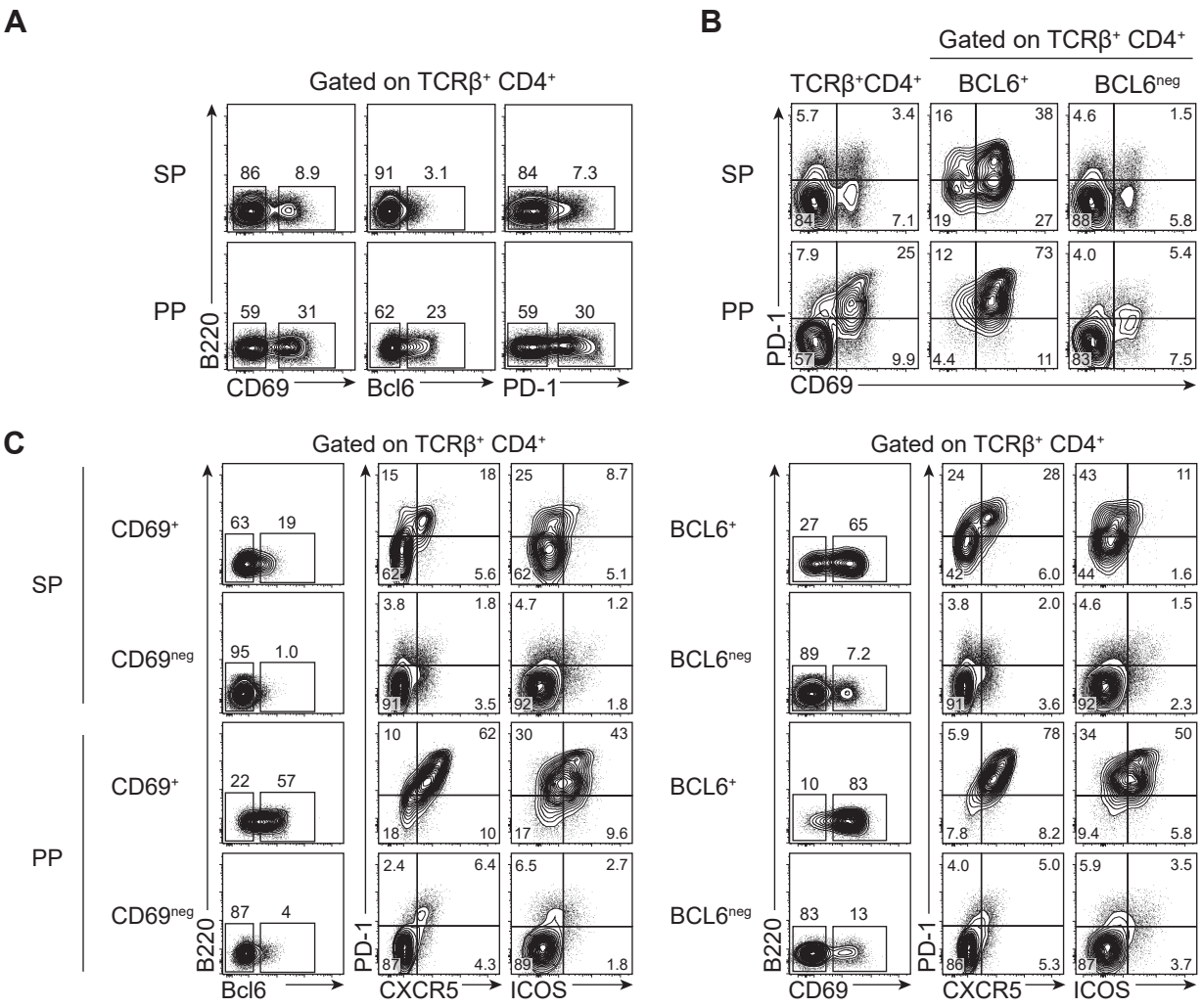

Figure 15. Endogenous polyclonal CD4$^+$Bcl6$^+$ cells in wildtype mice recapitulate certain phenotypes observed with Group 2 clones.

(A-C) Spleens and Peyer's patches were isolated from 6-8 week male C57BL/6 mice, and TCRβ $^+$CD4$^+$ T cells were assessed for expression of Bcl6, CD69, PD-1, CXCR5, and ICOS via flow cytometry. Numbers denote the percentage of cells falling within each quadrant or gate. SP, spleen; PP, Peyer's patches. Data are representative of one experiment.

In contrast, of the $CD69^+$ cells in the $CD4^+$ Tconv cell compartment of the spleen, only ~19% of these cells expressed Bcl6 (Figure 15C, left). This suggests that Bcl6 may not represent all self-reactive T cells that exhibit TCR signaling. Therefore, the endogenous repertoire likely harbors self-reactive specificities that display alternative phenotypes that have yet to be defined.

Conclusions

In summary, our study reveals a number of novel findings regarding the nature of a subset of self-reactive Tconv cell clonotypes in the endogenous repertoire. By employing deep-TCR sequencing approaches, we identified a group of self-specific $CD4^+$ T cell clones that are recurrently enriched in non-lymphoid organs following Treg cell-ablation. Furthermore, by generating retrovirally expressed TCRs to track and study the biology of self-specific Tconv clones *in vivo* and *ex vivo,* we revealed that these self-reactive Tconv clonotypes exhibit reactivity to widespread antigens yet routinely escape clonal deletion. In the periphery, these cells express numerous hallmarks of Tfh-like cells, including high expression of Bcl6 and PD-1. Although these clones harbor a Tfh-like phenotype at steady state, they do not appear to promote aberrant activation of autoreactive B cells. Our data provides some indication that these self-reactive TCR clonotypes do not appear to promote B cell help, and may potentially harbor the capacity to induce B cell deletion. An extrapolation of our findings to the polyclonal wildtype repertoire supports the notion that Bcl6 can identify a population of endogenous T cells exhibiting Tfh-like characteristics at steady state. In this regard, the self-reactive TCR clones identified in this study serve as a useful tool with which to track and elucidate the mechanism by which self-reactive Tfh-like cells are regulated at steady state and the immunological consequences that arise when their regulation is breached.

CHAPTER IV

DISCUSSION

Overview

In this study, we utilized deep-TCR sequencing approaches to identify self-specific $CD4^+$ Tconv clones that infiltrate non-lymphoid organs when released from Treg-mediated suppression. Using retrovirally expressed TCRs to track a group of self-specific Tconv clonotypes, we defined the nature of self-reactivity, anatomical distribution, and phenotype of these cells at steady state. Our study provides evidence of at least four naturally occurring $CD4^+$ T cell clonotypes that are widely self-reactive yet routinely escape negative selection. Strikingly, these clones harbor a T follicular helper-like phenotype at steady state, but do not appear to promote aberrant activation of autoreactive B cells.

The self-reactive clonotypes in our study exhibit TCR signaling in the thymus at a level sufficient to drive hallmarks of negative selection, suggesting that their affinity to self-ligands in the thymus is likely substantial. However, these cells routinely escape deletion and enter the periphery. Our results contradict models proposed by previous studies suggesting robust clonal deletion of T cells specific for ubiquitously expressed self-antigens[96,97]. The clones identified in our study are clearly reactive to ubiquitous antigens, yet they are not culled from the peripheral repertoire. Intriguingly, a closer examination of the data reported by Moon et al. reveals that, while specificities reactive to ubiquitous antigens appear to be deleted to a larger extent than those reactive to tissue-restricted antigens, they are nonetheless detected in the spleen and lymph nodes and can expand upon immunization. Further, analysis of effector function revealed that a subset of self-reactive $CD4^+$ T cells in these systems exhibited hallmarks of T follicular helper

cells, such as expression of CXCR5, PD-1[96], and Bcl-6[97]. These same self-reactive specificities also harbored a subset of Foxp3$^+$ Treg cells. Thus, the observations reported by other groups are in fact in-line with observations made in our study.

These studies also concluded that high-affinity clonotypes reactive to ubiquitous self-antigens were deleted, and that cells that remain in the peripheral repertoire are largely low-affinity clonotypes[96,97]. The conclusions were made based on mean fluorescence intensity of tetramer-positive cells. In our study, because the cognate antigens are not known, we are not able to assess the affinity and avidity of our TCR clonotypes to self-antigens. However, we used expression of the transmembrane receptor CD5 as a rough estimation of affinity, as CD5 expression level has been previously correlated with the affinity of TCRs with their known agonist peptides[209]. By fluorescence intensity, our data demonstrates that Group 2 self-reactive clonotypes express levels of CD5 comparable to those of polyclonal Treg cells (Figure 16). In contrast, Group 1 clonotypes express much lower levels of CD5, below those of polyclonal Treg cells and in the lower range of polyclonal Tconv cells. Therefore, our preliminary assessment suggests that the TCRs expressed by Group 2 clones that escape deletion likely exhibit considerable affinity for self-antigens, at levels comparable to those of polyclonal Treg cells.

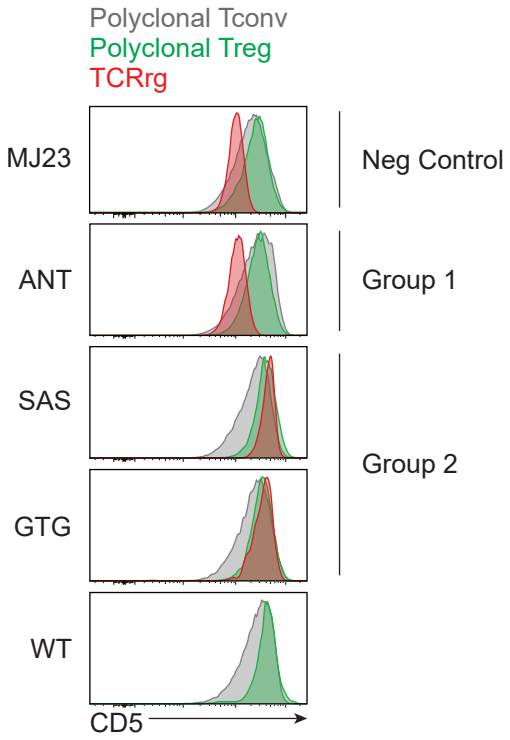

Figure 16. Group 2 TCRrg cells display high expression of CD5.
5-7 weeks after primary TCRrg hosts were generated, secondary lymphoid organs were isolated from primary TCRrg hosts, and polyclonal Tconv ($TCR\beta^{+}CD4^{+}Foxp3^{neg}$), polyclonal Treg ($TCR\beta^{+}CD4^{+}Foxp3^{+}$), or TCRrg ($TCR\beta^{+}CD4^{+}Thy1.1^{+}$) cells were assessed for surface expression of CD5. Histograms depicting fluorescence intensity of CD5 are shown. Data is representative of one experiment. Gray, polyclonal Tconv; green, polyclonal Treg; red, TCRrg.

It should be noted that our approach to evaluate TCR affinity/avidity is limited by the variability in the level of TCR expression on the cell surface due to the nature of retroviral transduction systems. To quantitatively define the affinity of these TCRs for their agonist self-ligands, a careful biochemical analysis should be performed using recombinant TCRs[210]. This task is hindered by a lack of knowledge regarding the identity of agonist self-ligands recognized by Group 2 TCRs. In collaboration with Eric Huseby's group, we intend to employ a high-throughput peptide-MHC display library method[211] to identify cognate peptides recognized by Group 2 TCRs.

Signals required for the adoption of Tfh-like phenotype by Group 2 clones

Our studies of retrovirally expressed TCRs (TCR retrogenic, TCRrg cells) revealed that Group 2 clones adopt a T follicular helper-like phenotype in the periphery at steady state, characterized by expression of Bcl6, PD-1, CXCR5, and ICOS (Figure 10). In the thymus, while we observed high Bcl6 expression in both polyclonal host cells and TCRrg cells at the pre-TCR stage, expression of Bcl6 was downregulated at the TCRβ^{hi} stage of development and was not maintained in mature Group 2 TCRrg thymocytes (Figures 10E and 10F). These observations suggest that Bcl6 expression is likely upregulated in the periphery upon peripheral TCR encounter with self-antigens, as opposed to upregulation upon self-antigen encounter during thymic development. Is recognition of a self-ligand required for the adoption of a Tfh-like phenotype?

It was shown that *in vitro* co-culture of Group 2 clones with DCs isolated from H2-DM-deficient mice abolished proliferation of these cells (Figure 2E and 2F), suggesting that these DCs lack presentation of the appropriate endogenous self-peptides necessary for Group 2 TCR

signaling. Accordingly, if TCR signaling by self-pMHC is required for the adoption of a Tfh-like phenotype, we would expect that Group 2 TCRrg cells generated in H2-DM-deficient hosts should fail to adopt the Tfh-like phenotype at steady state.

Because Group 2 clonotypes exhibited TCR signaling in the thymus at a level sufficient to drive hallmarks of negative selection, we reasoned that they must have encountered agonist self-ligands in the thymus prior to exiting. To confirm this, *in vitro* co-culture of Group 2 TCRrg cells with dendritic cells isolated from the thymus would reveal whether thymic DCs can present agonist ligands recognized by Group 2 clonotypes in an MHC class II-dependent manner. To what extent does thymic self-antigen encounter play a role in the differentiation fate of these cells later in the periphery? Is the adoption of a Tfh-like phenotype solely a result of peripheral signals or is this fate determined early during thymic development by some undefined mechanism?

If peripheral self-antigen encounter were sufficient to drive the Tfh-like phenotype, we would expect that Group 2 TCRrg cells would upregulate the Tfh-like phenotype upon adoptive transfer from the H2-DM-deficient primary hosts to wildtype H2-DM-sufficient hosts. However, if self-antigen expression in the thymus played a critical role in the fate of Group 2 TCRrg cells, grafting experiments of wildtype thymi into H2-DM-deficient hosts should facilitate the adoption of Tfh-like phenotype by Group 2 clones. It is also possible that expression of the agonist self-ligand is necessary in both the thymus and the periphery for the upregulation and maintenance of the Tfh-like phenotype. Lastly, it remains unclear whether Group 2 clones need to interact with B cells presenting the corresponding self-antigen in order to adopt a Tfh-like phenotype in the periphery, or whether interaction with DCs bearing self-peptides is sufficient. This question could be resolved by analyzing the phenotype of Group 2 TCRrg cells generated in B cell-

deficient mice. If B cell interaction were critical for the adoption of a Tfh-like phenotype, Group 2 clones should lack the Tfh-like phenotype in B cell-deficient hosts.

Lastly, given that Group 2 clones recognize ubiquitously expressed self-antigens, it remains unclear whether these self-ligands are presented across all antigen-presenting cell subsets, or whether Group 2 clones specifically interact with a particular dendritic cell subset or B cell subset. Are Group 2 clones encountering the same agonist ligands both in the thymus and the periphery? Do these cells interact predominantly with thymic epithelial cells or bone marrow-derived dendritic cells in the thymus? If the latter, do Group 2 clones interact with a particular subset of dendritic cells in the thymus and periphery? Where are the agonist ligands expressed in the thymus (i.e. cortex, medulla)? And what is the level of expression of these antigens in the thymus and the periphery, compared to other types of self-antigens (such as tissue-restricted antigens)? The role of thymic epithelial cells versus bone marrow-derived antigen-presenting cells can be evaluated via mixed bone marrow chimera approaches, by comparing lethally irradiated mice reconstituted with MHC class II-deficient versus wildtype bone marrow, or by comparing hosts harboring MHC class II-deficient versus wildtype radioresistant thymic epithelial cells. In addition, to assess the roles of unique DC subsets in facilitating the adoption of Tfh-like phenotype by Group 2 clones, Group 2 TCRrg cells could be generated in several mouse DTR strains that allow for depletion of specific DC subsets, or in transcription factor knock-out mouse strains that are deficient in certain DC subsets[212].

Escaping clonal deletion in the thymus

How do Group 2 clonotypes escape negative selection? For cells that escape thymic deletion—such as thymic Treg cells—upregulation of certain lineage-specific genes (e.g. Foxp3,

CD25) coincides with survival signals that protect these cells from apoptosis[98,99]. In this regard, there likely exists a delicate balance of survival and death signals during thymic development that is key to imparting certain self-reactive lineages with a survival advantage. Since Bcl6 is not expressed in mature Group 2 thymocytes (Figures 10E and 10F), it would be interesting to trace Group 2 clones across thymic maturation stages and assess whether Group 2 clones can be distinguished from negatively selected clones by any transcriptional or epigenetic profile. The identification of highly differentially expressed or epigenetically modified genes between Group 2 and control thymocytes may reveal molecules that might play a critical role in facilitating the deletional escape of self-reactive clones.

A peripheral niche for Group 2 clones?

Do all self-reactive $CD4^+$ T cells express Bcl6 and adopt a Tfh-like phenotype, or is there something specific to the nature of antigens recognized by Group 2 clones that promotes this fate (e.g. pattern of antigen expression, antigen dose, molecular structure translating to quality/strength/duration of TCR signaling)? Alternatively, are there secondary factors that play a role in determining the differentiation fate of self-reactive T cells into Th1, Th2, Th17, Tfh, Tfr, or Treg subsets upon self-antigen encounter? The expression of certain self-antigens could also coincide with distinct secondary factors (e.g. DC subset, localized cytokine niche), thus dictating the fate of these specificities.

Studies from Kalekar et al. have reported a polyclonal population of endogenous self-reactive cells that exhibit a $FR4^{hi}CD73^{hi}$ phenotype[195]. Furthermore, gene expression profiling of these $FR4^{hi}CD73^{hi}$ phenotype cells revealed high expression of Bcl6, c-Maf, CXCR5, CXCR4, and PD-1[195], consistent with a Tfh-like phenotype. However, other studies of endogenous $CD4^+$

T cells using model self-antigens have also shown that the same self-reactive specificities can differentiate into Th1, Th17, Treg, and Tfh subsets upon immunization with the model self-peptide[97]. Therefore, while these specificities recognize the same cognate antigen, they can differentiate into various different subsets upon immunization, suggesting that the nature of the self-antigen is not the sole factor in dictating the differentiation fate of these self-reactive T cells. What factors, in addition to the nature of the antigen, might play a role in the differentiation fate of these cells?

Within the secondary lymphoid organs, the geographic concentration of distinct DC subsets supports niche-restricted T cell differentiation by establishing specialized microenvironments[158]. These microenvironments differ in regards to the abundance of particular cytokines, co-stimulatory signals, and strength of antigenic stimulation. For example, the segregation and geography of Th1 cells and Th2 cells coincide with the positioning of conventional type 1 DCs (cDC1) and type 2 DCs (cDC2) within the lymph nodes, respectively. Th1 and cDC1 cells are often found deep within the T cell zone, whereas Th2 and cDC2 cells are located in the interfollicular zones near B cells. This segregation is largely guided by differential expression of the chemokine receptor CCR7, which is high on Th1 cells and cDC1 cells. The CCR7 ligands CCL19 and CCL21 are rich in the deep T cell zone, hence Th1 and cDC1 cells are typically localized there. cDC1 cells intrinsically produce more IL-12 upon stimulation than other DC subsets. In addition, accessory cells and stromal cells in the microenvironment also affect T cell fate through production of cytokines and chemokines. These accessory cells together with cDC1 cells create a milieu rich in IFNγ, IL-12, and TNF in the deep T cell zone to favor a Th1 differentiation fate and promote activation of $CD8^+$ CTL cells.

As introduced in previous chapters, Tfh induction occurs in two phases: interaction with DCs induce the pre-Tfh phase (which includes temporary upregulation of Bcl6), but pre-Tfh cells must then migrate toward B cells and stably interact with cognate B cells to receive conditioning signals (co-stimulation: CD40 and OX40, and cytokines: IL-6, IL-12, IL-21) to solidity their fate as Tfh cells[166]. The geography of this interaction is relevant. Studies show that the induction of the pre-Tfh phase occurs at the T cell-B cell border or at the interfollicular zones. These areas are largely populated by conventional type 2 DCs (cDC2), which preferentially present antigens on MHC class II molecules. As a result, homing of cDC2 cells to the T cell-B cell border allows these DCs to bring T cells and B cells together, thus creating a powerful niche for pre-Tfh development and commitment. Because the localization of Th2 versus Tfh cells and cDC2 cells is very similar, the factors and signals that determine T cell differentiation fates between Tfh and Th2 cells remain unclear. It has been established that the strength of TCR signaling plays a role in the regulation of T cell differentiation fate. Specifically, high antigen dose and strong TCR signaling has been shown to favor Tfh cell differentiation, while low antigen dose and TCR signaling seems to favor Th2 cell differentiation[158,213,214].

Why might Group 2 TCRrg cells preferentially interact with a particular DC subset? Distinct DC subsets may vary in the repertoire of self-antigens presented on their cell surface. This could be due to differences in their methods of antigen uptake and lysosomal processing, or differences in levels of peptide-loaded MHC class I versus MHC class II molecules presented on the cell surface. Therefore, intrinsic functional differences between DC subsets, along with differences in physical location of DC subsets within secondary lymphoid organs, may all contribute to creating immunologically distinct compartments that prompt distinct induction of T cell fates for Group 2 TCRrg cells. Our immunofluorescence analysis of spleens isolated from

primary TCRrg mice support this notion. $CD4^+$ $Bcl6^{hi}$ cells in the spleen of Group 2 TCRrg hosts were closely associated with B cell follicles and were not found within deep T cell zones (Figure 12). This localization was more pronounced in the spleen than in the lymph nodes, which coincides with a stronger Tfh-like phenotype observed for Group 2 clones in the spleen (Figure 10) than in the lymph nodes (data not shown) by flow cytometry. While lineage-specific niches in the spleen are thought to be organized in a similar fashion as those found in lymph nodes, the corresponding niches are less understood in the spleen and merit further investigation.

Because the Thy1.1 marker was ineffective in staining the tissue samples, we were not able to verify that the $CD4^+$ $Bcl6^{hi}$ T cell clusters in these sections corresponded directly to $Thy1.1^+$ cells. We hope to bypass this issue by staining for congenic markers CD45.2 (donor) and CD45.1 (host), or by optimizing staining conditions to enhance the Thy1.1 signal. That being said, the localization pattern of $CD4^+$ $Bcl6^{hi}$ T cell clusters in the spleens of unimmunized primary Group 2 TCRrg hosts is very intriguing. Specifically, why do these cells cluster together within a single B cell follicle instead of seeding multiple B cell follicles within a given white pulp? Is this a result of clonal expansion by a single clone that encountered its cognate self-antigen, or a convergence of multiple cells toward a single locus in response to some rare signal or local factors?

Clustering and clonal homogeneity has been described extensively for B cells undergoing affinity maturation during a germinal center (GC) reaction as a result of interclonal and intraclonal B cell competition[133,215,216]. Thus, mature GCs are thought to be "islands" dominated by one or a few clones that differ from neighboring GCs, simulating a "founder effect"[133,135,215,216]. The dynamics of Tfh cells in the GC are just beginning to be elucidated, but much less is known about these cells compared to GC B cells. In contrast to GC B cells, GC Tfh

cells are highly polyclonal within a given GC, and the same clones can be found across many different GCs, which suggests that they are continuously exchanged between GCs[137]. In addition, newly activated Tfh cells can infiltrate ongoing GCs and provide help to cognate B cells within these GCs. Currently, it remains unknown why GCs are open to free exchange of Tfh cells, while restricting B cell exchange. Our study focused on $CD4^+$ $Bcl6^{hi}$ cells at steady state, and GL-7 staining showed that GCs were largely absent in primary TCRrg hosts (data not shown). Therefore, current understanding of Tfh dynamics during a GC reaction cannot directly translate to explain the clustering phenomenon observed for $CD4^+$ $Bcl6^{hi}$ T cells in primary B cell follicles in our study. It would be of interest to evaluate how localization of $CD4^+$ $Bcl6^{hi}$ cells changes in the context of a GC reaction.

It should be noted that our analyses here involved mice receiving total body irradiation. It will be important to perform the same analyses in wildtype mice receiving adoptive transfer of Group 2 clones to evaluate whether this phenomenon holds true in non-irradiated mice. It would also be of interest to evaluate how localization of $CD4^+$ $Bcl6^{hi}$ cells changes in the context of inflammation, such as infection, immunization, or Treg cell depletion. If these scenarios create a disturbance in the microenvironment of lineage-defining niches within the spleen and lymph nodes (such as change in cytokine niches, DC activation status, surface MHC levels), one might expect that the localization and differentiation fates of self-reactive cells in these settings may be altered. Specifically, it has been proposed that IL-2 sinks promote Tfh cell responses, since IL-2 can downregulate CXCR5 expression on T cells[153,155-157,217]. The expression of the IL-2 receptor α-chain (CD25) on Treg cells, B cells and cDC2 cells may thus act together to create a suitable microenvironment for Tfh differentiation. However, depletion of Treg cells or activation of B

cells and cDC2 cells may impact this phenomenon. Consequently, the regulation of these niches may be essential to maintaining tolerance of self-reactive cells during inflammatory contexts.

The notion of lineage-defining niches in the periphery may also relate to our observations in which the emergence of the $FR4^{hi}CD73^{hi}$ phenotype in Group 2 clones appears inversely correlated with the frequency of Group 2 TCRrg cells that is present within the peripheral $CD4^{+}$ T cell compartment (Figure 8). The limiting factor was likely independent of the availability of cognate antigen interaction, because a large fraction of Group 2 cells (>60%) express the TCR signaling marker CD69 regardless of TCRrg cell frequency within the repertoire (Figure 8A). Thus, there likely exists at least one or more additional factor(s) that is required for the peripheral adoption of the $FR4^{hi}CD73^{hi}$ phenotype. These factors may involve cytokine niches or competition for cognate B cells at the B cell-T cell borders. While the expression of FR4 and CD73 may not directly equate to Bcl6 expression or Tfh phenotype or function, it may reflect the commitment of pre-Tfh cells to the Tfh-lineage, which requires productive engagement with cognate B cells. Accordingly, the loss of FR4 and CD73 expression by Group 2 TCRrg cells upon host Treg cell depletion could suggest a change in the T helper differentiation state or Tfh-like status of Group 2 clones in this setting (Figure 17D). It will be of interest to assess how these changes in FR4 and CD73 expression correlate with changes in Bcl6 expression or Tfh-like phenotype and function in Group 2 clones following Treg depletion.

Lastly, for the Group 3 clones that demonstrated “mild” self-reactivity (Figures 4A and 4B), it may be revealing to assess their localization within spleen and lymph nodes as well. We envision at least two possibilities to explain why they displayed milder indications of self-reactivity compared to Group 2 clones: 1) antigens recognized by Group 3 clones are less abundant in the periphery, or 2) Group 3 clones bind their ubiquitous antigens with lower

affinity/avidity. The effects of these potential differences on T helper lineage fate may be worth investigating. The same can be said for the MJ23 TCR clonotype, which is specific for a prostate-associated (tissue-restricted) self-antigen, and not a ubiquitous self-antigen.

Function of Tfh-like Group 2 clones

What might be the purpose of maintaining self-reactive Tfh-like cells in the endogenous repertoire? As discussed in the previous chapter, we envision the following possibilities: 1) Group 2 clones could be poised to promote the deletion of autoreactive B cells reactive to a linked self-antigen. Specifically, Group 2 clones might be poised to eliminate newly arising B cell specificities that bear self-reactive B cell receptors (BCRs) generated from somatic hypermutations (SHM) during germinal center (GC) reactions. 2) Group 2 clones could exist to provide an endogenous source of IL-2 (to support Treg cell homeostasis), 3) Group 2 clones could represent a population of suppressor cells (or a source of T follicular regulatory cell precursors), 4) Group 2 cells could have no function—deletion could simply be inefficient and these cells could be adequately controlled by Treg cells in the periphery, and 5) Group 2 clones could play a central role in the proper formation of lymphoid follicles within the spleen, such as the white pulp (perhaps through productive interactions with local dendritic cells and B cells to create the appropriate chemokine gradients).

Our study revealed that polyclonal B cells co-cultured with polyclonal T cells or Group 1 TCRrg cells led to a small fraction (~1%) of B cells that remained viable after 7 days in culture, but this was not observed when B cells were co-cultured with Group 2 TCRrg cells (Figure 13C). These findings suggest two potential explanations: 1) Group 2 TCRrg cells are incapable of providing B cell help, or 2) Group 2 TCRrg cells are actively deleting B cells that survive in

culture. We aim to determine whether Group 2 TCRrg cells have the capacity to delete B cells through Fas signaling. If this were the case, we would predict that addition of anti-Fas or anti-FasL blocking antibody in culture should be sufficient to prevent deletion and maintain B cell viability in groups containing Group 2 TCRrg cells. Furthermore, if Group 2 clones are indeed deleting B cells in culture, we predict a dominant effect, in which the addition of Group 2 TCRrg cells would induce the death of B cells that survived in other groups, despite receiving help by other T cells (polyclonal or Group 1). This experimental approach would be significantly enhanced by the identification of the cognate self-antigens recognized by Group 2 clones. Identification of the relevant antigens would allow us to select antigen-specific B cells using peptide-MHC tetramers and evaluate the effects of Group 2 clones on their corresponding specificity of endogenous self-reactive B cells, both *in vitro* and *in vivo*.

Lastly, the difference in morphology of lymphoid follicles between the spleens of Group 1 and Group 2 TCRrg hosts by immunofluorescence (Figure 12) suggests that Group 2 clones may play a role in promoting the organization of T cell zones and B cell zones within splenic white pulps. Similar roles have been established for lymphoid tissue inducer (LTi) cells in promoting the formation of lymph nodes and gut-associated lymphoid tissues (GALTs)[218]. LTi cells are a type of innate lymphoid cells that are the first to colonize developing lymph node anlagens during embryonic development, and are required for the proper formation of lymph nodes and Peyer's patches[218,219]. They depend on the expression of the orphan transcription factor RORγt[220], and promote lymphoid structure organization by providing the tumor necrosis factor (TNF) superfamily ligands lymphotoxin α1β2 and RANKL[218]. Interestingly, the proper formation of organized splenic white pulp does not require LTi cells. In RORγt$^{-/-}$ and Id2$^{-/-}$ mice that reportedly lack all LTi cells, spleens have demonstrably segregated T and B cell areas[221].

Further, $Rag^{-/-}$ mice that harbor LTi cells but lack T and B lymphocytes display very little expression of CCL21 and CXCL13, homeostatic chemokines that are responsible for the segregation of T and B cell zones, indicating that their expression is dependent on lymphocytes[222].

In mice, lymphocytes start to appear in the spleen shortly after birth, but are initially poorly segregated. CCL21 is the first chemokine to appear, and its appearance is coincident with the development of rings of B cells surrounding cores of T cells[221,223]. At this time, B cell follicles do not yet express discrete CXCL13. The development of splenic B cell follicles is dependent on expression of lymphotoxin by B cells, which occurs a few days after the appearance of CCL21[222,223]. Finally, the marginal zone develops one week after birth and is also dependent on lymphotoxin signals. Interestingly, it has been demonstrated that the formation of T cell zones is B cell-dependent[223,224]. Specifically, B cell expression of LTα1β2 is required for inducing the expression of CCL21 by stromal cells in the spleen. Extrapolating these processes to our observations of TCRrg hosts, it is possible that Group 2 self-reactive T cells interact with self-reactive B cells to promote proper expression of lymphotoxin and CCL21 in the spleen. To test this, we can adoptively transfer polyclonal B cells into lymphopenic $Rag^{-/-}$ hosts, along with either Group 1 or Group 2 TCRrg $CD4^+$ T cells, and evaluate the extent to which proper white pulp structures are formed in the spleens. Alternatively, it is possible that Group 1 clones simply do not respond appropriately to CCL19 and CCL21 gradients. The inclusion of polyclonal T cells (or polyclonal T cells lacking Bcl6-expressing cells) may be informative in this case.

Intriguingly, there is a growing body of evidence suggesting that the expression of the TNF ligands OX40L and CD30L on LTi cells is critical for the generation of CD4 memory cells, through the provision of OX40 and CD30 signaling on primed $CD4^+$ T cells[225]. It is unclear

whether LTi cells interact with antigen-experienced self-reactive $CD4^+$ T cells (such as Group 2 TCRrg cells), but the presence of LTi cells across T cell and B cell zones of adult mouse spleens suggests that this interaction may be worth investigating.

Function of Bcl6

In our study, we discovered that all four self-reactive Group 2 TCR clonotypes distinctly expressed Bcl6 and PD-1 (Figures 10A-D). To investigate the role of Bcl6 in Group 2 clonotypes, it is worth discussing the molecular mechanisms underlying its functions in B cells and T cells. Originally identified in diffuse large B-cell lymphoma, Bcl6 is a transcriptional repressor and a member of the BTB-POZ zinc finger family of transcription factors[226,227]. In recent years, Bcl6 has been identified as the master regulator for the differentiation of $CD4^+$ T follicular helper (Tfh) cells, and is also important for the differentiation of germinal center (GC) B cells[146]. Studies have also implicated Bcl6 in pre-GC B cell and T cell events[149]. Employment of Bcl6-reporter mice and high-resolution intravital cellular imaging demonstrated an early role for sustained Bcl6 expression during B cell and Tfh cell interaction within the interfollicular zone prior to GC entry[216]. Extrafollicular antibody responses resulting from early T:B cell interactions have also been shown to be dependent on $Bcl6^+$ pre-GC Tfh cells[148,149]. In addition, Bcl6 is required for the development of $Foxp3^+$ T follicular regulatory (Tfr) cells, which originate from thymic $Foxp3^+$ Treg cells, and appear to control the magnitude of the GC response[228-230].

Outside of its roles in GC B and T cells, Bcl6 has been shown to be upregulated in pre-B cells undergoing V(D)J recombination, protecting cells from DNA-damage-induced apoptosis[231]. Bcl6 also harbors checkpoint functions, and enables cell cycle exit during the late pre-B cell

stage through repression of *Myc* and *Ccnd2*[232]. Multiple genes targeted by Bcl6 in Tfh cells have been defined via analysis of genome-wide Bcl6 occupancy, together with transcriptome profiling[226]. ChIP-seq analysis for Bcl6 in Tfh cells revealed that most Bcl6 binding sites were localized to intron (41%) and intergenic (46%) regions, while ~7% were located to promoter regions[226]. Interestingly, only a minor fraction of Bcl6-bound genes were shared between B cells, T cells, Tfh cells, and macrophages, suggesting that Bcl6 gene regulation occurs in a cell type-specific manner[226]. Furthermore, it was revealed that the top Bcl6 binding motifs in T cells, Tfh cells, and B cells are largely different[226]. Thus, Bcl6 likely provides divergent functions among each cell type.

A primary function of Bcl6 in $CD4^+$ Tfh cells is the suppression of genes that are required to drive the differentiation of alternative lineages. For example, competition between Bcl6 and T-bet (a driver of T helper type 1 (Th1) cells) is important for determining the commitment of transitional T helper cells toward the Tfh phenotype[153,157,158]. Importantly, Bcl6 appears to promote Tfh cell differentiation in part through antagonizing the IL-2-induced IL-7R-STAT5 signaling axis[153,157,226]. This can be partly explained by the fact that the core element in Bcl6 binding consensus in Tfh cells is highly similar to that of the STAT5 binding motif, differing in only one nucleotide[226]. Therefore, Bcl6 and STAT5 likely compete in regulating Tfh cell differentiation, and serve reciprocal roles in Tfh vs non-Tfh polarization, respectively.

One of the primary molecular functions of Bcl6 involves a role in transcriptional regulation. Since Bcl6 is a transcriptional repressor, there are expectedly more downregulated target genes than upregulated genes[226]. Interestingly, one of the genes upregulated by Bcl6 (*Mxd4*) encodes a protein that is able to inhibit IL-2 expression in activated T cells[226]. This particular function is of interest to our experimental observations of Group 2 TCRrg cells, and

could explain why Group 2 clones could not proliferate *in vitro* without exogenous IL-2. In Tfh cells specifically, Bcl6 target genes were more likely involved in cell death and survival, cell cycle, and growth[226]. In addition, Bcl6 target genes were strongly associated with cellular activation and maintenance, signal transduction, cell adherence and metabolism. Bcl6 targets were also specifically enriched for genes involved in calcium and MAPK signaling pathway, JAK-STAT signal transduction, cytokine/cytokine receptors, and purine metabolism. This last pathway may explain why Group 2 TCRrg clones in our studies expressed high levels of CD73, an ectoenzyme that converts extracellular AMP to adenosine (purine metabolism). Future work should involve transcriptional profiling of Group 2 versus Group 1 TCRrg cells via RNA-sequencing to assess the extent to which the differentially expressed genes in these two groups coincide with the aforementioned repertoire of Bcl6-regulated genes in canonical Tfh cells, or whether Bcl6 expression in Group 2 clones imparts a novel function.

Plasticity of Tfh-like cells

Although initially viewed as terminally differentiated, it is now appreciated that T helper subsets, including Tfh cells, display substantial plasticity in their characteristics. Specifically, while T helper subsets exhibit permissive active chromatin conformations at genetic loci encoding subset-specific cytokines (and repressive marks in alternate subsets), such modes of regulation do not appear to be the case for loci encoding "master regulator" transcription factors[150,166,226]. Thus, rather than expression in an all-or-none fashion, the various lineage-defining transcription factors all exist in a poised state that can be readily induced for expression. The defining type of response occurs as a result of a balance between competing signals, and is dependent on the ratio and timing of the various transcription factors expressed.

Correspondingly, a growing body of data demonstrates that Tfh cells can express other transcription factors (T-bet, GATA3) and cytokines (IFNγ, IL-4, IL-17) characteristic of Th1, Th2, and Th17 effector populations, respectively, albeit at lower levels[150]. Thus, by assuming Tfh1, Tfh2, or Tfh17 cell characteristics contingent on the context of activation, Tfh cells may maintain fluidity in their ability to tune distinct types of humoral responses required to manage the specific type of infectious agent at hand.

Bcl6 also plays a role in the epigenetic modification of its DNA binding targets. Genome-wide epigenetic modification profiling of Bcl6 binding sites reveals that Bcl6 binding leads to decreased 5hmC modification (methylation) of its binding targets[226]. 5hmC refers to a type of epigenetic DNA modification in which the cytosine is methylated to become 5-hydroxymethylcytosine (5hmC)[233]. It remains unclear what role 5hmC plays in chromatin accessibility, histone modifications, and regulation of gene expression in Tfh cells. However, the impact of Bcl6-mediated epigenetic modifications may influence the plasticity or stability of lineage-committed Tfh cells that express Bcl6. Fittingly, Bcl6 was previously shown to be important for the generation and maintenance of effector and memory $CD8^+$ T cells and memory $CD4^+$ T cells. It has also been shown that certain Tfh cells can differentiate into memory Tfh cells.

In regards to Group 2 clones in our study, it would be interesting to determine whether these cells possess any epigenetic "memory" as a result of imprinting encoded at multiple levels, such as methylation of DNA, modifications of histones, organization of nucleosomes, and expression of non-coding RNAs. These epigenetic marks would persist through cell division and would be maintained throughout memory development, allowing memory T cells to "remember" and retain an active or repressive chromatin state at specific sites. These processes serve as the

molecular basis for the robust and rapid execution of effector functions (or robust maintenance of functional unresponsiveness) seen in memory T cells (or anergic cells) upon antigen re-encounter[234]. If the self-reactive Group 2 clones in our study do not possess a stable lineage-committed or anergic phenotype, they may be susceptible to conversion into pathogenic inflammatory lineages. This is especially challenging in the context of GC reactions in response to infection or vaccination strategies.

Taking this into account, the stability of Group 2 Tfh-like cells remains to be determined. A preliminary assessment of this question could involve the evaluation of Bcl6 expression and Tfh characteristics upon adoptive transfer into lymphopenic hosts, H2-DM-deficient hosts, or upon systemic Treg cell ablation. In our study, the adoptive transfer of Group 2 clones at low frequencies into T cell-deficient hosts (along with polyclonal filler cells) led to an increased ability to produce IL-21 (Figures 17A and 17B). Concurrently, the expression of Bcl6 was less pronounced in this setting, as compared to cells in the primary TCRrg hosts (data not shown).

Systemic Treg depletion led to the downregulation of the Tfh-associated markers FR4 and CD73 by Group 2 clones. It remains to be determined to what extent systemic Treg ablation can induce a switch in lineage-commitment by Group 2 clones (in relation to expression of transcription factors and production of effector cytokines), and what implications would result in regards to autoimmunity. Interestingly, systemic Treg cell depletion was also associated with a striking emergence of $Foxp3^{+}$ cells in the Group 2 subset (~20-30% of Group 2 subset) (Figures 18A and 18B). At this time, it is unclear whether this population emerged from the expansion of a pre-existing rare population of $Foxp3^{+}$ Group 2 cells, or whether they arose as a result of peripheral conversion from $Foxp3^{neg}$ Group 2 cells. It is possible that Group 2 clones maintain permissive chromatin conformations at their Foxp3 loci that are readily induced for expression.

This could be evaluated through bisulfite sequencing of the Foxp3 locus and other Treg-associated loci in Group 2 cells.

Because Group 2 clones in primary TCRrg hosts did not appear to exhibit overt production of effector cytokines, we investigated whether immunization with a strong adjuvant could induce a change in this phenotype. Group 2 clones in mice immunized with complete Freund's adjuvant did not appear to alter any cytokine production, though the expression of Bcl6 and PD-1 did appear to decrease marginally, both at the draining lymph node and in the spleen (data not shown). These results suggest that Group 2 Tfh-like clones may not be a very stable state. Thus, these cells could be highly susceptible to changes in the delicate balance of signals and cues surrounding its immediate environment, underscoring the importance of maintaining a tight regulation on these cells.

Potential function of CD73 in Group 2 Tfh-like cells

What might be the purpose of CD73 expression on Group 2 clones (and on Tfh-like cells in general)? Functionally, it is well established that the CD39 and CD73 ecto-enzymes play strategic roles in calibrating the duration, magnitude, and chemical nature of purinergic signals delivered to immune cells through the conversion of extracellular ADP/ATP to AMP, and AMP to adenosine, respectively[235]. The levels of extracellular ATP and adenosine serve as mediators that signal metabolic disturbance or insults in a cell, as well as the health of a cell[235,236]. ATP is released by damaged and dying cells. The released extracellular ATP in-turn binds purinergic (P2) receptors on its own cell or neighboring cells, which induces a pro-inflammatory response that includes the release of pro-inflammatory cytokines, mitochondrial damage, cellular disintegration, and apoptosis[237]. Cells that express CD39 and CD73 induce dephosphorylation of

ATP into ADP/AMP, then finally into adenosine. In contrast to ATP, adenosine mediates immune suppression by engaging A2A receptors on effector T cells, which in-turn downregulates NF-κB activation and thus reduces the release of pro-inflammatory cytokines and chemokines. The activities of CD39 and CD73 therefore act as "immunological switches" that drive a shift away from an ATP-driven pro-inflammatory environment to an adenosine-mediated anti-inflammatory milieu. By converting ATP/ADP/AMP to adenosine, cells that express CD39 and CD73 maintain a purinergic "halo" of immunosuppressive microenvironment in an autocrine and paracrine fashion.

For lymphocytes, CD73 is highly expressed on Treg cells, and its catabolic activity appears to synchronize with the activation status of these cells[238,239]. In addition, it was speculated that the enhanced activity of CD39 and CD73 following TCR signaling allows the entrance of Treg cells into inflamed regions, where it reduces the extracellular level of ATP, thereby decreasing ATP-mediated Treg cell death[240]. CD73 expression has also been associated with Tfh cells[197]. The activity of CD73 on Tfh cells may play an important role when it enters and surrounds GC regions in which B cell death is abundant. Therefore, the expression of CD73 on Tfh cells may reflect its role in functioning in a pro-inflammatory environment surrounded by dying neighboring cells. Taken together, our observations that Group 2 clones express CD73 may shed light on the type of environment in which these cells commonly operate, and thus may provide clues to their evolutionary purpose.

Functional unresponsiveness of Group 2 TCR clonotypes

In the periphery, our *in vitro* and *in vivo* analyses of Group 2 clones reveal conflicting observations regarding the functional responsiveness of these cells. These cells do not proliferate

in vitro without addition of recombinant IL-2, and they do not produce effector cytokines when re-stimulated with PMA and Ionomycin. As such, these findings are consistent with common descriptions of functional unresponsiveness (anergy). However, these cells express the proliferation marker Ki67 within primary TCRrg hosts, and can produce IL-21 when transferred at low frequencies into lymphopenic hosts with polyclonal wildtype filler cells. Thus these cells can exhibit effector functions in certain conditions, but do not appear to do so within primary TCRrg hosts.

How do we reconcile our findings that Group 2 clones express Ki67 *in vivo* yet display features of functional anergy when re-stimulated *ex vivo*? First, it should be noted that the use of Ki67 expression as a measure of cell proliferation has its limitations. The Ki67 antigen is expressed during all phases of the cell cycle (G_1-S-G_2-M) but not in quiescent cells in the G_0 phase or in cells stimulated to enter the G_1 phase[241]. Because Ki67 expression can be retained in nonproliferating cells arrested at the G_1/S or G_2/M phases of cell cycle[241], its expression is of limited use when determining whether Group 2 clones are actively proliferating *in vivo* at steady state or whether they are arrested at a particular phase of cell cycle upon TCR ligation. In this regard, adoptive transfer of CellTrace-Violet (CTV) labeled TCRrg cells into wildtype hosts would best reveal the extent to which Group 2 clones undergo cell division *in vivo*. Finally, there is a formal possibility that sufficient levels of IL-2 is made available to Group 2 clones *in vivo,* which allows these cells to proliferate *in vivo*, despite their inability to do so *in vitro* in the absence of IL-2. In any case, while it remains unresolved whether Group 2 clones are actively dividing *in vivo,* they do not appear to exist in an irreversible anergic state, and have the capacity to proliferate and produce effector cytokines when given adequate signals.

Regulation of Group 2 clones

Our studies thus far reveal that Group 2 TCRrg cells adopt a Tfh-like phenotype, yet do not appear to promote aberrant self-reactive B cell activation in primary TCRrg mice. However, adoptive transfer of Group 2 clones at low frequencies into lymphopenic hosts along with polyclonal wildtype lymphocytes was sufficient to promote production of IL-21 by Group 2 cells (Figures 17A and 17B), suggesting that these cells have the capacity to produce effector cytokines given the proper environmental context. It remains unknown what factors are required to facilitate activation of Group 2 clones. As mentioned before, the clonal frequency of Group 2 clones appears to influence the effector phenotype of these cells. Therefore, it is possible that the factors limiting effector phenotype of Group 2 clones involve the availability of certain homeostatic cytokines such as IL-2 or IL-7.

An alternative possibility is that Group 2 clones are efficiently maintained in a dormant state through peripheral tolerance mechanisms such as Treg-mediated suppression. Consistent with our initial TCR repertoire sequencing analysis (Figure 1), adoptively transferred Group 2 TCRrg cells infiltrate the prostate and salivary glands of Foxp3^{DTR} recipients following systemic Treg cell ablation (Figure 17C). Treg cells may maintain peripheral tolerance of Group 2 clones through a number of mechanisms: 1) by maintaining the homeostasis and localization of DCs within spleen and lymph nodes, 2) by serving as cytokine sinks for IL-2, thereby promoting signals that favor Bcl6 expression as opposed to T-bet expression, 3) by directly interacting with Group 2 clones through expression of CTLA-4, 4) by creating an immunosuppressive microenvironment near Group 2 clones through the release of anti-inflammatory cytokines or other molecules, or 5) by directly competing with Group 2 clones for binding of self-peptide-MHC molecules on antigen-presenting cells.

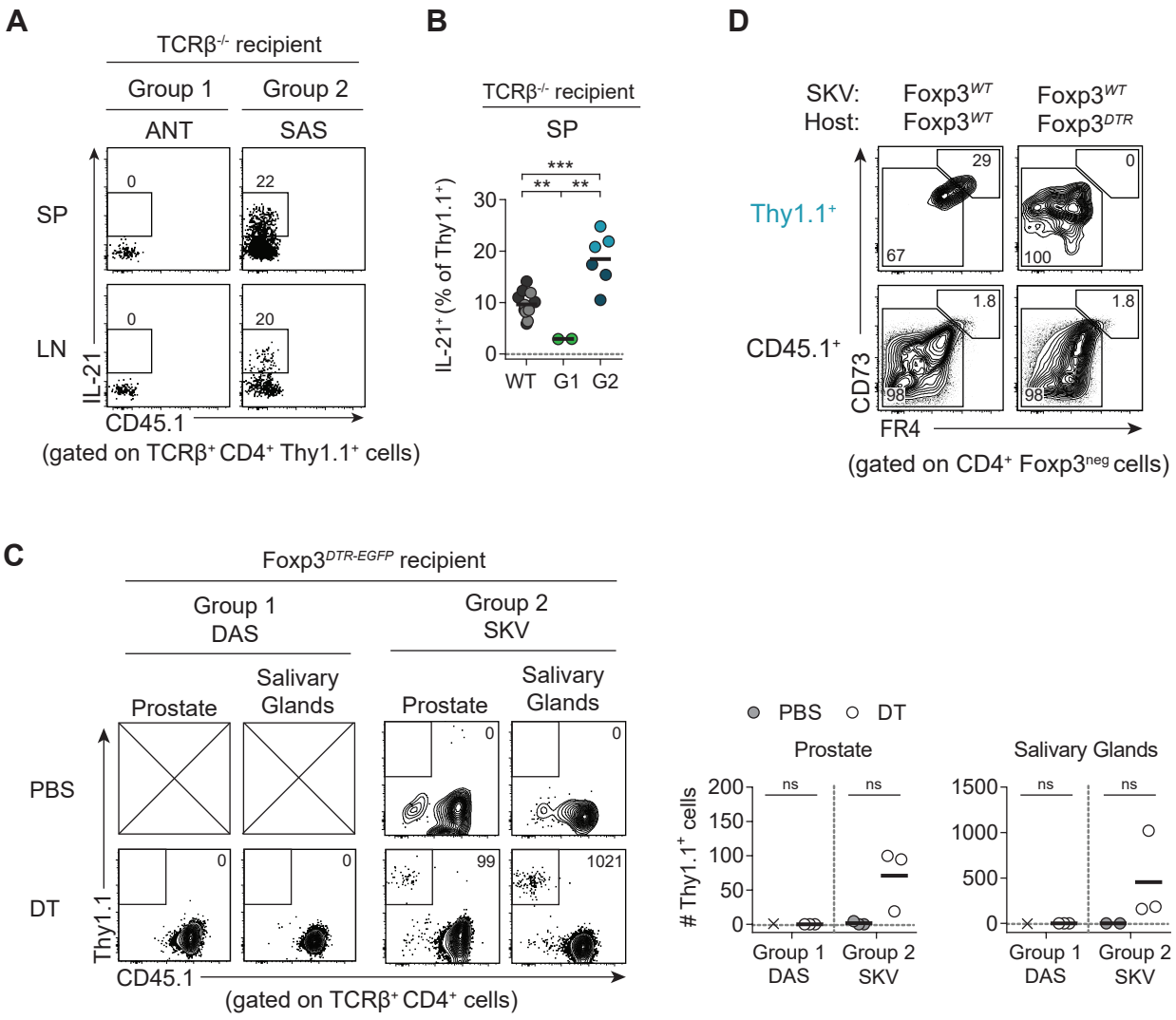

Figure 17. Group 2 clones exhibit a change in phenotype upon Treg-depletion or transfer into lymphopenic hosts.

Figure 17, continued.
(A) Secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^+$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. ~1 x 10^5 sorted TCRrg cells were co-transferred along with 10 × 10^6 B6.SJL bulk pooled spleen and lymph node cells into $TCR\beta^{-/-}$ female mice, such that TCRrg cells constituted ~0.5-1% of the inoculum. 3 weeks post-transfer, secondary lymphoid organs were isolated from reconstituted $TCR\beta^{-/-}$ recipients, and TCRrg ($TCR\beta^+CD4^+Thy1.1^+$) or co-transferred polyclonal B6.SJL ($TCR\beta^+CD45.1^+Thy1.1^{neg}$) cells were assessed for intracellular IL-21 production after a 5-hour stimulation with PMA/Ionomycin and monensin at 37°C via flow cytometry. Numbers denote the percentage of IL-21^+ cells. SP, spleen; LN, pooled lymph nodes (inguinal, axillary, brachial, cervical, periaortic).
(B) Quantification of (E). Data are representative of one independent experiment. WT, polyclonal B6.SJL; G1, Group 1 TCRs (light green = ANT); G2, Group 2 TCRs (light blue = SAS, dark blue = GTG).
(C) Secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^+$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. ~1 x 10^5 sorted TCRrg cells were adoptively transferred into 6-8 week-old male congenically marked CD45.1 $Foxp3^{DTR\text{-}EGFP}$ recipients. Diphtheria toxin (DT) or control PBS was administered to CD45.1 $Foxp3^{DTR}$ recipients as described in Methods. On day 12, the prostate and salivary glands were isolated from Treg-depleted mice, and the frequency of TCRrg cells within each organ was determined via flow cytometry (Left). Numbers denote the percentage of cells falling within each gate. (Right) Quantification of TCRrg cells within the prostate and salivary glands of Treg-depleted versus Treg-replete mice. PBS, phosphate-buffered saline; DT, diphtheria toxin.
(D) Secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^+$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. ~1 x 10^5 sorted TCRrg cells were adoptively transferred into 6-8 week-old male congenically marked CD45.1 $Foxp3^{DTR\text{-}EGFP}$ recipients. Diphtheria toxin (DT) was administered to CD45.1 $Foxp3^{DTR}$ recipients as described in Methods. On day 12, secondary lymphoid organs were isolated from CD45.1 $Foxp3^{DTR}$ recipients, and TCRrg ($TCR\beta^+CD4^+Thy1.1^+$) or polyclonal host ($TCR\beta^+CD45.1^+Thy1.1^{neg}$) cells were assessed for expression of FR4 and CD73 via flow cytometry. Numbers denote the percentage of cells falling within each gate.

Interestingly, Group 2 clones seem to lose their $FR4^{hi}CD73^{hi}$ phenotype following chronic Treg cell ablation (Figure 17D), suggesting a reversible or unstable phenotype. It remains to be determined whether this change is associated with changes in expression of Bcl6 (or other T helper transcription factors) and effector function, such as an increased production of effector cytokines. In addition, other studies have demonstrated that deletion of CTLA-4 on Treg

cells leads to spontaneous germinal center formation and elevated levels of autoantibody production[208]. It will be of interest to us to determine whether Group 2 clones represent the endogenous self-reactive population responsible for the promotion of GC formation and high-affinity autoantibody production in this setting. Similarly, because Group 2 clones express high levels of PD-1, there is interest in evaluating the effects of PD-1 blockade on Group 2 clones.

Lastly, we noted a puzzling finding that depletion of host Treg cells is associated with a striking emergence of $Foxp3^+$ cells in the Group 2 subset (~20-30% of Group 2 cells) (Figures 18A and 18B). At this time, it is unclear whether this population emerged from the expansion of a pre-existing rare population of $Foxp3^+$ Group 2 cells, or whether they arose as a result of peripheral conversion from $Foxp3^{neg}$ Group 2 cells. To test this, we would sort $Foxp3^{neg}$ Group 2 TCRrg cells using a Foxp3-reporter strain (but lacking the $Foxp3^{DTR}$ allele), and perform adoptive transfer of these Tconv cells into $Foxp3^{DTR}$ recipients to assess the emergence of newly arising Treg cells from this donor population following host Treg cell depletion.

Sequencing analyses of Group 2 clones had shown that many of these clonotypes were detected in both the polyclonal Tconv and Treg cell repertoires of secondary lymphoid organs isolated from fixed TCRβtg mice (Figure 1; Figure 19), suggesting that they can exist as $Foxp3^+$ cells at steady state. However, when Group 2 TCRrg mice were generated, their TCRs did not appear to facilitate efficient differentiation into Treg cells during thymic development or peripheral Treg conversion, even at low precursor frequencies (Figure 6). Future work remains to determine what molecular signals are required to induce the emergence of this $Foxp3^+$ population, and what functions they serve. Interestingly, the adoptive transfer of Group 2 clones alone into lymphopenic hosts led to the engraftment of Group 2 cells containing a high proportion of $Foxp3^+$ cells (data not shown).

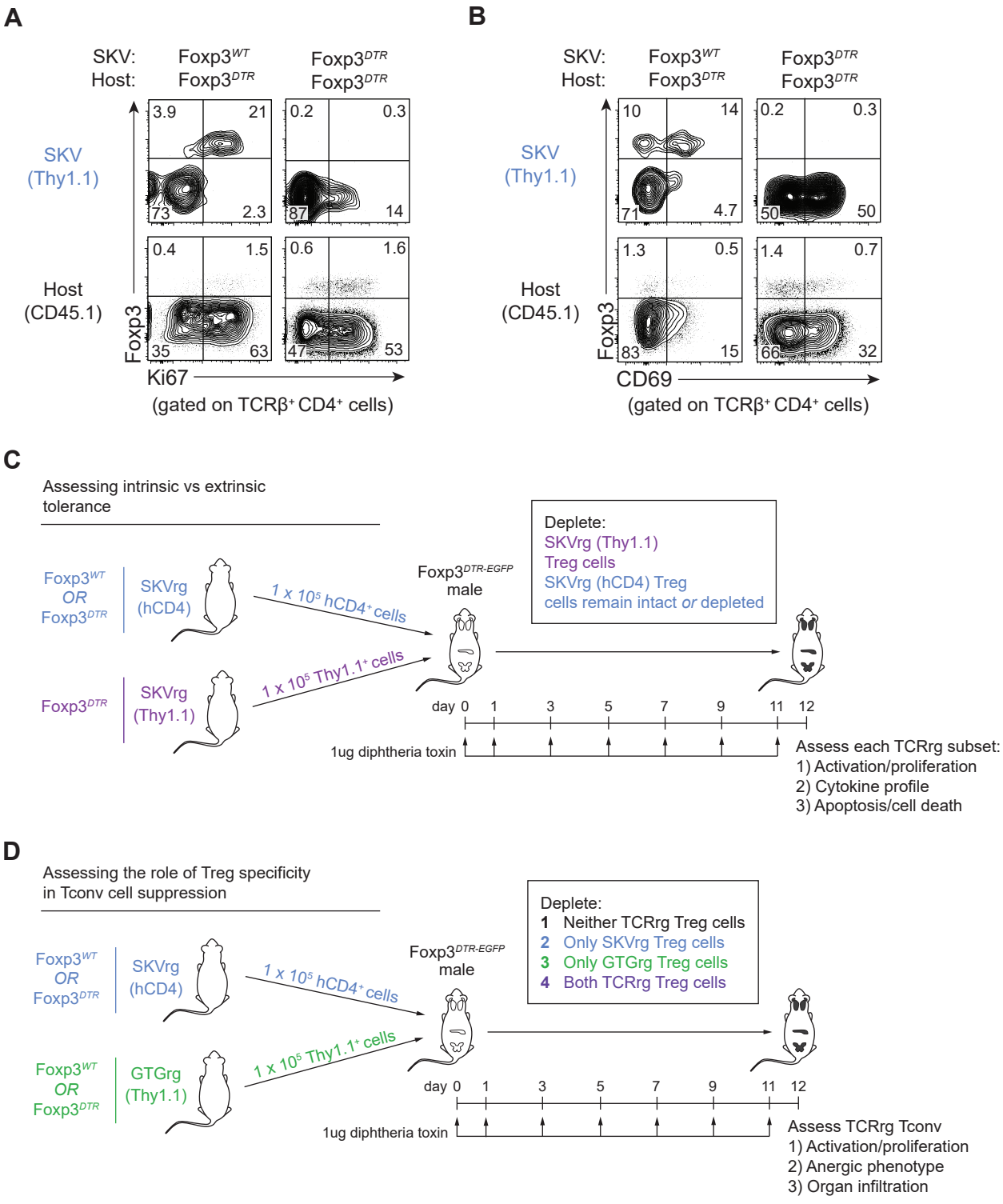

Figure 18. Group 2 clones are detected as Foxp3⁺ cells in Treg-depleted settings.

Figure 18, continued.
(A-B) Secondary lymphoid organs were isolated from primary TCRrg hosts, enriched for $CD4^+$ T cells via MACS kit, and sorted for TCRrg cells ($CD8\beta^{neg}CD45.1^{neg}Thy1.1^+$) via FACS. ~1 x 10^5 sorted TCRrg cells were adoptively transferred into 6-8 week-old male congenically marked CD45.1 $Foxp3^{DTR\text{-}EGFP}$ recipients. Diphtheria toxin (DT) was administered to CD45.1 $Foxp3^{DTR}$ recipients as described in Methods. On day 12, secondary lymphoid organs were isolated from CD45.1 $Foxp3^{DTR}$ recipients, and TCRrg ($TCR\beta^+CD4^+Thy1.1^+$) or polyclonal host ($TCR\beta^+CD45.1^+Thy1.1^{neg}$) cells were assessed for expression of Foxp3, Ki67 (left), and CD69 (right) via flow cytometry. Numbers denote the percentage of cells falling within each gate.
(C) Experimental approach to assess the intrinsic versus extrinsic nature of Treg-mediated suppression.
(D) Experimental approach to assess the role of Treg cell clonotype in its ability to exert immune suppression on Tconv cells of a different clonotype *in trans*.

It is possible that Group 2 clones are amenable to differentiating into $Foxp3^+$ cells in a lymphopenic context, or that rare $Foxp3^+$ cells engraft and expand in this setting with a competitive advantage to their $Foxp3^{neg}$ counterpart. Similarly, studies by Kalekar et al. have shown that anergic phenotype $FR4^{hi}CD73^{hi}$ cells (which are presumably self-reactive) adoptively transferred into lymphopenic hosts readily differentiate into $Foxp3^+$ Treg cells, thus serving as a source of Treg cells[195]. How could we reconcile our incongruous findings between TCR sequencing analyses and TCR retrogenic studies? Perhaps Group 2 clones generated during the neonatal period led to the accumulation of some $Foxp3^+$ cells in the host (as neonatal mice are largely lymphopenic[66], hence their identification via sequencing analyses of unmanipulated fixed-TCRβtg mice. Accordingly, the differentiation of $Foxp3^+$ Group 2 clones would be expectedly more rare in studies of lymphoreplete adult mice.

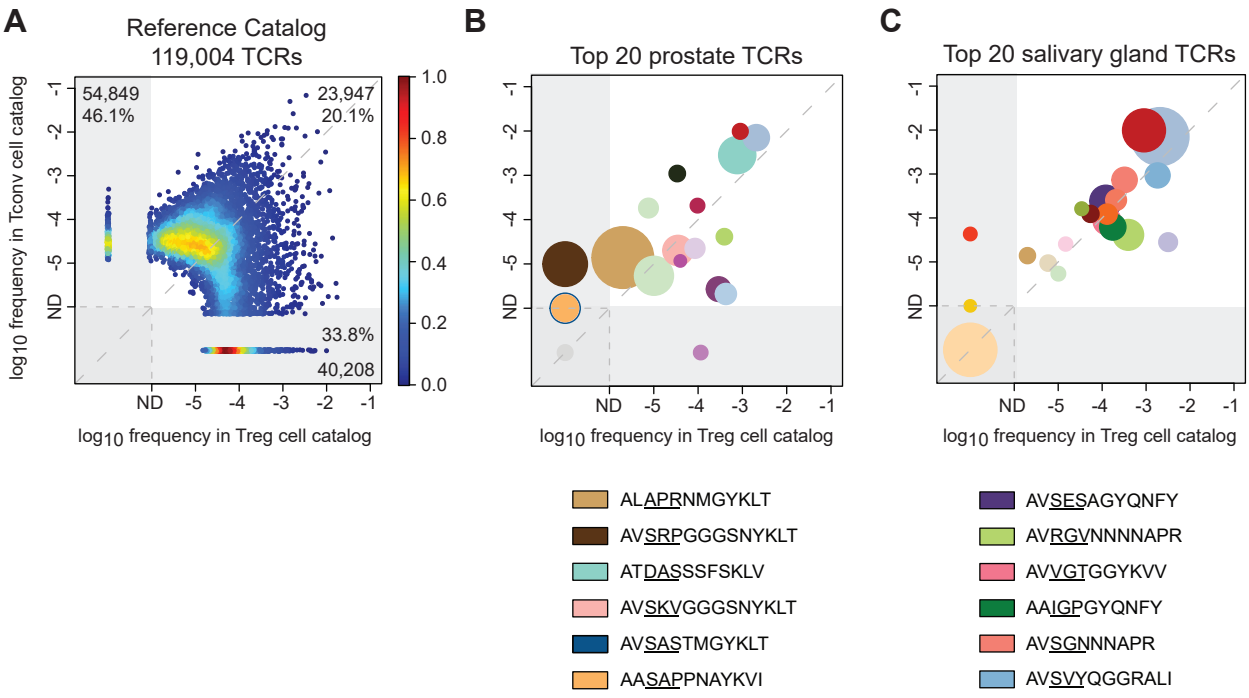

Figure 19. Many TCRs expressed by Tconv cell clonotypes detected in non-lymphoid organs of Treg cell-depleted mice appear to be found in both Treg and Tconv compartments of healthy mice.

(A) $CD4^{+}Foxp3^{+}$ Treg cells and $CD4^{+}Foxp3^{neg}$ Tconv cells were purified by FACS from pooled spleen and lymph nodes of four 9-week-old TCRβtg $Foxp3^{GFP}$ males, and subjected to complete TCRα sequencing. The mean frequencies of TCR clonotypes in the Tconv cell and Treg cell catalogs are plotted on the y-axis and x-axis, respectively. The density of TCR distribution on the plot is depicted by a gradient color scale from most dense (red = 1) to least dense (blue = 0). Areas of the plot that are colored in warmer colors reflect a larger number of TCRs found distributed in those positions of the plot. Of the 119,004 TCRs shown, 40,208 (33.8%) are biased to the Treg cell lineage, 54,849 (46.1%) are biased to the Tconv cell lineage, and 23,947 (20.1%) are found in both compartments, although there appears to be a number of TCRs skewed to either lineages even when found in both compartments.

(B-C) Five 6-week-old TCRβtg $Foxp3^{DTR\text{-}EGFP}$ males were treated with DT every other day for 10-12 days. Following Treg cell-depletion, Tconv cells were FACS-purified from the prostates or salivary glands of Treg cell-depleted mice, and subjected to complete TCRα sequence analysis, as described in Figure 1. These data were then compared to TCR frequency data from "catalogs" described in Figure 1D. For the twenty most prevalent Tconv cell TCRs in the prostate (B) and salivary gland (C) samples, the average frequency (log10) of each TCR in the Tconv cell catalog is plotted versus the Treg cell catalog. The size (area) of each data point is proportional to the frequency of this TCR within the Tconv cell subset of the Treg-depleted prostate (B) or salivary gland (C) sample. Data points centered within the dashed boxes were not detected in both the Tconv cell and Treg cell catalogs. ND, not detected in catalog.

When Foxp3^{+} cells in the Group 2 subset emerged following host Treg cell depletion, we noticed a striking observation that the Foxp3^{+} and Foxp3^{neg} subsets of the same clonal specificity exhibited a stark difference in expression of Ki67 and CD69 (Figure 18A and 18B). The Foxp3^{+} fraction expressed high levels of both CD69 and Ki67, while the Foxp3^{neg} fraction expressed negligible amounts of both markers. Further, when the Foxp3^{+} population of Group 2 TCRrg cells was also depleted (using donor mice bearing the Foxp3^{DTR} allele), the Foxp3^{neg} fraction then began expressing some Ki67 and CD69. This was surprising because the Foxp3^{+} and Foxp3^{neg} fractions of Group 2 TCRrg cells presumably possess the same antigen specificity. Therefore, their divergent behavior cannot be readily explained by a difference in their specificity, antigen-presenting cell (APC) binding partners, or localization/microenvironment.

Is this difference in phenotype a result of disparities in intrinsic qualities or extrinsic factors between the two subsets? First, the Foxp3^{+} fraction may be suppressing their Foxp3^{neg} counterpart, thereby inhibiting the TCR signaling and proliferation of the Foxp3^{neg} fraction. Along a similar vein, the Foxp3^{+} fraction may possess a competitive advantage in binding to the same pMHC molecules, thereby blocking access of Foxp3^{neg} cells to their cognate antigen. The competitive advantage in pMHC binding between the Foxp3^{+} and Foxp3^{neg} subsets could be due to differences in: a) surface density of TCRs, b) ability to cluster TCRs at the immunological synapse, c) chemokine expression or kinetics in trafficking to the site of antigen presentation. Similarly, Foxp3^{+} and Foxp3^{neg} subsets may be predisposed to respond differently to antigenic signals due to differences in transcriptional or epigenetic profiles. Third, as mentioned before, the Foxp3^{+} cells may actually be newly arising cells that converted from the Foxp3^{neg} fraction upon antigen encounter and proliferation. Therefore, Foxp3^{neg} cells are simply cells that have not yet encountered antigen and thus have not yet proliferated or converted.

To test the proposed possibilities, we can co-transfer equal ratios of two populations of cells expressing the same Group 2 TCR into Foxp3^{DTR} recipients (Figure 18C). Half of the cells would bear the Foxp3^{DTR} allele while the other half would bear the Foxp3^{WT} allele—the two populations would be distinguished by reporter markers (Thy1.1 versus human-CD4). Selective depletion of Treg cells in one-half of the donor group would reveal whether the same outcome is observed in the Foxp3^{neg} fractions of both donor populations. Does Treg cell depletion only affect the Foxp3^{neg} cells that came from the Foxp3^{DTR} donor pool, or do both Foxp3^{neg} populations exert the same change in phenotype? If the difference in expression of Ki67 and CD69 were caused by cell-intrinsic factors as a result of bearing the Foxp3^{DTR} allele, we would expect the Foxp3^{neg} cells to display different phenotypes between the two donor populations in response to Treg cell depletion. However, if the Foxp3^{+} cells were in fact exerting a cell-extrinsic suppressive effect on their Foxp3^{neg} counterparts, we would expect both Foxp3^{neg} populations to behave similarly. If the latter were true, we would then investigate whether antigen-specificity plays a role in this effect by performing the same experiment using two populations bearing different Group 2 TCRs (Figure 18D), assuming the antigen specificity of the two TCR clonotypes are different.

Lastly, it is known that Foxp3 can be transiently expressed by activated effector T cells[242]. Therefore, careful phenotyping of Group 2 Foxp3^{+} cells would be required to confirm that these are indeed bona fide Treg cells (such as expression of CTLA-4, Helios, Nrp-1, production of IL-10 and TGF-β, epigenetic profiling at Treg-associated genetic loci, and capability to exert suppression *in vitro*). As Treg cells have been recently described to also harbor other functions, such as promoting tissue repair, it may be interesting to evaluate whether Group 2 Treg cells express the ST2 receptor for the alarmin cytokine IL-33, and whether they

produce amphiregulin in response to tissue damage[243-246].

Polyclonal Bcl6^{hi}CD69^{hi}PD-1^{hi} cells in the endogenous repertoire

Our study was predicated on a goal to determine the frequency of self-reactive specificities that exist in the endogenous repertoire. Based on our findings that self-reactive Group 2 clones exhibit Tfh-like characteristics (with expression of Bcl6 and PD-1 being the most pronounced), it is of interest to us to determine the frequency, phenotype, specificity, function, and regulation of the Bcl6^{hi}CD69^{hi}PD-1^{hi} polyclonal population that exists in mice at steady state. Preliminary assessment of the CD4^{+} Foxp3^{neg} Tconv cell compartment in unmanipulated wildtype mice revealed that ~3% of the spleen harbor Bcl6^{+} cells within the endogenous repertoire (Figure 15A). The presence of Tfh cells reactive to microbial antigens has been well-described in the Peyer's patches at steady state[208]. These sites harbored ~23% of Bcl6^{+} cells within the CD4^{+} Foxp3^{neg} Tconv cell compartment, which exhibited a higher resemblance to canonical Tfh cells in regards to robust expression of PD-1, CXCR5, and ICOS (Figures 15B and 15C).

In contrast, Bcl6^{+} cells identified in the spleen display a lower fluorescence intensity of Bcl6, and only a fraction of these cells exhibit expression of PD-1, CXCR5 and ICOS (Figures 15B and 15C), consistent with our observations of Group 2 TCRrg clones (Figure 10). This could be explained in at least three ways: 1) the Bcl6^{+} cells identified in the spleen are pre-Tfh cells that transiently upregulated expression of Bcl6 upon engagement with self-pMHC displayed on DCs (first phase of Tfh differentiation), but require stable interaction with cognate B cells in order to fully commit to the Tfh lineage (second phase of Tfh differentiation), 2) the Bcl6^{+} cells identified in the spleen are "memory" Tfh cells that retain expression of Bcl6 and

PD-1, but downregulate expression of CXCR5 and ICOS as they circulate the body, or 3) although this population expresses the Tfh master regulator Bcl6, it exists as a separate lineage from conventional Tfh cells and serves a novel function that remains to be defined. These scenarios can be tested preliminarily via adoptive transfer of Bcl6-expressing T cells into secondary wildtype hosts or B cell-deficient hosts to determine the stability of this phenotype, and to determine the extent to which Bcl6-expressing cells are committed to the Tfh-lineage.

To confirm whether the endogenous $Bcl6^{hi}CD69^{hi}PD\text{-}1^{hi}$ polyclonal population is representative of Group 2 clones identified in our study, we can perform a number of analyses. First, we could sequence the TCRα chains of this population isolated from fixed TCRβtg mice. We would expect to identify our Group 2 clones in this population, and not in the $Bcl6^{lo}CD69^{lo}PD\text{-}1^{lo}$ population. Second, if we performed *in vitro* stimulation of the $Bcl6^{hi}CD69^{hi}PD\text{-}1^{hi}$ polyclonal population, we would expect that these cells would proliferate upon co-culture with splenic $CD11c^{+}$ DCs in the presence of IL-2, since many of these specificities may be ubiquitously self-reactive. In addition, these cells should proliferate when co-cultured with germ-free DCs, but not when co-cultured with MHCII-deficient DCs, H2-DM-deficient DCs, or when MHC class II-blocking antibody is added.

We do not expect these cells to produce effector cytokines at steady state, since Group 2 clones did not appear to do so in primary TCRrg hosts, but co-culture of $Bcl6^{hi}CD69^{hi}PD\text{-}1^{hi}$ polyclonal cells with polyclonal B cells may reveal whether these cells are capable of deleting B cells in a Fas-dependent manner, or whether these cells can provide B cell help *in vitro*. Additionally, immunofluorescence staining of endogenous $Bcl6^{hi}CD69^{hi}PD\text{-}1^{hi}$ cells in the spleen and lymph nodes of unmanipulated wildtype mice may reveal whether they localize to distinct B cell follicles in a similar fashion as that observed in Group 2 primary TCRrg hosts. Furthermore,

it may be of interest to evaluate whether these cells play an important role in promoting the proper formation of white pulp in the spleen by co-transferring these cells with polyclonal B cells into Rag-deficient hosts (compared to the co-transfer of polyclonal B cells with a $Bcl6^{lo}CD69^{lo}PD\text{-}1^{lo}$ population). Lastly, it will be important to evaluate: 1) whether the $Bcl6^{hi}CD69^{hi}PD\text{-}1^{hi}$ population possesses a higher capacity to infiltrate non-lymphoid organs upon systemic host Treg cell depletion, compared to the $Bcl6^{lo}CD69^{lo}PD\text{-}1^{lo}$ population, and 2) how these cells respond to treatment with PD-1 blockade.

Group 1 clones

As described in the previous chapter, all of our available data suggest that Group 1 TCRs do not exhibit self-reactivity in adult mice (Figures 2-4). At the present time, it remains an enigma why the clones in this category were recurrently detected in the prostate following Treg cell ablation. A few possibilities can be hypothesized as follows. First, fulminant systemic immune activation may have lowered the threshold required for TCR stimulation and organ-infiltration, or revealed previously sequestered self-antigens. This possibility does not seem likely because a) *in vitro* stimulation of Group 1 clones with DCs co-cultured with prostatic extracts isolated from Treg-depleted males was not able to stimulate these cells, and b) adoptive transfer of Group 1 clones into Treg-depleted hosts did not recapitulate observable infiltration into organs (Figure 17C). Second, these clones may have infiltrated non-lymphoid organs as passengers. Alternatively, these sequences may have been expressed as a secondary TCR α-chain in driver clones in which allelic exclusion was not complete. These also do not seem likely, since these clones were detected at high frequencies recurrently across multiple mice. Therefore, the infiltration of these clones does not seem TCR-independent or random. Third, these TCRs may

require certain post-translational modifications that could not be recapitulated through the expression of engineered TCRs via the retroviral system. And lastly, perhaps these cells are innate-like but require development at low precursor frequencies to acquire the proper phenotype and capabilities. It would be of interest to phenotype Group 1 clones for markers associated with innate-like cells.

Conclusion

Based on our findings in this study, we present a model to describe the nature of a subset of self-reactive, MHC class II-restricted $CD4^+$ αβ Tconv cells in the endogenous repertoire that possesses reactivity to widespread self-antigens. These cells undergo positive selection in the thymus ($CD5^{hi}CD69^{hi}$) and upregulate phenotypes characteristic of negative selection ($DP^{dull}PD\text{-}1^{hi}$), but many escape clonal deletion. Upon migration out of the thymus as mature $CD4^+$ αβ Tconv cells, these cells circulate throughout secondary lymphoid organs as antigen-experienced $CD69^{hi}$ $Bcl6^{hi}$ $PD\text{-}1^{hi}$ T follicular helper-like cells, and are not peripherally deleted. Depending on the frequency of these cells within the repertoire, many also adopt an $FR4^{hi}CD73^{hi}$ phenotype in the periphery and express the proliferation marker Ki67. Furthermore, in the spleen, these cells localize toward—and often within—primary B cell follicles of white pulps. Although these clones harbor a Tfh-like phenotype, they do not appear to promote aberrant activation of autoreactive B cells, spontaneous germinal center formation, or production of IL-21 or other effector cytokines at steady state. Following systemic Treg cell depletion, many of these self-reactive clones infiltrate multiple non-lymphoid organs, and are not restricted to a single tissue site. Further, a proliferating population of $Foxp3^+$ Treg cells emerges within this subset of self-reactive clonotypes.

Future systematic dissection of this subset of self-reactive Tfh-like cells may reveal whether these cells serve any function in immunity or homeostasis to warrant an evolutionary benefit to maintaining these cells in the peripheral repertoire. In addition, because these cells are self-reactive and have the capacity to infiltrate non-lymphoid organs under certain immunological conditions, an understanding of the mechanisms that lead to their activation may shed light on the processes underlying certain autoimmune diseases. Given that these self-reactive clones exist as Tfh-like cells at steady state, they are especially relevant to the study of diseases that manifest as a result of autoantibody production caused by dysregulated T follicular helper cells and their corresponding B cell counterparts. These processes may hold wide-reaching implications to human autoimmune diseases that include systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, type I diabetes, and many more.

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