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IDENTIFICATION AND CHARACTERIZATION OF REGULATORY T CELLS THAT RECOGNIZE NATURAL EPITOPES

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List of Abbreviations

Aire	Autoimmune regulator
APC	Antigen presenting cells
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome
BAC	Bacterial artificial chromosome
BMC	Bone marrow chimera
CDR3	Complementarity determine region
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DN	Double negative
DP	Double positive
EAE	Experimentally induced autoimmune encephalomyelitis
Foxp3	Forkhead box P3
IPEX	immune dysregulation polyendocrinopathy enteropathy X-lined syndrome
iTreg	Induced regulatory T cell
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MOG	Myelin oligodendrocyte glycoprotein
mTEC	Medullary thymic epithelial cell
Nrp-1	Neuroplin-1
PGE	Promiscuous gene expression

PLZF	Promylocytic leukemia zinc factor
pMHC	peptide-major histocompatibility complex
pTreg	Peripherally derived regulatory T cell
Rg (rg)	Retrogenic
SP	Single positive
Tconv	Conventional T cell
TCR	T cell receptor
TEC	Thymic epithelial cell
Tg (tg)	Transgenic
TRA	Tissue restricted antigen
Treg	Regulatory T cell
tTreg	Thymicly derived regulatory T cell

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Abstract

Regulatory T (Tregs) cells expressing the transcription factor Foxp3 play an important role in tolerance and prevention of autoimmunity. Thus, there is a need to understand their molecular mechanisms of development and homeostasis, yet the role of antigen recognition by Tregs remains incompletely defined due to the gap in knowledge of natural Treg ligands. Here we identify natural Aire-dependent ligands recognized by endogenous prostate-associated Tregs. We found that recurrent Treg cell clones from independent sequencing studies recognize distinct non-overlapping peptide epitopes derived from the same prostate-specific protein. Here, we investigate the role of cognate antigen expression in the thymus and periphery through the study of T cells reactive to a natural Aire-dependent peptide ligand in wild-type and gene-targeted mice lacking this epitope. Previous evidence suggests that Aire can promote both clonal deletion of antigen-specific T cells and the differentiation of such cells into the Foxp3 regulatory lineage. We demonstrate that antigen expression in the thymus does not impact clonal deletion of polyclonal antigen-specific T cells, but instead is required to direct a substantial number of antigen-specific T cells into the Treg lineage. Further, we show that this skewing to the Treg fate is required to prevent T cell infiltration of the prostate in the absence of inflammation. In all, our data demonstrates that Aire-dependent expression of endogenous self-peptide in the thymus restricts organ-specific autoimmunity by skewing antigen-specific cells to the Treg lineage.

Chapter I: Introduction

The adaptive immune system has the distinct ability to generate diverse receptors to mount a response against potential pathogens. In T cells, this diversity is generated during development through the stochastic rearrangement of germline encoded gene segments to produce the T cell receptor (TCR). While this provides a mechanism for protection against immunological insults encountered throughout life, it also creates the potential of generating autoreactive T cells. Thus, there is a critical need to control or prevent self-reactivity in order to maintain immune tolerance, defined by a state of unresponsiveness or non-reactivity toward a substance that would normally elicit an immune response. Breakdown of tolerance is associated with numerous autoimmune diseases such as lupus, diabetes, and rheumatoid arthritis. The immune system employs several mechanisms that collectively operate to prevent inappropriate responses to self. For example, Foxp3-expressing regulatory T cells (Tregs) are central to the prevention of autoimmunity by exerting dominant tolerance over potentially pathogenic T cells[1]. However, the suppression of T cell responses by Treg cells can have detrimental effects in the settings of cancer or infection. Thus, understanding the development and homeostasis of these cells is essential. A major barrier in the field has been the knowledge of the natural antigens recognized by endogenous Tregs that drive their thymic development and maintenance in the periphery throughout life.

The T cell receptor is a heterodimeric protein composed of two unique polypeptide chains, linked by a disulfide bond[2]. The majority of T cells express TCRs consisting of

an α and β chain. A minor population of T cells express TCRs made up of γ and δ chains; these cells have unique immune functions and selection requirements. All $\alpha\beta$ T cells recognize peptide antigens in the context of major histocompatibility molecules (MHC). CD8 T cells recognize peptide antigens presented by MHC class I molecules, that present short peptides (9-11 amino acids in length) that are largely derived from cytosolic proteins transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) protein complex[3]. CD4 T cells on the other hand, recognize longer peptide antigens presented by MHC class II molecules that are derived from proteins acquired through the process of endocytosis or phagocytosis that are present within the endosomal or lysosomal compartments. Recognition of the peptide-MHC (pMHC) complexes by the TCR is in part facilitated by the coreceptors, CD4 and CD8, that bind to conserved determinates on their respective MHC molecules.

$CD4^+$ and $CD8^+$ T cells get activated in the periphery upon encounter with cognate pMHC molecules expressed by activated dendritic cells (DC). T cell activation requires 2 signals: TCR-pMHC engagement and binding of CD28 on the T cell with B7-1 (CD80) or B7-2 (CD86) expressed on a DC or another antigen presenting cell (APC). Activation of naïve T cells occurs within secondary lymphoid organs resulting in effector T cells that are able to enter tissue sites. Upon activation, $CD8^+$ cells develop into cytotoxic T cells and typically target cancerous cells or cells infected with viruses. $CD4^+$ cells become helper T cells and express cytokines that help activate other immune responses, including B cells, eosinophils, and macrophages. For most $CD4^+$ and $CD8^+$ cells, activation is triggered in response to pathogen-derived peptides, however, the stochastic nature of

TCR gene recombination results in the generation of autoreactive TCRs. A T cell expressing a self-reactive TCR can become activated in the periphery if it encounters cognate pMHC on activated APCs resulting in attack of host tissues. Thus, there is a requirement for the immune system to prevent the emergence or activation of self-reactive T cells.

T Cell Tolerance

The immune system has evolved several mechanisms to prevent the potentially deleterious response that self-reactive T cells can have throughout the body. These mechanisms include deletion, T cell ignorance, generation of Tregs, and induction of anergy, a state of T cell unresponsiveness. Tolerance mechanisms can broadly be broken down into dominant (Treg-mediated) and recessive (deletion or functional inactivation) tolerance. Both dominant and recessive tolerance operate in the thymus, through generation of thymic Treg cells (tTregs) and negative selection of autoreactive cells, respectively. These will both be discussed in detail below. Additionally, peripheral tolerance through induction of anergy, generation of peripherally induced Tregs (pTreg), peripheral deletion, and immunological ignorance work collaboratively to prevent autoimmunity.

Immunological ignorance occurs when T cells bind to self antigens at very low affinity or rarely encounter antigen resulting in an absence of stimulation *in vivo*. However, if these cells encounter a high level of antigen, they can be induced to respond. Peripheral tolerance mechanisms act as a second line of defense against self-reactive cells that

escape negative selection in the thymus. Anergy is the process by which lymphocytes are rendered unable to respond to stimulation because of previous encounter with strong antigen in the absence of costimulation (i.e. no CD28/B7 engagement)[4]. Though these cells are unresponsive to stimulation, they can persist in the periphery long term. Interestingly, a recent study found that $CD4^+$ anergic cells that expressed CD73, FR4, and Neuroplin-1 (Nrp-1) could convert to pTregs, linking anergy and Treg-mediated tolerance[5]. Peripheral deletion can also occur in response to strong TCR signaling with insufficient costimulation. Often this comes in the form of abortive proliferation, in which T cells initially undergo a few rounds of proliferation, but are not maintained and are deleted from the repertoire[6].

Recognition of self antigen can lead to the induction of anergy or deletion due to strong TCR signaling, alternatively, it can lead to the development of both tTregs and pTregs. However, what drives the development of Tregs as opposed to deletion or anergy is not well defined. Thus, there is a critical need to identify the natural self ligands recognized by Tregs to determine the differences that induce these cell fates.

Identification of Regulatory T Cells

Tregs, constituting approximately 10% of the peripheral $CD4^+$ pool, were first reported by Sakaguchi and colleagues[1]. Seminal work defining these cells used the expression of the high affinity interleukin 2 (IL-2) receptor α chain (CD25) as a cell surface maker differentiating Tregs from conventional T cells (Tconv). The requirement for these cells was shown by transfer of bulk splenocytes into BALB/$c^{nu/nu}$ mice; recipient mice

developed multi-organ autoimmunity only when $CD4^+CD25^+$ cells were depleted prior to transfer, indicating that $CD25^+$ cells have the ability to suppress $CD25^-$ cells and prevent organ infiltration in a dominant fashion. Subsequent studies by Sakaguchi showed that neonatal thymectomy of 3 day old mice led to a similar multi-organ autoimmune disorder as was observed in the transfer studies[7]. Further, they showed that $CD25^+$ cells emerge from the mouse thymus starting at day 3, suggesting that the resulting autoimmune disease was associated with the absence of $CD25^+$ cells. Transfer of $CD25^+$ cells into neonatal mice immediately after thymectomy prevented development of autoimmunity indicating that these cells were the preventers of autoimmunity[7].

Hemizygous male scurfy mice succumb to a fatal autoimmune disease within 3 weeks of life[8]. This mouse line arose as a spontaneous mutation in 1949 and was the first X-linked disease reported in mice that caused a lethal phenotype[9]. These mice are characterized by the development of a severe lymphoproliferative disorder mediated by $CD4^+$ T cells. Similar to the disease observed in scurfy mice, the human immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome results in the development of multisystem autoimmunity including early onset type 1 diabetes mellitus, atopy, and food allergies, characterized by a hyper Th2 ($CD4^+$) T cell phenotype[10].

The Foxp3 protein, originally named scurfin, was first mapped to a 500-kilobase region identified by 4 overlapping bacterial artificial chromosomes (BACs). RT-PCR of thymic RNA from scurfy and wildtype mice revealed a 2-bp insertion in the coding region of the Foxp3 gene that results in a truncated protein[11]. At the same time, the IPEX disease was mapped to loss-of-function mutations in the FOXP3 gene[10,12,13]. Together these

results indicated that Foxp3 could be important in immune homeostasis. Foxp3 (Forkhead box P3) is a member of the forkhead/winged-helix family of transcriptional regulators that is present on the X-chromosome.

The cloning of *Foxp3* enabled further research on the development and function of Tregs that had previously been hindered due to the use of CD25 expression as a maker of Tregs. While Tregs are characterized by high expression of CD25, it is also expressed on all activated T cells to some extent[14], requiring a better method of identification of Tregs. Subsequent studies showed that Foxp3 was expressed at high levels specifically in the $CD4^+CD25^+$ T cell subset, and to a much lower degree in $CD8^+$ and $B220^+$ cells, linking this transcription factor to the Treg cell phenotype[15,16]. Targeted deletion of several exons in the Foxp3 locus enabled loss-of-function studies that demonstrated loss of Foxp3 expression leads to an activated T cell phenotype and a total loss of suppressive function[16]. Similar to the rescue of thymectomized mice[7], transfer of $CD4^+CD25^+$ cells into scurfy mice eliminated the lethal autoimmunity normally observed, indicating the scurfy phenotype results from a deficiency in $CD4^+CD25^+$ cells and not a cell-intrinsic defect in $CD4^+CD25^-$ cells[16]. Further, the forced expression of Foxp3 by retroviral transduction into Tconv cells is sufficient to confer a suppressive phenotype, indicating that Foxp3 is the master regulator of the Treg cell program[15,16].

Tregs are required throughout life to maintain tolerance as demonstrated by chronic depletion of $Foxp3^+$ cells in mice expressing the $Foxp3^{DTR}$ gene. Administration of the diphtheria toxin results in deletion of all cells expressing Foxp3 which results in multi-organ autoimmunity and death within 14 days[17]. The development of fatal

autoimmunity in the absence of Tregs highlights the dominant role these cells play in maintaining tolerance.

The important role that Treg cells play in maintaining tolerance lead to interest in the development, function, and maintenance of these cells to understand how they can be used to prevent autoimmunity or be inactivated during cancer or infections. However, a limiting factor in understanding these cells has been the nature of the ligands that Treg cells recognize within the body.

Mechanisms of Treg Suppression

As described above, it is well appreciated that Tregs play a critical role in suppressing activation of Tconv cells. This suppression is indispensable in preventing autoimmunity driven by pathogenic Tconv cells, however, this can also prevent the immune system from mounting a strong response to tumors. For this reason, understanding how Tregs prevent activation of other cells is critical. Many potential mechanisms of Treg suppression have been defined through *in vitro* studies[18]. These include secretion of inhibitory cytokines or cytolytic molecules, direct cell-cell contact, and indirect suppression by preventing maturation of DCs. Importantly, the requirement for any one mechanism *in vivo* remains unknown.

Secretion of the inhibitory cytokines IL-10 and TGF-β[19], and recently described IL-35[20] can act on Tconv cells to prevent helper T cell function. Tregs preferentially express IL-35, however, other immune cells including $\gamma\delta$ T cells, $CD8^+$ T cells, and APCs may also express IL-35[20]. In addition to secretion, TGF-β has been shown to be tethered to Tregs

cells facilitating suppression of Tconv in a contact dependent manner[18]. Several studies have shown Tregs can induce apoptosis of effector T cells or APCs by secretion of granzyme-A and granzyme-B as well as perforin[21,22]. Tregs have also been shown to induce apoptosis due to cytokine deprivation. In this model, the high expression of CD25 on Tregs acts as an IL-2 sink preventing effector T cells' access and limiting expansion of these cells[23]. Additionally, the expression of the ectoenzymes CD39 and CD73 that convert AMP to adenosine, has been shown to play a role in inhibiting effector T cell function through the expression of the adenosine receptor on Tconv cells[24].

DCs are important mediators of the immune response. Thus, the ability of Tregs to act on DC function could play an important role in effector T cell responses. Cytotoxic T lymphocyte associated molecule-4 (CTLA-4), is constitutively expressed on Tregs. CTLA-4 binds CD80 and CD86 (B7-1 and B7-2) expressed by DCs with high affinity. This interaction may prevent activation by limiting access of CD28 on the naïve effector cells to the necessary costimulation by CD80/CD86 on the DC by occupying the binding site, or by the direct removal of these receptors by transendocytosis[25,26]. Interestingly, CTLA-4 was shown to be required for proper immune control before its role on Tregs was realized[27,28] because of its role in regulating Tconv responses after activation. Deletion of CTLA-4 lead to systemic autoimmunity and death of mice within 3 weeks of birth, similar to that of the scurfy mice. This result could have been due to the inability to regulate effector T cell responses, or due to the loss of regulatory T cell function, as it was later shown that expression of CTLA-4 is required for Treg function and development[26].

In addition to directly inhibiting effector T cell functions, adenosine, generated by CD73 and CD39, can impact DC maturation, thus promoting a more tolerogenic phenotype. A third mechanism by which Tregs can limit DC maturation is ligation of LAG3, a CD4 homolog, with high affinity for MHC-II molecules[29]. The high affinity interaction of LAG3 with MHC-II prevents effector T cell activation by both blocking access to MHC-II and inducing an inhibitory signal in the DC[30]. Finally, Nrp-1, expressed by Tregs to varying degrees, has been shown to increase Treg-to-DC contact time[31].

Heterogeneity of Treg cells likely contributes to the difficulty in elucidating the necessary mechanisms for suppression. This phenotypic heterogeneity is demonstrated by the different targets of pTregs and tTregs as well as the differences of Tregs found in lymphoid tissues versus non-lymphoid tissues, as described by work in the Campbell lab[32]. The functional differentiation of Tregs allows for restraint of particular types of immune responses, such as the requirement for T-bet expression in Tregs to prevent Th1 mediated inflammation[33]. These findings indicate that additional transcriptional programs can be used in coordinating suppression of distinct effector T cells.

Given the fact that many of these mechanisms have been defined *in vitro*, the extent to which each of these processes controls Treg cell suppression *in vivo* is still largely unknown. It is likely that different microenvironments and stimuli play a role in shaping the response of Tregs. Further, multiple mechanisms of suppression may act concurrently to prevent autoimmunity or inhibit tumor immunity, however, specific programs may be required at different sites. For example, the selective loss of IL-10 in Tregs leads to reduced Treg function, with deleterious results in the gut, but does not

result in systemic autoimmunity like that seen in scurfy mice. Importantly, the role of antigen in driving these responses is largely unknown. The limited overlap in TCRs expressed by Tregs and Tconv cells[34] further begs the question of how targeted suppression by Tregs occurs, while limiting collateral damage.

Generation of T Cell Receptor Diversity

Unique receptors form during thymic development by V(D)J recombination within the TCRα and TCRβ loci. The human TCRα chain locus is comprised of variable (V) genes, joining (J) genes and one constant (C) gene. The TCRβ chain locus is similarly comprised of V and J genes, as well as 2 diversity (D) gene segments and 2 C genes. The random selection of each V(D)J gene and imprecise joining of these by the RAG1/RAG2 recombinase generates diversity in the TCR at the complementarity determining region (CDR3), the primary region of antigen recognition. TCRs are further diversified in the CDR3 region during recombination by the addition or deletion of N-nucleotides at the junctional site of each gene segment during the joining process leading to different antigen recognition by each TCR. Importantly, during TCR rearrangement, TCRβ chains are rearranged first. Once a TCRβ is successfully expressed the locus is closed establishing allelic exclusion. Next the TCRα chain undergoes rearrangement to produce a fully functional $\alpha\beta$TCR.

$\alpha\beta$TCRs recognize their peptide antigens in the context of MHC molecules with a conserved docking mode. The TCR binds the pMHC complex with a diagonal orientation in the range of 44-115˚. In this setting, the CDR3 loop, where diversity is the greatest, is

located over the peptide contributing to ~30-70% of the buried surface area, while the other more conserved regions of the TCR contact the MHC molecule[2,35]. However, Tconv cells bearing TCRs associated with autoimmunity have been shown to exhibit unusual docking orientation[35,36]. Thus, it is important to determine the natural ligands recognized by Tregs to determine if they interact with pMHC molecules using the conserved docking mode, or if they use a structurally distinct configuration that may influence thymic development or suppressive functions *in vivo*.

The diversity generated during the recombination process can produce approximately 10^{15} unique $\alpha\beta$TCRs in humans. However, this far exceeds the number of T cells in a human body (10^{12}). As there cannot be more TCR specificities than T cells in the body, the actual diversity found in humans is far lower than the theoretical possibility. The TCR repertoire is shaped by positive and negative selection within the thymus, which limits diversity. Additionally, pathogen encounter leads to the generation of memory cells that are clonally expanded, further limiting the actual diversity found. The peripheral $\alpha\beta$TCR diversity in humans is estimated at 2.5 x 10^7 naïve TCRs and 1-2 x 10^5 memory T cells[37]. Similarly, the mouse TCR repertoire has been estimated at 2 x 10^6 unique TCRs, among 1-2 x 10^8 total T cells. Though the actual diversity in the repertoire is far lower than the theoretical calculations, the diversity present still allows for the generation of immune responses against pathogens and the potential to respond to self antigens. The process of T cell selection is required to educate T cells to respond appropriately and quell the generation of autoreactive T cells.

T Cell Selection

T cell selection is the process by which T cells are educated within the thymus. Selection of αβ T cells largely results in the generation of CD4 and CD8 T cells. Classically, T cell selection is characterized by a two step process – positive selection, which ensures that developing thymocytes express receptors that can recognize antigens presented by self MHC molecules, followed by negative selection (also called clonal deletion). These selection steps occur in a temporal manner and in spatially distinct regions of the thymus. Positive selection occurs in outer, cortex region, while negative selection mainly occurs within the inner, medullary region of the thymus. These distinct regions of the thymus contain discreet cell populations that play critical roles in driving selection and deletion.

TCR rearrangement starts during the double negative (DN) stage of T cell development. Successful recombination of the TCRα and TCRβ loci results in the expression of the mature TCR in combination with both CD4 and CD8 coreceptors during the double positive (DP) stage. Within the cortex, DP thymocytes encounter pMHC complexes presented by cortical thymic epithelial cells (cTECs) and undergo positive selection if they are able to recognize self MHC molecules or death by neglect if they are unable to recognize self. Cells that are positively selected commit to either the CD4 or CD8 T cell lineage and migrate to the medulla as single positive (SP) cells due to expression of the chemokine receptor CCR7[38]. Within the medulla, CD4SP and CD8SP cells associate with medullary thymic epithelial cells (mTECs) and DCs that express high levels of costimulatory molecules and host ligands. Upon encounter with self ligands in

the medulla, T cells that bear a self-reactive TCR can undergo clonal deletion or be directed into the Treg lineage. Importantly, the factors driving these alternative fates are unknown, highlighting a need to define natural Treg ligands.

Originally, thymic deletion was thought to be dependent upon encounter with superantigens that cross-linked TCRs with MHC molecules independently of the peptide expressed by the MHC[39]. However, further studies on clonal deletion indicated that T cell recognition of specific pMHC complexes could drive T cell deletion. This was first shown to occur in mice that expressed a transgenic TCR specific for the male H-Y antigen[40]. In this system, TCRs specific for the H-Y antigen were deleted in the thymus of male mice, but not female mice. The authors found that the reduced cellularity in the male thymus was due to a dramatic reduction in $CD4^+CD8^+$ DP cells with a high frequency of DN cells, leading to the conclusion that deletion occurs during the DP phase of T cell development. However, cells expressing the TCR specific for the H-Y antigen were found in the lymph nodes of transgenic males, indicating that negative selection is an imperfect process[40]. A subsequent study using the same TCR found that clonal deletion in male mice occurred at the single positive stage within the medulla[41]. This discrepancy is attributed to inappropriate expression of the TCRα chain during the DN stage of thymocyte development in the original transgenic model, as opposed to expression of the TCRα chain during the DP stage in the latter study, stressing the importance of correct expression of the TCR during selection.

The individual contributions of positive and negative selection on the T cell repertoire remain controversial. One study attempted to quantify the number of T cells

undergoing negative selection. Mice lacking the proapoptotic molecule Bim (bcl2l11)[42] were analyzed as a method for tracking cells that should have undergone deletion. The *Bim*$^{-/-}$ mice also expressed Nur77^{GFP}. Nur77 is an early gene up-regulated in response to TCR stimulation[43]. Thus, GFP expression correlated with TCR signal strength in all T cells[44]. Using these mice, the authors found that ~65% of T cells in the thymus get deleted at the DP stage, with a minor fraction of cells undergoing clonal deletion at the SP stage[45]. This was an early indication that far fewer cells get positively selected than undergo antigen-mediated deletion in the thymus, suggesting that clonal deletion may not represent a major mechanism of tolerance. Further, this study showed that the cells that get deleted were largely GFP high, indicating that deletion was due to strong TCR signaling, consistent with the affinity hypothesis discussed below. A more recent study used an unbiased screen of preselection TCRs to determine the contribution of positive and negative selection[46]. These authors found that the vast majority (85%) of cells do not undergo selection but instead die by neglect. Of the 15% of TCRs that get selected, half of these undergo negative selection. Unexpectedly, this study showed that negative selection largely targets TCRs that are crossreactive to different MHC haplotypes and classes, opposed to the classic model of pMHC driven deletion.

Despite clear evidence that some self-reactive T cells are eliminated in the thymus by clonal deletion, a substantial body of evidence suggests that the removal of auto-reactive T cells from the endogenous repertoire is an incomplete process. This is evident in the systemic autoimmunity that is induced by chronic ablation of Tregs[17] indicating that autoreactive T cells are present in the repertoire, but normally kept in check by Tregs.

Additionally, several groups have used pMHC tetramers to show the presence of autoreactive T cells in the periphery. For example, MHC-I tetramers specific to the male Smcy3 antigen showed that between 25-40% of self-reactive clones escape deletion in male mice[47]. Similarly, a report using tetramers against the human SMCY antigen demonstrated that the frequency of tetramer specific $CD8^+$ T cells in males was only 3-fold lower than in females who don't express the antigen. This study showed that these cells were as frequent as naïve $CD8^+$ T cells that recognize foreign antigens[48]. Self-reactive $CD4^+$ T cells have also been identified using pMCH-II tetramers. One such study used mice, termed Act-2W, that were engineered to ubiquitously express the model antigen 2W1S. By comparing the frequency of $tetramer^+$ cells within WT mice that lack expression of the antigen and Act-2W mice, the authors demonstrated that ~30% of cells escaped clonal deletion[49]. Altogether, these studies show that negative selection is an imperfect process and indicate that additional tolerance mechanisms are needed to prevent the activation of these cells in the periphery.

The recognition of agonist ligands in the thymus can drive clonal deletion or the differentiation of T cells into "innate-like" lineages, such as NKT cells and CD8$\alpha\alpha$ IELs, or direct tTreg development. NKT cells represent a unique subset of $\alpha\beta$ T cells selected by ligands presented by the non-classical MHC-I like molecule CD1d. NKT cells have innate-like properties and express the invariant chain Vα14-Jα18 in mice and Vα24-Jα18 in humans. Their unconventional selection also requires the expression of the transcription factor promyelocytic leukemia zinc finger (PLZF)[50], but does not require coreceptors. CD8$\alpha\alpha$ IELs were recently found to derive from cells undergoing negative

selection in response to strong interactions with self ligands, demonstrated by elevated TCR signaling hallmarks including CD69, PD-1, and Nur77[51]. Retrogenic TCRs used in this study were selected from cells within the $CD4^{lo}CD8^{lo}$ (DP^{dull}) pool of cells that have previously been shown to be undergoing negative selection. This is consistent with previous work that showed CD8αα IELs were the result of thymocytes that had escaped negative selection[52]. NKT and CD8αα IELs have been shown to derive from cells with high TCR signaling. Generation of Tregs, which will be discussed in depth below, also occurs in response to agonist selection, however what drives negative selection versus Treg generation is still undefined.

Altogether, these data emphasize the need to identify the natural antigens recognized by Tregs, so the extent TCR signaling plays in Treg development and clonal deletion can be determined.

Models of T Cell Selection

The affinity model of thymocyte selection is a common paradigm in the field describing the potential impact of TCR affinity for pMHC complexes in driving T cell development. According to this model, high-affinity TCR-pMHC interactions result in negative selection by apoptosis, whereas low level TCR-pMHC interactions protect cells from death by neglect and promote positive selection. In this model, cells that undergo agonist selection, such as NKT, CD8αα, and Tregs, have strong TCR-pMHC signaling, but undergo lineage diversion instead of deletion. Thus, in this model, Tregs express TCRs that have higher affinity interactions than cells that are positively selected, and

lower or similar affinity as cells that get clonally deleted[53]. What drives the stochastic overlap in affinity that directs Treg development as opposed to clonal deletion is unknown. Importantly, however, the affinity of natural Treg TCRs for their cognate ligands has never been measured, resulting in an incomplete picture of TCR affinity in selection.

Since NKT, CD8$\alpha\alpha$, and Tregs all undergo agonist selection, the Nur77^{GFP} reporter mouse, described above, was generated to study the affinity of TCR signaling in polyclonal T cells[44]. Based on studies with these mice, Tregs express higher levels of GFP in the thymus, indicating they perceive strong TCR signals[44]. This finding was extended using *Bim$^{-/-}$ Nur77^{GFP}* mice[45] which demonstrated Tregs and cells rescued from deletion had similarly high GFP expression, suggesting that both Tregs and cells undergoing deletion receive strong TCR signaling. While these studies of polyclonal Tregs support the affinity model, a large body of data supporting this hypothesis has been derived from studies of double transgenic mice expressing TCR transgenes and cognate antigen in the thymus[54-58].

Early double transgenic model systems showed that expression of cognate ligand in the thymus was sufficient to induce some Treg development in cells that expressed high affinity TCRs[56]. The TCR affinity was important in these models, because when a second transgenic TCR of low affinity was used, the same ligand did not drive Treg development. Additionally, the cells with high affinity TCRs, but not low affinity TCRs expressed high levels of CD69 and CD5, indicating that Treg induction occurs in response to strong TCR signaling[56]. A study using tetracycline regulated expression of antigen in the thymus showed that both Treg generation and negative selection occur concurrently

when cells are exposed to high levels of antigen in the thymus[58]. Further, the authors of this study showed that increasing the expression of antigen did not result in increased numbers of $CD4^+CD25^+$ cells. Instead, an increase in the frequency of $CD4^+CD25^+$ cells in relation to the $CD4^+CD25^-$ cells was observed. Based on this, the authors concluded that Treg cells are more resistant to deletion than Tconv cells to agonist ligand encounter, leading to the appearance of Treg differentiation[58]. However, a later study found that Tregs and Tconv cells that have high affinity TCRs for the same antigen are deleted with similar efficiency, indicating that there is no cell-intrinsic bias promoting survival of $Foxp3^+$ Tregs[55].

While these studies indicate that affinity of the TCR for pMHC is important in driving T cell fate, these studies did not directly measure the affinity of the TCRs used. One major weakness of the use of the double transgenic mouse models is that different promoters were used to drive the expression of antigen in different systems[58]. Thus, different levels of antigen expression in each model (i.e. ubiquitous versus Aire-dependent) could play a role in directing T cells to undergo clonal deletion or diversion into the Treg lineage.

A more recent report used TCRs specific for the model antigen OVA with a variety of different affinities[57]. TCR reactivity of these clones was measured by tetramer binding as well as response to antigen stimulation by hybridomas expressing the different TCRs. The authors found that Treg generation in response to antigen could occur over a wide range of TCR affinities, with more Tregs developing from high affinity TCRs compared to the low affinity TCRs[57]. Importantly, these results indicate that high affinity TCRs are

biased to the Treg lineage, while low affinity TCRs for the same antigen can escape deletion even if they are self-reactive.

The affinity model provides a framework to think of T cell selection, however, there are some critical limitations in the available evidence testing this concept. The use of model antigens as neo-self antigens, coupled with high affinity TCRs[54-58] may not recapitulate expression of natural Treg ligands. Furthermore, many of the TCRs used in these studies are not derived from naturally occurring Treg clones, but instead are derived from Tconv clones that do not routinely drive Treg generation[34,59]. The affinity of endogenous Treg TCRs has never been directly measured due to the gap in knowledge of the identity of the natural antigens sensed by Tregs. Tregs are hypothesized to be selected by agonist ligands, indicating high affinity TCR-pMHC interactions, but it is not known where the affinity falls within the affinity range of common TCRs reactive to agonist ligands (1 - 100 μM K_D)[60].

Without knowledge of the natural antigens sensed by endogenous Tregs, the affinity of Tregs cannot be determined, preventing a complete understanding of T cell selection.

Thymic Treg Development

Treg development in the thymus has clearly been established[7,61], yet the complete set of requirements for development remains elusive. As discussed above, TCR affinity and self-reactivity have been implicated as primary drivers of this cell fate[44,45,54,56,58].

However, without knowledge of the natural antigens recognized by tTregs, the molecular mechanisms driving Treg development can not be completely defined.

Two models of tTreg development have been proposed – the instructive model and the stochastic or selective model[59]. The instructive model, like the affinity model[53], relies on TCR affinity in driving Treg development. In contrast, the stochastic model proposes that Treg fate may be affected by TCR-independent factors prior to selection[62]. In this model, Foxp3 induction occurs stochastically at the DN stage of development, then coupled with high TCR reactivity leads to Treg differentiation in the presence of Foxp3 expression or clonal deletion in its absence[59].

Early studies of thymic Treg development demonstrated that expression of MHC molecules is required[63], suggesting that TCR-pMHC interaction is required for thymic development. These data, in combination with studies that showed development of Tregs by cells expressing high affinity TCRs in response to neo-self antigen expression in the thymus[54-58], indicate that TCR specificity is crucial for Treg development. Further support for the role of TCR specificity in tTreg development is demonstrated by the generation of transgenic mice bearing Treg TCRs identified though sequencing studies[64,65]. Initially, these studies showed that cells expressing Treg TCRs did not develop into Tregs in the thymus when crossed onto the RAG-deficient background. However, intrathymic injection and low frequency bone marrow chimera (BMC) experiments revealed that a proportion of these cells develop into Tregs when present at low clonal frequency, suggesting that antigen availability is a limiting factor in Treg development[64,65]. The results of these two studies showed that there is an inverse relationship between clonal frequency and Treg

development, with improved Treg development at low clonal frequencies. This relationship led to the niche hypothesis of Treg development which suggests that antigen availability is a limiting factor in Treg development. Additionally, these studies[64,65] showed that the number of Treg cells able to develop reaches a plateau, further indicating that antigen is a limiting factor and TCR affinity alone is not a sufficient indicator of Treg development[59].

Several studies using double transgenic mice attempted to determine the role of antigen expression in thymic development of Tregs. Jordan et al.[56] showed that expression of antigen in the thymus was sufficient to drive Treg differentiation in a proportion of TCR transgenic cells reactive to a model antigen. Several subsequent studies using double transgenic mice supported these initial observations[54-56,58]. One report attempted to directly visualize competition for high affinity ligands using a naturally occurring Treg TCR clone, G113, of unknown specificity[55] crossed to the Nur77^{GFP} reporter mouse[44]. The G113 cells were able to efficiently develop into Tregs at low frequencies and high GFP expression correlated with lower T cell frequency, suggesting these cells were competing for strong TCR interactions[44]. While antigen appears to be a key factor in tTreg development, there are antigen-independent factors that are important in tTreg development including the number and identity of APCs in thymus, expression of co-stimulatory factors CD80(B7-1) and CD86(B7-2), and the presence of the common gamma chain (γc) cytokines IL-2 and IL-15[59].

T cells encounter different microenvironments throughout development within the thymus. Further, APCs differ in identity and antigen presentation machinery, leading to

presentation of different antigens in each compartment. As T cells undergo development, they migrate from the cortex to the medulla, constantly surveying pMHC complexes throughout the process. The primary APC within the cortex, cTECs, express a unique subunit of the proteasome, β5t, referred to as the thymoproteasome, that plays a role in presentation of distinct MHC class I peptides[53]. cTECs also express two unique lysosomal proteases, cathepsin L, and the thymus-specific serine protease (TSSP) that lead to presentation of different MHC class II peptides[53]. In addition to the cTECs, SIRPα$^+$ CD8α^{neg} migratory dendritic cells (SIRPα$^+$ DCs) can present antigens acquired in the periphery to T cells within the cortex[53].

T cells that survive the cortex migrate to the medulla where they can encounter a different array of self antigens. This is facilitated by the promiscuous expression of tissue-restricted antigens (TRAs) by medullary thymic epithelial cells (mTECs)[66,67]. This promiscuous gene expression in mTECs is driven by the transcription factors Aire[68] and Fezf2[69]. In addition to expressing TRAs, mature mTECs express high levels of co-stimulatory molecules such as CD80[67] that are important for T cell selection. Both SIRPα$^+$ migratory DCs and thymic resident CD8α$^+$ DCs are present within the medulla and can acquire antigens from dead or dying mTECs. Importantly, two studies recently showed that both mTECs and bone marrow derived DCs are necessary within the thymus for proper selection of Aire-dependent T cell selection, though there may be a differential requirement for the SIRPα$^+$ and CD8α$^+$ DCs[70,71]. Integrating these data, the location of antigen encounter and presentation by the correct APC likely plays a role in driving deletion versus Treg generation.

The CD28/B7 axis has been shown to be critically important in the development of tTregs[72]. In the NOD mouse model of type 1 diabetes, deletion of B7 leads to an increased incidence of diabetes that can be controlled by transfer of $CD4^+CD25^+$ cells from control mice, indicating that $CD4^+CD25^+$ (Treg) cells require expression of CD80/CD86 for proper development[72]. Furthermore, CD80/CD86 expression by bone marrow-derived APCs is required for the development of the prostate associated Treg clone MJ23[71]. Further, $CD8\alpha^+$ DCs were dispensable for the development of the MJ23 clone, suggesting the identity of the APC inducing costimulation in the developing thymocytes is also important for Treg induction. Importantly, this study showed that transfer of Aire-dependent antigens to bone marrow derived DCs is required for the development of a tissue-specific Treg clone, indicating that both mTECs and DCs are required for proper Treg development[71].

The requirement for IL-2 and to a lesser extent IL-15 has been demonstrated by the enrichment of Treg precursors in the $Foxp3^-CD4^+CD25^+$ thymocyte population[59]. Providing IL-2 or IL-15 stimulation to these cells is sufficient to drive Foxp3 expression and lead to Treg development without further TCR stimulation[73]. Thus, it has been proposed that Treg development occurs in two steps: a TCR dependent (i.e. antigen dependent) step followed by a TCR independent (i.e. cytokine dependent) step.

Altogether, these data demonstrate the complexities of tTreg development. TCR recognition, coupled with antigen-dependent and -independent factors must harmonize to ensure the correct development of Treg cells in the thymus. Identifying natural ligands will provide a better understanding of how all of these factors work together to promote Treg development.

Promiscuous Gene Expression in the Thymus

Protection of distant organ sites from attack by autoreactive T cells is driven in part by expression of TRAs in the thymus. Expression of these antigens occurs exclusively within mTECs[66], and is driven in part by the transcription factor Aire[67,68,74]. In addition, recent work suggests a role for Fezf2 in promoting promiscuous gene expression in the thymus[69]. Loss-of-function mutations in *AIRE/Aire* lead to the autoimmune disease APECED (Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome) in humans, and lead to a similar organ specific autoimmunity observed in Aire-deficient mice[68]. In mice, this autoimmunity is characterized by T cell infiltration of target organs specific to the genetic background of the mouse strain[75] indicating that additional genetic factors play a role in Aire-associated autoimmunity. Deletion of Fezf2 in mTECs also results in targeted organ infiltration and autoantibody expression, though the target organs differ from those targeted in Aire-deficient mice[69]. The difference in targeted organs may be due the different structure of the transcription factors and resulting differences in gene regulation[67,69,74].

Early work suggested that Aire-dependent promiscuous gene expression enforces immune tolerance by driving the clonal deletion of thymocytes reactive to Aire-dependent self-peptides[76]. This hypothesis is supported by the findings that Aire-deficient mice produce comparable levels of $Foxp3^+$ cells that can suppress colitis when transferred into lymphopenic hosts. Furthermore, thymic grafting studies showed that co-grafting one $Aire^{+/+}$ thymus with an $Aire^{-/-}$ thymus was not able to prevent disease. However, grafting 4 $Aire^{+/+}$ thymi with one $Aire^{-/-}$ thymus was sufficient to prevent disease[76]. Further support

for deletion as the primary role of TRA expression by Aire is largely based on data from TCR transgenic systems that expressed model antigens under the rat insulin promoter as a method to test the requirement for Aire-dependent promiscuous gene expression[76-78]. However, a study that looked at the thymic frequency of an endogenous $CD4^+$ T cell clone reactive to an Aire-dependent eye antigen, showed that deletion is incomplete with only a ~2-fold decrease in the frequency of antigen-specific cells in WT mice[79].

A series of recent studies has challenged the idea that Aire enforces tolerance solely by driving clonal deletion. A study by Savage and colleagues[80] showed that Aire is required for the thymic development of a prostate-specific Treg cell clone, MJ23, that is enriched in mice bearing prostate tumors. This finding supported previous data showing that ectopic expression of a model antigen by Aire-expressing cells can promote Treg development of TCR transgenic Treg cells[81]. Subsequently, a study to determine the dual role of Aire in enforcing tolerance used TCR repertoire analysis to demonstrate that Aire deficiency leads to the generation of "Trogue" cells, Tconv cells bearing TCRs normally found in the Treg repertoire in Aire-sufficient mice[82]. In the absence of Aire expression, Trogue cells are not diverted to the Treg lineage, and enter the periphery as autoreactive Tconv cells that are able to drive organ-specific autoimmunity[82]. Taking this into account and revisiting the thymic grafting experiments described above[76], the ability of 4 $Aire^{+/+}$ thymi to prevent autoimmunity driven by one $Aire^{-/-}$ thymus is likely due to the requirement for enough Aire-dependent Tregs to reach their target organs before Tconv cells of the same specificity are able to expand.

Importantly, findings by Mathis and colleagues[83] demonstrated that Aire expression is not required throughout life, but is dispensable after 3 weeks of age. Using a doxycycline-regulated transgene that targeted Aire, the authors could control the expression of Aire temporally. Ablation of Aire expression after the perinatal period of life did not result in development of the severe autoimmune disease that was observed when Aire was deleted at earlier time points[83]. This result indicates that Aire-induced tolerance rapidly develops after birth and is maintained throughout life. These data in combination with the requirement for Aire expression in driving the development of organ-specific Tregs, implies that Treg specificities that emerge early in life play a significant role in preventing autoimmunity. This hypothesis was confirmed by a recent study that showed Aire-dependent Tregs that develop during the perinatal period represent a stable population that is required to prevent the development of autoimmunity[84]. Transfer of perinatally marked Tregs, but not bulk or adult Tregs, into newborn Aire-KO mice was sufficient to prevent pathology, indicating that Tregs generated during the perinatal period of life have distinct functional properties and are required for protection against autoimmunity[84].

Thus, recent evidence is consistent with the idea that the major role of Aire is to express tissue-restricted antigens that are required to divert self specific clones into the Treg lineage. However, the knowledge of the nature of Aire-dependent antigens that drive Treg generation in unknown.

Nature of Antigens Recognized by tTreg Cells

A wide body of evidence suggests that tTregs are reactive to self-ligands. As previously mentioned, several studies have demonstrated that the expression of neo-self antigens in the thymus of mice with TCRs specific for that antigen is sufficient to drive tTreg development[54-56,58]. Early TCR repertoire studies demonstrated that Tregs and Tconv cells express diverse TCRα chains that only partially overlap indicating antigen driven selection[34]. Additionally, T cells engineered to expressed TCRs isolated from the $CD4^+CD25^+$ Treg subset are more likely to proliferate in lymphopenic hosts than T cells expressing TCRs from the $CD4^+CD25^-$ Tconv subset, indicating that these cells may be reactive to self-antigen. In similar work by Gavin et al.[85], cells transcribing a null form of Foxp3 conferred autoimmunity when transferred to lymphopenic hosts, again suggesting that these cells may be reactive to self-antigens. Moreover, recent studies have demonstrated that some Tregs require the expression of TRAs by Aire for their development within the thymus[70,80]. While these studies all suggest tTregs recognize antigens, none have formally shown Treg development in response to a natural antigen. Therefore, reactivity to commensal or environmental antigens cannot be excluded. Indeed, a fraction of $CD4^+$ cells reactive to foreign pMHC-II complexes are Tregs, suggesting some TCRs may be crossreactive to self ligands that promote Treg development[49].

Interestingly, in models of experimental autoimmune encephalomyelitis (EAE), immunization with a self-peptide specific for myelin oligodendrocyte glycoprotein (MOG) drives tissue-specific autoimmunity and the emergence of Tconv and Treg cells specific

to the peptide[86,87]. This system has been cited as an example of naturally occurring Tregs[87], however, the Tregs present in these mice represent a minor fraction of the peptide-specific cells, and are unable to prevent the development of autoimmunity, indicating that these cells are not efficiently directed into the Treg lineage. Importantly, it is unknown if tTregs reactive to MOG are present in the endogenous repertoire at steady state or if these are pTregs generated in response to inflammation in the CNS. Thus, although some Tregs reactive to MOG exist in inflammatory CNS lesions in response to immunization[86-88], these do cells not represent a specificity that is naturally skewed to the Treg cell lineage, and are incapable of preventing organ-specific autoimmune reactions.

In all, the nature of the self antigens recognized by endogens Treg cells remains unknown. Moreover, it is not known if a single antigen is required for development or if Tregs are broadly cross-reactive leading to selection by diverse peptides[49]. Identification of natural Treg ligands will shed light on these lingering questions of Treg antigen recognition.

Peripheral Treg Development

Treg cells do not only originate in the thymus, but can also result from a process in which naïve $CD4^+$ T cells differentiate into Tregs in the periphery in response to a variety of signals ranging from non-inflammatory to inflammatory[89]. Conversion of naïve T cells to pTregs in non-inflammatory conditions can occur in response to prolonged exposure to low-doses of antigen[90] or in response to high levels of TGF-β, IL-2, and retinoic acid (RA) in the gut leading to oral tolerance to food antigens and prevention of

colitis[91]. Development of pTregs under inflammatory settings has also been observed, such as the generation of Tregs in response to aeroantigens in the setting of asthma[92,93]. Though foreign antigens are typically the targets of pTregs, they can also develop in response to self antigens as evident by the generation of pTregs against tumor antigens that prevent tumor rejection[89].

The ability to distinguish between tTregs and pTregs is critical for determining if they play non-redundant, cooperative roles in immune tolerance. However, markers to distinguish these subsets remain incompletely defined. Helios, a transcription factor of the Ikaros family was proposed to be expressed at high levels on thymic derived Tregs[94]. However, a subsequent study found that Helios expression could be transiently induced during T cell activation regardless of the T cell population studied, indicating that it is not an exclusive maker for tTregs[95]. Two studies published in 2012 proposed that Nrp-1 could be used as a surface marker to differentiate pTregs (low Nrp-1) from tTregs (high Nrp-1)[96,97]. However, the faithfulness of Nrp-1 as a strict maker of tTreg origin has been called into question[98,99], indicating further studies will be needed find makers that can differentiate pTregs from tTregs.

Importantly, the contribution of pTregs and tTregs in maintaining tolerance is unclear due to the inability to fully distinguish between the two subsets. Deletion of the CNS1 region of the Foxp3 locus was shown to be required for generation of pTregs, but not tTregs[100]. However, a subsequent study demonstrated that only generation of pTregs in the gut was affected[101] by this deletion, indicating pTreg development may be required at mucosal sites, but not other tissues. Whether pTregs and tTregs play non-redundant

roles, or act in concert with each other to prevent autoimmunity will be important to determine.

Homeostasis and Maintenance of Treg Cells in the Periphery

Not much is known about the factors driving enrichment, and activation of Tregs in the periphery, however TCR recognition is required for Treg function and maintenance[102,103]. Several findings indicate that Treg recognition of antigen in the periphery is important for coordinating Treg localization. In the periphery, Treg cells proliferate and can perceive strong TCR signals at steady state indicating that they are actively recognizing antigen[44,104,105]. This observation, together with the requirement of TCR expression[102,103] indicates that cognate antigen expression in the periphery may be required for maintenance of Tregs.

TCR analysis of prostate tumors[80], muscle[106], and visceral adipose tissue (VAT)[107,108] have demonstrated oligoclonal enrichment of Treg populations providing further evidence for antigen driven enrichment throughout the body. These more recent studies are consistent with an earlier TCR repertoire analysis that revealed asymmetric distribution of Treg TCRs throughout secondary lymphoid sites, indicating antigen-driven enrichment of distinct clones in regional lymph nodes[109]. Further support for antigen dependent enrichment was demonstrated by transfer studies in which Tregs isolated from organ-draining lymph nodes were better at suppressing organ specific autoimmunity than Tregs from distant lymph nodes[110-112]. Additionally, the enrichment of an organ-specific Treg clone can be abrogated by the removal of the antigen source, as demonstrated by

the redistribution of the prostate specific Treg clone, MJ23, after castration and prostate involution; provision of testosterone and prostate regeneration reversed this effect[71]. Altogether these findings indicate that antigen expression in the periphery plays a key role in Treg homeostasis.

Antigen presenting cells play a critical role in driving the oligoclonal enrichment of Treg populations in the periphery. Leventhal et al.[71] demonstrated that CCR7-expressing migratory DCs are required for enrichment of a prostate-specific Treg clone in the draining lymph node indicating that trafficking of DCs from tissues to lymph nodes is important for proper Treg distribution. However this does not exclude the requirement for other signals such as the expression of the chemokine receptors CCR4[113] and CCR7[105] or the expression of a G-coupled protein receptor GPR15[114] on Tregs, and the ability to sense IL-2 or IL-15[32]. The different expression of these molecules drives Treg localization to distinct areas of the body (i.e. CCR4 drives homing to the lungs, GRP15 drives localization of the lamina propria)[32,114] and identifying Treg antigens will reveal if these signals are sufficient to drive localization and maintenance of Treg populations, or if antigen is also required.

Treg Stability

Stability of Tregs has been a topic of debate in the literature. Ablation of Foxp3 in Treg cells after development leads to a loss of the Foxp3 transcriptional profile, and loss of Treg function[115], demonstrating the requirement for Foxp3 expression in maintenance of Treg cells. Several studies suggest that Foxp3 expression in Tregs is unstable in

lymphopenic or inflammatory conditions and can lead to the expression of proinflammatory cytokines such as TNF-α, IL-6, and IL-17[116,117]. These data demonstrated that under certain conditions, Tregs exhibited plasticity. In further support for plasticity of Treg fate, one study showed a population of "ex-Treg" cells, in which Foxp3 has been expressed and lost, mediated autoimmune pathology[117]. However, using in vivo labeling of Foxp3 expression, Treg were shown to be stable long term *in vivo*, even after challenges such as radiation-induced lymphopenia and cytokine-induced inflammation[118]. Principally, this study showed that Treg cell fate is a stable identity, but the mechanisms that maintain this identity in the periphery are unknown.

Thus, Treg development represents a fully differentiated state, but the factors that drive their maintenance and localization in the periphery remain incompletely defined. The identification of natural Treg antigens will facilitate studies on the role of antigen in this process and may shed light on mechanisms that could be coopted to promote proper localization of Tregs in the treatment of autoimmunity.

Conclusion

In the more than 20 years since the identification of Tregs, much has been learned about their biology and their essential role in tolerance has been established. However, many questions still remain. What is the nature of the self antigens tTregs recognize? How does expression of the antigen in the thymus influence Treg development? Does Treg development occur concurrently with negative selection? If so, is this driven by TCR-

pMHC affinity or other qualitative factors? What signals are required in the periphery for Treg maintenance? How does antigen expression in the periphery shape the repertoire?

In subsequent chapters, we describe the identification of endogenous peptide ligands recognized by naturally occurring Treg clones, and address several of these major gaps in knowledge. We demonstrate that two recurrent Treg cell clones, MJ23 and SP33, identified in independent sequencing studies, are reactive to distinct non-overlapping peptides derived from a single prostate-specific protein, Tcaf3. This indicates that protection against organ infiltration may be dependent upon recognition of a limited number of autoantigens that are most susceptible to autoimmune attack.

Definition of the peptide antigens recognized by the MJ23 and SP33 clones enabled us to generate pMHC tetramers that were used to study the endogenous polyclonal repertoire of Aire-dependent, tissue-specific Treg populations in various settings of health and disease. Further, using the knowledge of the peptide recognized by MJ23 cells, we generated mice that have a selective deletion of this peptide, allowing us to study the role of an Aire-dependent antigen in a wildtype setting. Using these mice, we tested the role of antigen expression in the thymus and periphery in the development and homeostasis of MJ23 Treg clone and the effect of global deletion of the antigen on the polyclonal Tcaf3-specific population.

The elucidation of natural antigens recognized by endogenous Treg clones has provided new insights into Treg biology and will lead to a better understanding of how Tregs develop, function, and persist as well as the factors that lead to a break in Treg-mediated tolerance.

Chapter II: Materials and Methods

Mice

Mice were bred and maintained in accordance with the animal care and use regulations of the University of Chicago with approval and oversight by IACUC. The following mice were purchased from the Jackson Laboratory: C57BL/6J (B6) mice, CD45$^{.1/.1}$ B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ mice, TRAMP C57BL/6-Tg(TRAMP)8247Ng/J mice, *Rag1*$^{-/-}$ B6.129S7-*Rag1*^{tm1Mom}/J mice, *Aire*$^{-/-}$ B6.129S2-Aire$^{tm1.1Doi}$/J mice, TCR$\alpha^{-/-}$ B6.129S2-Tcra^{tm1Mom}/J mice, CD4-Cre B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ mice, *Bim*$^{-/-}$ B6.129S1-Bcl2l11$^{tm1.Ast}$/J mice, TCR$\beta^{-/-}$ B6.129P2-Tcrb^{tm1Mom}/J, and *Foxn1*$^{nu/nu}$ B6.Cg-Foxn1^{nu}/J. OT-II transgenic CD57BL/6-Tg(TcraTcrb)425Cbn/Crl mice wre purchased from Charles River Laboratories. MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ and "TCRβtg" mice expressing a fixed TCRβ chain of sequence TRBV26-ASSLGSSYEQY were generated as described previously[80]. *Tcaf3*^{tm1} mice containing a genetic deletion of the region encoding the Tcaf3$_{646\text{-}658}$ peptide were generated as described previously[119]. Mice were not randomized for assignment to experimental group and experiments were not conducted in a blinded fashion.

Preparation of prostatic extracts

The procedure for the preparation of prostatic secretory extracts was adapted from Fujimoto et al.[120]. Prostates of tumor-bearing TRAMP male mice of 6 months of age were dissected to separate the anterior, ventral, and dorsolateral lobes. The dissected lobes were incubated separately in 1-2 mL PBS for 5 minutes at room temperature to extract

secreted proteins, and then spun for 5 minutes at 10,000 x g at 4°C. The supernatant was transferred to a fresh tube and spun again for 5 minutes at 13,200 x g at 4°C. The supernatant from this second spin was retained and total protein content was quantified by BCA protein assay (Pierce). These secretory extracts were flash frozen in liquid nitrogen and store at -80°C until use.

Recombinant protein production

Tcaf3 protein and the negative control protein (Tgm4) were produced recombinantly in High Five insect cells (Thermo Fisher Scientific) with an N-terminal gp67 secretion signal and 8xHis tag. Tagged proteins were purified from culture supernatant by nickel affinity chromatography, and their His tags were removed by cleavage with 3C protease. Purified proteins were exchanged into PBS using a Zeba desalting spin column (Thermo Fisher Scientific) and sterilized by 0.22 µm filtration.

In vitro T cell stimulation

$CD4^+$ T cells were isolated from MJ23tg *Rag1*$^{-/-}$ $CD45^{.1/.1}$ female or OT-IItg *Rag1*$^{-/-}$ $CD45^{.1/.1}$ donor mice and purified by MACS (Miltenyi Biotech) enrichment. $CD4^+$ T cells were CellTrace-Violet (Thermo Fisher Scientific) labeled per manufacturer instructions with slight modification. Briefly, cells were pelleted, resuspended in CellTrace-Violet (CTV) at 1:1000 dilution and incubated for 20 minutes at 37°C. The reaction was quenched by the addition of 13 mL of complete culture media. To isolate splenic dendritic cells, splenocytes were isolated from C57BL/6 $CD45^{.2/.2}$ mice and enriched for $CD11c^+$

cells by MACS-based (Miltenyi Biotech) positive selection. 1 x 10^4 CTV-labeled T cells were co-cultured with 5 x 10^4 $CD11c^+$ splenocytes, 100 U/mL recombinant mouse interleukin-2 (IL-2), and prostatic extract, proteins, or peptides as indicated. Additionally, anti-MHC-II antibody clone M5 from eBioscience (Thermo Fisher Scientific) or IgG2b, κ isotype control antibody (BD Pharmingen) was added to indicated cultures at a final concentration of 10 μg/mL. Cell cultures were set up in 384-well ultra low attachment, round-bottom plates (Corning). Dilution of CTV was assessed by flow cytometry on day 3 or day 5 as indicated.

In vivo T cell stimulation

$CD4^+$ T cells were isolated from MJ23tg *Rag1*$^{-/-}$ $CD45^{.1/.1}$ female or OT-IItg *Rag1*$^{-/-}$ $CD45^{.1/.1}$ donor mice and purified by MACS (Miltenyi) enrichment. $CD4^+$ T cells were CellTrace-Violet (Thermo Fisher Scientific) labeled as described above. Cells were resuspended in incomplete media and retro-orbitally injected into B6 $CD45^{.2.2}$ female hosts. 2 hour later mice were immunized i.v. with 5 μg of Tcaf3 or control protein in PBS, or with PBS alone. $CD4^+$ T cells from the spleen or pooled inguinal, axillary, and brachial lymph nodes were MACS-enriched, and dilution of CTV was assessed by flow cytometry on day 5.

Intrathymic injection of thymocytes

4 x 10^6 bulk thymocytes from MJ23tg *Rag1*$^{-/-}$ $CD45^{.1/.1}$ females were injected intrathymically into 4-8 week old Tcaf3-sufficient (*Tcaf3*$^{+/+}$ or *Tcaf3*$^{tm1/+}$) or Tcaf3-decient

(*Tcaf3*$^{tm1/tm1}$) mice and analyzed 7 days post-transfer. Donor thymocytes were Foxp3^{neg} due to intraclonal competition in MJ23tg *Rag1*$^{-/-}$ CD45.1/.1 females.

CFA immunization

Mice were given subcutaneous injection of 100 μl of CFA (InvivoGen) emulsion containing 100 μg of peptide (Genscript).

I-A^b-binding peptide prediction

Tcaf3-derived peptides most likely to bind I-A^b were predicted by two computational methods: the Immune Epitope Database (IEDB[121]) and an in-house peptide scoring script based on the amino acid position probabilities reported by Zhu et al.[122]. Top-scoring peptides from either method were synthesized and assayed as described below. Of note, the validated antigenic peptide (Tcaf3$_{646-658}$) was predicted by Zhu et al.[70], but not by the IEDB[121].

Peptides

The top 36 candidate peptides were synthesized in Sigma's PepScreen format, and the crude, unpurified peptides were screened for *in vitro* stimulation of MJ23tg T cells as described above. Truncation mutants were also synthesized in the PepScreen format and assayed as crude peptides. All other peptide experiments were performed with peptides synthesized and purified to >98% purity (GenScript).

Tcaf3 Western blot

Serum was isolated from Aire-sufficient (*Aire*$^{+/+}$ or *Aire*$^{+/-}$) or Aire-deficient (*Aire*$^{-/-}$) males at varying ages, from TCRb$^{-/-}$ host 8-10 weeks after transfer of CD4^{+} T cells from *Tcaf3*$^{+/+}$ or *Tcaf3*$^{tm1/tm1}$ mice and *Foxn1*$^{nu/nu}$ mice 6, 8, and 12 weeks after graft of *Tcaf3*$^{+/+}$ or *Tcaf3*$^{tm1/tm1}$ thymic lobe. 3 µg Tcaf3 protein was loaded onto a 1-well 4-20% SDS-PAGE gel (Bio-Rad; ~38 ng protein per mm lane width) and transferred to nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 3% w/v BSA in TBSt, and then assembled into a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad). Sera were diluted 1:400 in TBSt + 3% w/v BSA + 0.1% w/v sodium azide, loaded into separate channels of the Multiscreen Apparatus, and incubated overnight at 4°C. Channels were washed with TBSt in the Apparatus, then the membrane was removed from the Apparatus, washed again with TBSt, and blotted for 1 hour at room temperature with bovine anti-mouse-IgG HRP conjugate (Santa Cruz Biotechnology, sc-2371) diluted 1:10,000 in TBSt + 5% w/v nonfat dried milk. The membrane was washed with TBSt, incubated with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher) and imaged on a ChemiDoc imager (Bio-Rad).

I-A^{b} tetramer production

Tcaf3/I-A^{b} tetramers bearing the Tcaf3$_{646\text{-}658}$(648Y) peptide (THYKAPWGELATD) and 2W1S/I-A^{b} tetramers bearing the 2W1S peptide (EAWGALANWAVDSA) were produced using methods similar to those described previously[123]. I-A^{b} was expressed in Drosophila S2 cells, either using separate plasmids to encode the alpha and beta chains, as

described previously [123], or using a new vector encoding both alpha and beta chains joined by a P2A ribosome cleavage sequence (ATNFSLLKQAGDVEENPGP) (Szymczak et al., 2004). The latter construct was assembled from the pMT-puro vector (Addgene plasmid #17923) in order to enable rapid, stable selection of both I-A^b chains from a single plasmid. In both sets of constructs, the alpha chain contained at its C-terminus an acidic leucine zipper and a BirA biotinylation sequence, and the beta chain contained at its N-terminus the antigenic peptide plus a 13 amino acid linker, and at its C-terminus a basic leucine zipper and 6xHis tag. In the pMT-puro construct, the chains were ordered as follows: beta-P2A-alpha. Constructs were co-transfected into *Drosophila* S2 cells together with a plasmid encoding the BirA biotin ligase. Protein expression was induced with the addition of 0.8 mM $CuSO_4$, in the presence of 2 µg/mL D-biotin (Pierce #29129). Biotinylated I-A^b protein was purified from culture supernatant by nickel affinity chromatography (His Bind Ni-IDA resin; Millipore Sigma) and avidin affinity chromatography (Pierce Monomeric Avidin UltraLink Resin). Tetramers were formed by mixing biotinylated I-A^b with streptavidin-APC (Prozyme PJ27S) or streptavidin-PE (Prozyme PJRS34) at a slight molar excess of I-A^b to biotin binding sites. Saturation of the streptavidin conjugate was verified by non-reducing SDS-PAGE without boiling samples.

I-A^b tetramer staining

Tetramer staining was adapted from Tungatt et al.[124] and performed as previously described[119]. Briefly, cells were treated with dasatinib (AdooQ Bioscience) for 30 min at

37˚C. APC- and PE-labeled tetramers were added directly to dasatinib-treated cells and incubated for 1 hour at room temperature. Cells were washed and incubated with mouse anti-APC antibody (clone APC003, Biolegend) and mouse anti-PE antibody (clone PE001, Biolegend) for 20 min on ice. Cells were washed and stained for flow cytometric analysis as described below. Staining of cells with 2W1S/I-A^b tetramers was performed with 10 nM of each tetramer; staining of cells with Tcaf3/I-A^b tetramers was performed with 50-100 nM of each tetramer.

I-A^b tetramer-based enrichment (AutoMACS)

Tetramer enrichment was adapted from Legoux et al.[125]. Cells from naïve mice were isolated from the spleen, inguinal, axillary, brachial, cervical, mesenteric, and periaortic lymph nodes. Cells were co-stained with APC- and PE-labeled tetramer at a final tetramer concentration of 200 nM. Following staining, cells were incubated with anti-APC and anti-PE microbeads (Miltenyi Biotech) following manufacturer's protocol. Cells were magnetically enriched using an AutoMACS Pro Seperator (Miltenyi Biotech). The bound fraction was stained and analyzed as described below. The total number of tetramer-positive events was calculated.

I-A^b tetramer-based enrichment (StemCell)

EasySep positive selection (StemCell Technologies) enrichment was performed on mice after immunization with CFA plus peptide. Cells from immunized mice were isolated from the spleen, inguinal, axillary, brachial, cervical, mesenteric, and periaortic lymph nodes.

Cells were stained with either APC- or PE-labeled tetramer at a final tetramer concentration of 100 nM. Following staining, cells were incubated with APC or PE selection cocktail followed by magnetic nanoparticles following manufacturer's specifications. Cells were magnetically enriched using EasyEights EasySep magnet (StemCell Technologies). The bound fraction was stained and analyzed as described below. The total number of tetramer-positive events was calculated.

Antibodies and flow cytometry

All antibodies used were purchased from Biolegend, eBioscience (Thermo Fisher Scientific), or BD Biosciences. Cells were stained with conjugated antibodies specific for the following proteins (clone name in parentheses): CD4 (GK1.5), or CD8β (Ly-3), CD3 (17A2), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), Foxp3 (FJK-16s), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), and F4/80 (BM8). CD25 (PC61), CD44 (IM7), TCR (H57-597), PD-1 (RMP1-30), CD24 (M1/69), and active caspase-3 (C92-605). Cells were stained for 20 min on ice in staining buffer (PBS with 2% FCS, 0.1% NaN_3, 5% normal rat serum, 5% normal mouse serum, 5% normal rabbit serum [all sera from Jackson ImmunoResearch], and 10 μg/μl 2.4G2 antibody). Intracellular staining for Foxp3 and active caspase-3 was preformed using fixation and permeabilization buffers from eBioscience (Thermo Fisher Scientific). Flow cytometry was performed on an LSR Fortessa (BD Biosciences) and data was analyzed using FlowJo software (Tree Star).

Retrovirus production, infection, and generation of SP33rg mice

SP33rg mice were generated as described previously[51]. Briefly, the SP33 TCRα was cloned into a retroviral construct modified from Turner et al.[51,126]. Plat-E cells, also previously described[127], were used to generate retrovirus. TCR$\alpha^{-/-}$ CD4-Cre TCRβtg mice on a C57BL/6 background were injected with 5-fluorouracil (APP Pharmaceuticals) 3 days prior to bone marrow harvest. Bone marrow cells were cultured for 2 days in X-Vivo 10 (Lonza) containing 15% FCS, 1% penicillin/streptomycin, mouse SCF, mouse IL-3 and mouse IL-6 (Biolegend). Cells were infected with retrovirus by spinfection in the presence of 6 μg/mL polybrene (Millipore Sigma) and cultured for an additional 24 hours. 1 x 10^6 spinfected cells were then mixed with 5 x 10^6 freshly harvested bone marrow "filler" cells from *Rag1*$^{-/-}$ mice and injected into irradiated (800 rad) CD45$^{.1/.1}$ B6.SJL recipient mice to generate TCR "retrogenic" (rg) mice. SP33rg cells were isolated from retrogenic mice 6-8 weeks after bone marrow reconstitution. CD4$^+$ T cells were FACS-purified from SP33rg mice following staining with the following antibodies: anti-CD8β (Ly-3), anti-CD45.1 (A20), anti-CD45.2 (104), and anti-Thy1.1 (OX-7). Isolated cells were CellTrace-Violet labeled and used for *in vitro* and *in vivo* experiments as described above.

TCR gene usage and CDR3 sequences

The MJ23 TCR contains the alpha chain TRAV14-LYYNQGKLI, utilizing TRAJ23, and the beta chain TRBV26-ASSLGSSYEQY, utilizing TRBJ2-7. The SP33 TCR contains the alpha chain TRAV9D-ALSMSVNYQLI, utilizing TRAJ33, and the beta chain TRBV26-ASSLGSSYEQY, utilizing TRBJ2-7.

Bone Marrow Chimera generation

Bone marrow cells were harvested and T cell depleted using CD90.2 microbeads (Miltenyi Biotech). 5 x 10^6 cells were injected retro-orbitally injected into sublethally (500 rads) irradiated host mice. Low frequency bone marrow chimeras were comprised of 5% MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ bone marrow cells plus 95% B6 cells. All cells were taken from female donor mice. Mice were analyzed 6 weeks post engraftment.

CD4$^+$ T cell transfer

Bulk CD4$^+$ T cells were isolated from the spleen, inguinal, axillary, brachial, cervical, mesenteric, and periaortic lymph nodes of donor mice. CD4$^+$ T cells were magnetically isolated using MACS CD4 T cell isolation kit II (Milteny Biotech) per manufacturer's instructions. 5 x 10^6 CD4$^+$ T cells were retro-orbitally injected into *Rag1*$^{-/-}$ or *TCR*$\beta^{-/-}$ male hosts. Host mice were either immunized 3 weeks after CD4$^+$ T cell transfer and analyzed 14 days later as above, or left unimmunized and analyzed 8-10 weeks after CD4$^+$ T cell transfer.

Statistical analysis

Data was analyzed using Prism software (GraphPad). Significance testing was performed using the nonparametric Mann-Whitney test or the Student's t-test (two-tailed) or Extra sum-of-squares F test where indicated. No statistical methods were used to predetermine sample size.

Chapter III: Identification of Natural Regulatory T Cell Epitopes Reveals Convergence on a Dominant Autoantigen

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Leonard JD*, **Gilmore DC***, Dileepan T, Nawrocka WI, Chao JL, Schoenbach MH, Jenkins MK, Adams EJ and Savage PA. Identification of natural regulatory T cell epitopes reveals convergence on a dominant autoantigen. Immunity 47, 107–117 July 18, 2017.

SUMMARY

Regulatory T (Treg) cells expressing the transcription factor Foxp3 are critical for the prevention of autoimmunity and the suppression of anti-tumor immunity. The major self antigens recognized by Treg cells remain undefined, representing a substantial barrier to the understanding of immune regulation. Here, we have identified natural Treg cell ligands in mice. We found that two recurrent Treg cell clones, one prevalent in prostate tumors and the second associated with prostatic autoimmune lesions, recognized distinct non-overlapping MHC class-II-restricted peptides derived from the same prostate-specific protein. Notably, this protein is frequently targeted by autoantibodies in experimental models of prostatic autoimmunity. Based on these findings, we propose a model in which Treg cell responses at peripheral sites converge on those self proteins that are most susceptible to autoimmune attack, and we suggest that this link may be exploited as a

generalizable strategy to identify the Treg cell antigens relevant to human autoimmunity.

INTRODUCTION

The immune system generates a diverse repertoire of conventional $CD4^+$ T cell clones capable of responding to foreign antigens, while restricting immune responses directed at self antigens. Each $CD4^+$ T cell expresses a unique T cell receptor (TCR) capable of recognizing major histocompatibility complex class II molecules (MHC-II) complexed with short peptides, which are generated from intact proteins by antigen processing. In the process of negative selection, many $CD4^+$ T cells exhibiting strong reactivity to self peptide-MHC-II (pMHC-II) are eliminated from the conventional T (Tconv) cell repertoire by clonal deletion or differentiation into innate-like T cell lineages[53]. In contrast, some $CD4^+$ thymocytes exhibiting overt reactivity to self pMHC-II ligands differentiate into regulatory T (Treg) cells expressing the transcription factor Foxp3[59], which function in the periphery to maintain immune homeostasis and suppress autoreactive Tconv cells that evade negative selection. Thus, reactivity to self antigen is crucial for the establishment of two major tolerance mechanisms – negative selection and Treg cell development, which function in concert to restrict immune responses to self tissues. Beyond the role of self pMHC-II recognition in directing Treg cell development in the thymus, the continued recognition of self antigen outside the thymus is critical for orchestrating Treg cell differentiation, homeostasis, and suppressor activity. Given that self pMHC-II recognition is central to many facets of Treg cell biology, it is essential to identify the endogenous peptides that trigger Treg cell development in the thymus and

are engaged by Treg cells to coordinate immune suppression in the periphery. However, due to technical challenges associated with identifying MHC-II-restricted self peptides, the natural antigens recognized by thymus-derived Treg (tTreg) cells have remained undefined. Without this knowledge, it has not been possible to gain a complete understanding of why Treg cell-mediated suppression is subverted in autoimmune and inflammatory diseases, and how Treg cells are co-opted by developing cancers to suppress anti-tumor immunity.

The paradigm that self pMHC-II recognition via the TCR drives both the thymic development and peripheral function of Treg cells is supported by a large body of evidence. Early studies in mice utilizing TCR repertoire profiling revealed that the TCRs expressed by peripheral Treg cells are largely distinct from those expressed by Tconv cells[34,55,109,128], demonstrating that the formation of the Treg cell repertoire is an antigen-driven, TCR-dependent process. Consistent with this, developmental studies show that Treg cell-derived TCRs facilitate thymic differentiation into the Treg cell lineage, whereas Tconv-expressed TCRs are inefficient at directing this process[53,64,65]. Similarly, transgenic expression of a model antigen containing a pMHC-II-binding peptide in the thymus promotes the development or survival of antigen-specific Treg cells [59], indicating that TCR-dependent agonist signals promote thymic (t)Treg cell development. More recent studies demonstrate that the thymic development of some Treg cell specificities is dependent on the expression of Autoimmune regulator (Aire)[70,80], a transcription factor that drives the promiscuous expression of tissue-specific antigens in the thymus[67,68]. In the periphery, a substantial proportion of Treg cells proliferate[104,105] or perceive strong

TCR signals[44] at steady state, suggesting that many peripheral Treg cells actively recognize agonist pMHC-II ligands in the absence of inflammation. Likewise, T cells transduced with Treg-derived TCRs can proliferate following transfer into lymphopenic mice, suggesting that some Treg cell TCRs confer reactivity to self pMHC-II complexes[34]. Finally, recent work demonstrates that conditional ablation of the TCR on Foxp3$^+$ cells results in systemic autoimmunity[102,103], demonstrating an important role for TCR-dependent signals in Treg cell differentiation and function in the periphery.

The gap in knowledge regarding the identity of endogenous antigens recognized by Treg cells has restricted progress in several fundamental areas of Treg cell biology. First, it has not been possible to analyze the phenotype, frequency, anatomical distribution, and repertoire complexity of Treg cells reactive to natural self antigens that direct Treg cell differentiation in the thymus. Second, it is unknown whether tTreg TCRs recognize endogenous ligands with unique biochemical or structural characteristics, such as binding affinity, docking mode, or conformational changes, that might provide insight into the mechanisms of Treg cell-mediated suppression. Third, given the vast array of potential self peptides in the body, it is unclear whether the protection of tissues from autoimmune attack is dependent on the presence of Treg cells reactive to particular self pMHC-II complexes. Fourth, it has been challenging to clearly elucidate why some self-reactive T cells are purged by clonal deletion whereas others are directed into the Treg cell lineage, and whether the affinity of TCR-pMHC-II binding is a primary determinant of these alternate cell fates.

Here we report the identification of the peptide antigens recognized by two recurrent prostate-specific Treg cell clones, one prevalent in mouse prostate tumors and the second associated with autoimmune lesions of the prostate. These endogenous Treg cell clones recognize distinct non-overlapping MHC-II-restricted peptides from a prostate-specific protein that is frequently targeted by autoantibodies in multiple mouse models of autoimmunity. The link between the antigen specificity of Treg cells and that of autoantibodies that emerge in settings of immune dysregulation has implications for our understanding of Treg cell-mediated tolerance, and reveals a potential strategy for identifying additional Treg cell antigens in mice and humans.

RESULTS

Identification of a Self pMHC-II Antigen Recognized by MJ23 Treg Cells

To reveal the nature of self peptides recognized by tTreg cells, we aimed to identify a pMHC-II ligand recognized by a naturally occurring Treg cell clone named "MJ23", which we identified previously based on its predominance in mouse prostate tumor lesions[80]. The MJ23 TCR confers reactivity to a prostate-associated, MHC-II-restricted antigen of unknown identity, and mediates Treg cell development in the thymus via an Aire-dependent process[80]. To screen for antigenic activity, we used an *in vitro* culture system to monitor the proliferation of TCR transgenic T cells expressing the MJ23 TCR (MJ23tg T cells). Using this system, we found that splenic dendritic cells (DCs) cultured with protein extracts from the dorsolateral or anterior (but not ventral) lobes of the prostate

induced proliferation of MJ23tg T cells (Figure 1A), demonstrating that the relevant peptide can be isolated and detected using T cell-based assays.

We reasoned that the MJ23 peptide antigen would fit three criteria: 1) the protein containing the peptide would be exclusively or preferentially expressed in the prostate; 2) the gene encoding this protein would be a transcriptional target of Aire in medullary thymic epithelial cells[67,68,74]; 3) this gene would not be located on the Y chromosome, because MJ23tg Treg cells undergo thymic development in both male and female mice[80].

Using data from a transcriptional profiling study[74], we identified 20 candidate genes fitting these criteria (Table 1), and expressed the corresponding proteins recombinantly in insect cells. We cultured DCs with individual proteins, and assayed for their capacity to induce proliferation of MJ23tg T cells (Figure 1B and Figure 2A-B). One of these candidates, encoded by *TRPM8 channel-associated factor 3* (*Tcaf3*, also known as *Eapa2* or *Fam115e*), induced robust stimulation of MJ23tg T cells *in vitro* (Figure 1B). Stimulation was abolished by anti-MHC-II blocking antibody, and Tcaf3 did not induce stimulation of transgenic T cells of an irrelevant specificity[129] (Figure 1B). Immunization with recombinant Tcaf3 protein, but not with another Aire-dependent prostatic protein (transglutaminase 4, Tgm4; Table 1), induced robust proliferation of MJ23tg T cells *in vivo* (Figure 1C), demonstrating that the Tcaf3-derived peptide can be efficiently processed and presented *in vivo*. To identify the peptide epitope recognized by MJ23tg T cells, we used *in silico* approaches[121,122] to generate a list of Tcaf3-derived peptides predicted to bind I-A^b, the only functional MHC class II gene expressed in these mice. Of these

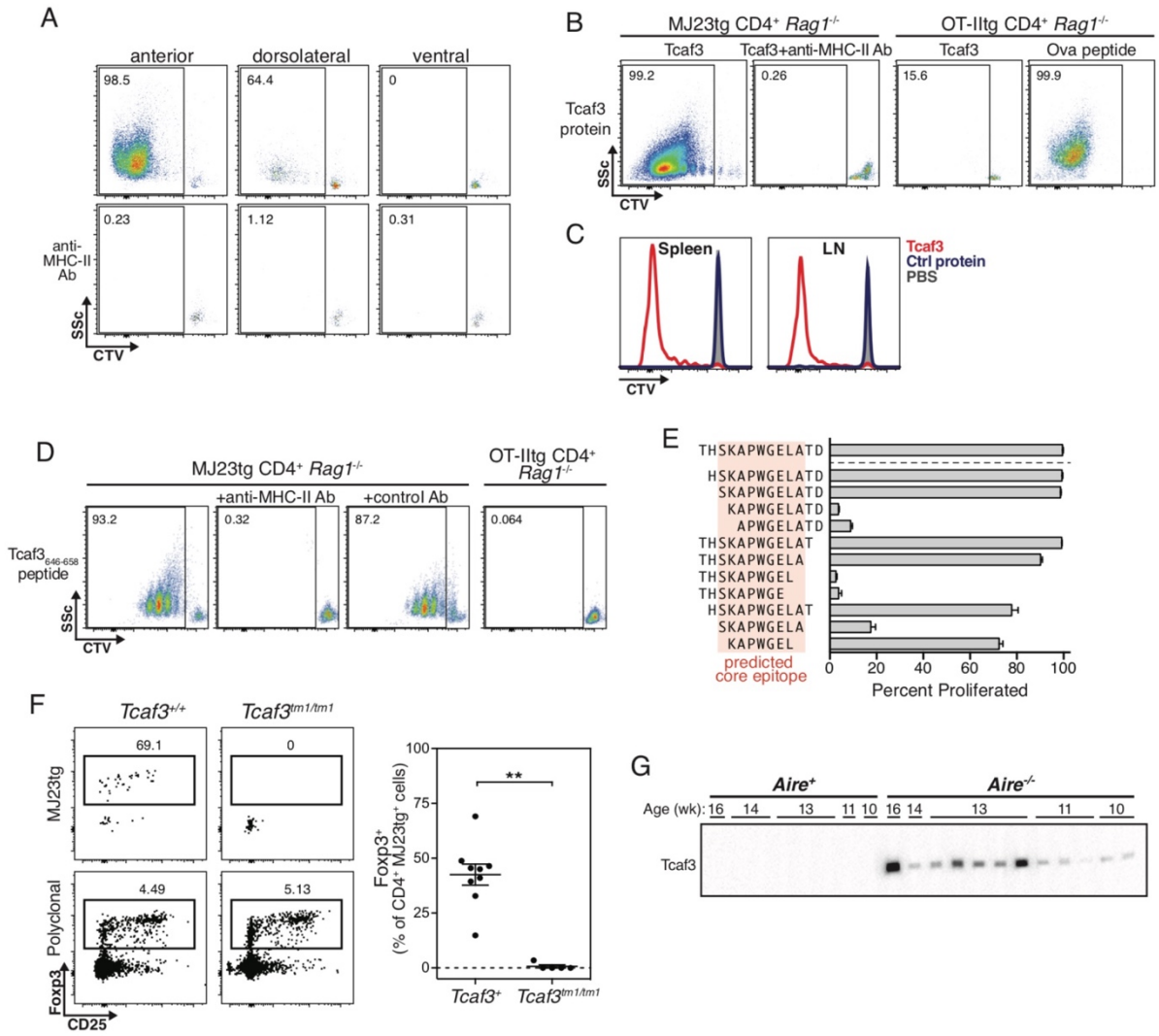

Figure 1: MJ23 T cells recognize an antigen derived from the prostatic protein Tcaf3. $CD4^+$ T cells were isolated from MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ or OT-IItg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ mice, labeled with CellTrace-Violet (CTV), and used as a probe for antigen. (A) *In vitro* stimulation of MJ23tg T cells by prostatic secretory extracts. 1 x 10^4 MJ23tg T cells were cultured with 5 x 10^4 $CD11c^+$ cells from B6 spleen, plus secretory extracts prepared from the anterior, dorsolateral, or ventral prostate lobes of tumor-bearing TRAMP males, with or without anti-MHC-II blocking antibody. Dilution of CTV was assessed by flow cytometry on day 5. (B) *In vitro* stimulation of MJ23tg T cells by Tcaf3 protein. As in (a), MJ23tg or OT-IItg T cells were stimulated *in vitro* with 2 µg/mL recombinant Tcaf3 protein or 1 µM $Ova_{323-339}$ peptide, and assayed on day 5. (C) *In vivo* stimulation of MJ23tg T cells by Tcaf3 protein. 1 x 10^5 MJ23tg T cells were transferred i.v. into congenically disparate B6 female hosts. 2 hours after transfer, recipients were immunized with 5 µg Tcaf3 protein, 5 µg Tgm4 protein, or PBS alone. T cells from the spleen (left panel) and pooled skin-draining lymph nodes (right panel) were analyzed for CTV dilution on day 5. (D) *In vitro* stimulation of MJ23tg T cells by Tcaf3 peptide. As in (A), MJ23tg or OT-IItg T cells were stimulated *in vitro* with 5 nM $Tcaf3_{646-658}$ peptide, with or without anti-MHC-II Ab or isotype control. Dilution of CTV was analyzed on day 3. (E) $Tcaf3_{646-658}$ peptide truncation analysis. As in (A), MJ23tg T cells were stimulated *in vitro* with 33 nM $Tcaf3_{646-658}$ peptide variants, comprising serial truncations from the N- and/or C-termini. Dilution of CTV was analyzed on day 3. The core nonamer epitope

Figure 1 continued: predicted computationally is denoted by red shading. Percent of cells proliferated is shown as the mean ± SEM of three replicates. (F) $Tcaf3_{646-658}$ peptide is required for the thymic development of MJ23tg Treg cells. Bulk thymocytes from MJ23tg *Rag1*$^{-/-}$ CD45.1/.1 females were transferred into 4-6-week-old *Tcaf3*$^{+/+}$, *Tcaf3*$^{+/tm1}$, or *Tcaf3*$^{tm1/tm1}$ hosts, both male and female, and analyzed at day 7 for expression of CD25 and Foxp3. Left panels, representative flow cytometric analysis of Foxp3 and CD25 expression on MJ23tg and polyclonal thymocytes for recipients of the indicated genotype. Right panel, summary plot of data for MJ23tg thymocyte transfer into *Tcaf3*$^{tm1/tm1}$ or *Tcaf3*$^{+}$ (*Tcaf3*$^{+/+}$ and *Tcaf3*$^{+/tm1}$) recipients. Significance testing was performed using the Student's t-test. ** indicates $p < 0.01$. (g) Tcaf3-specific autoantibodies can be detected in the serum of *Aire*$^{-/-}$ males. Recombinant Tcaf3 protein was resolved by SDS-PAGE, and subjected to Western blotting using serum from Aire-deficient (*Aire*$^{-/-}$) or *Aire*$^{+}$ (*Aire*$^{+/+}$ or *Aire*$^{+/-}$) littermates of the indicated ages. Data are representative of multiple independent experiments: (A) $N = 4$, (B) $N = 3$, (C) $N = 3$, (D) $N = 5$, (E) $N = 3$, (F) $N = 2$, (G) $N = 3$. See also Figure 2.

Table 1: Candidate MJ23 antigens

Gene	Synonyms	NCBI transcript	Uniprot
5430419d17rik	DMBT1-like protein	NM_175166.3	http://www.uniprot.org/uniprot/Q8BZE1
Abo	NAGAT	NM_030718.5	http://www.uniprot.org/uniprot/P38649
Msmb	PSP94	NM_020597.3	http://www.uniprot.org/uniprot/O08540
Odam	Apin	NM_027128.2	http://www.uniprot.org/uniprot/A1E960
Pate4	Svs7	NM_020264.4	http://www.uniprot.org/uniprot/Q09098
Pbsn		NM_017471.2	http://www.uniprot.org/uniprot/O08976
Sbp		NM_011321.3	http://www.uniprot.org/uniprot/P15501
Sbpl	Sbp-like	NM_001077421.1	http://www.uniprot.org/uniprot/Q3TUY3
Smr2	Msg2, Vcs2	NM_021289.2	http://www.uniprot.org/uniprot/O35985
Spinkl		NM_183123.2	http://www.uniprot.org/uniprot/Q8CEK3
Svs1		NM_172888.3	http://www.uniprot.org/uniprot/Q6WIZ7
Svs2	Sem1g	NM_017390.4	http://www.uniprot.org/uniprot/Q8BS30
Svs3a		NM_021363.2	http://www.uniprot.org/uniprot/F2Z472
Svs3b		NM_173377.2	http://www.uniprot.org/uniprot/Q8BZH8
Svs4	Svp2	NM_009300.3	http://www.uniprot.org/uniprot/P18419
Svs5	Svp5	NM_009301.2	http://www.uniprot.org/uniprot/P30933
Svs6	SVSP99	NM_013679.2	http://www.uniprot.org/uniprot/Q64356
Tcaf3	Fam115e, EAPA2	NM_203396.1	http://www.uniprot.org/uniprot/Q6QR59
Tgm4	EAPA1	NM_177911.4	http://www.uniprot.org/uniprot/Q8BZH1
Wfdc3	Wap14	NM_027961.1	http://www.uniprot.org/uniprot/Q14AE4

Table 1. Related to Figure 1. A published dataset of Aire-dependent transcripts in medullary thymic epithelial cells[74] was curated for genes whose peripheral expression is restricted to the prostate, according to the BioGPS database[130]. Alternate gene names, NCBI transcript numbers and UniProt entries are listed.

candidate peptides, we identified one peptide of sequence THSKAPWGELATD, spanning residues 646-658 of Tcaf3, that robustly stimulated MJ23tg T cells *in vitro* (Figure 1D). Subsequent analysis of peptide truncations revealed the minimal core epitope to be the nonamer SKAPWGELA (residues 648-656) (Figure 1E), the same core sequence that was predicted computationally to bind to I-A^b. Taken together, our results identify $Tcaf3_{646-658}$ as the I-A^b-restricted peptide recognized by prostate-specific MJ23 Treg cells.

To verify that $Tcaf3_{646-658}$ is the antigenic peptide recognized by prostate-specific MJ23 Treg cells *in vivo*, we generated mice with targeted deletion of the region encoding the $Tcaf3_{646-658}$ 13-mer peptide (hereafter referred to as *Tcaf3*^{tm1} mice). To test the requirement for this epitope in driving MJ23 Treg development *in vivo*, we isolated thymocytes from MJ23tg *Rag1*$^{-/-}$ females, which harbor no Foxp3$^+$ cells due to intraclonal competition[80], and transferred these cells into new hosts by intrathymic injection. Donor MJ23tg thymocytes readily differentiated into Foxp3$^+$ cells in *Tcaf3*$^{+/+}$ hosts, but failed to do so in *Tcaf3*$^{tm1/tm1}$ mice (Figure 1F). Consistent with this, prostatic extracts isolated from *Tcaf3*$^{tm1/tm1}$ mice failed to stimulate MJ23tg T cells *in vitro* (Figure 2C), demonstrating a specific requirement for $Tcaf3_{646-658}$ peptide. These data provide loss-of-function evidence that the $Tcaf3_{646-658}$ peptide is a natural epitope recognized by Treg populations *in vivo*, and demonstrates that the thymic development of this tissue-specific Treg cell clone requires thymic expression of a single self peptide.

Mouse Tcaf3 is a 102-kDa protein of unknown function that is exclusively expressed in the dorsolateral and anterior lobes of the mouse prostate[120], consistent with our MJ23tg T cell stimulation data (Figure 1A). Of note, previous studies have

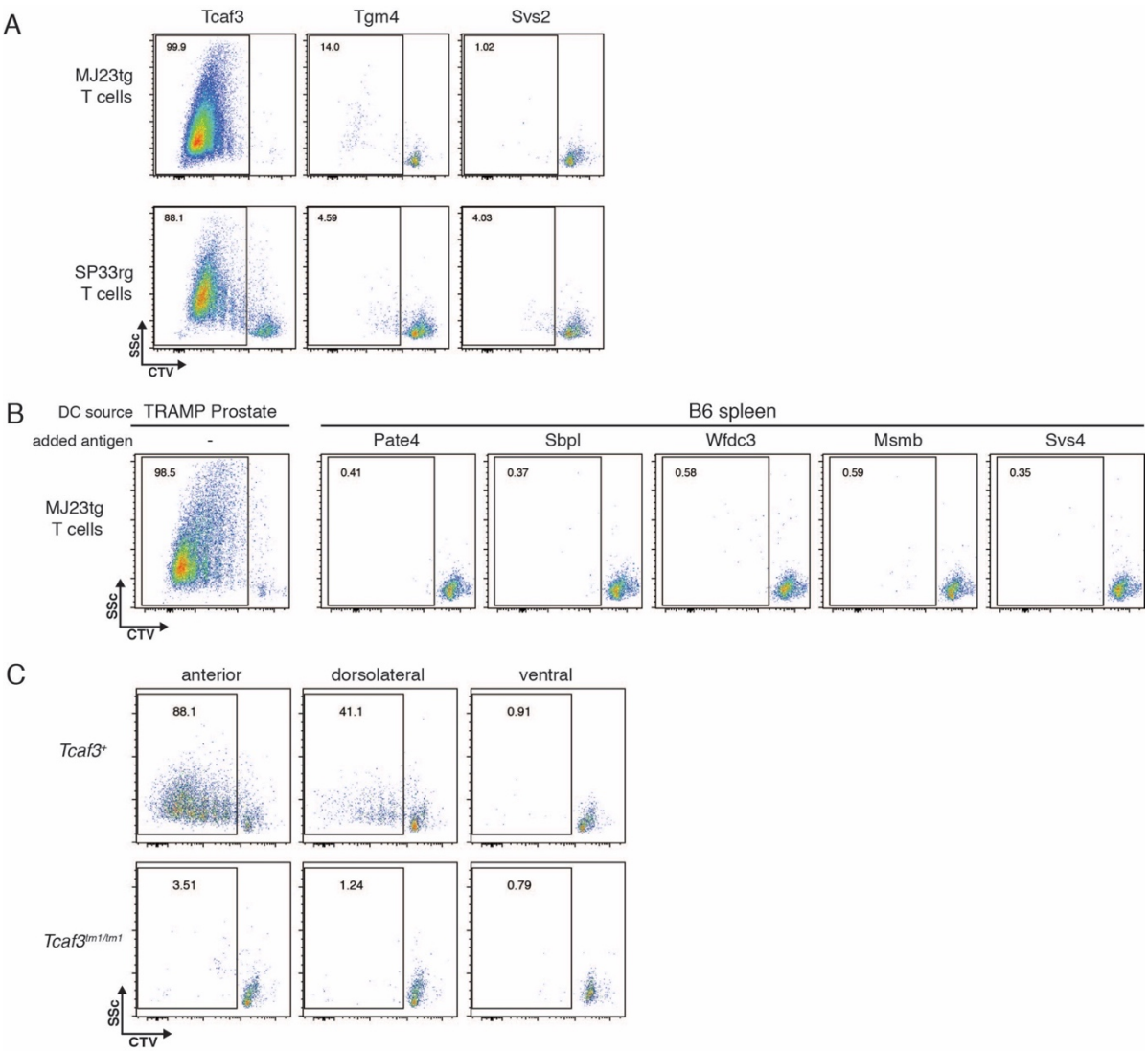

Figure 2: Specific stimulation of MJ23tg and SP33rg T cells by co-culture with dendritic cells and recombinant proteins or prostatic extracts. $CD4^+$ T cells were purified from MJ23tg $Rag1^{-/-}$ $CD45^{.1/.1}$ mice (as in Figure 1) or SP33rg mice (as in Figure 4), labeled with CellTrace-Violet (CTV), and used as a probe for antigen. T cells were co-cultured with 5 x 10^4 $CD11c^+$ cells from B6 spleen (unless otherwise indicated), plus recombinant prostate-associated proteins or prostate secretory extracts. Dilution of CTV was assessed at day 5. (A) In vitro culture of MJ23tg or SP33rg T cells with $CD11c^+$ cells plus 2 µg/mL of the indicated recombinant protein (Tcaf3, Tgm4, or Svs2) produced in insect cells. (B) In vitro culture of MJ23tg T cells with $CD11c^+$ cells plus 10 µg/mL of the indicated recombinant protein (Pate4, Sbpl, Wfdc3, Msmb, and Svs4) produced in E. coli. The leftmost panel depicts the positive control in which MJ23tg T cells were cultured with 1 x 10^4 $CD11c^+$ dendritic cells (DCs) sorted from TRAMP prostate tumors. See also Table S1. (C) In vitro activation of MJ23tg T cells by prostatic secretory extracts. 5 x 10^3 MJ23 T cells were cultured with 5 x 10^4 $CD11c^+$ cells from B6 spleen, plus secretory extracts prepared from the anterior, dorsolateral, or ventral prostate lobes of $Tcaf3^+$ or $Tcaf3^{tm1/tm1}$ males, as indicated. Dilution of CTV was assessed at day 5.

rare antigen-specific T cells were expanded *in vivo* by immunization with peptide demonstrated that Tcaf3 is targeted by autoantibodies in multiple experimental mouse models characterized by prostatic autoimmunity, including neonatal thymectomy[111], castration[131], and Aire deficiency[111]. Concordant with these published results, we recurrently detected serum antibodies reactive to recombinant Tcaf3 protein in Aire-deficient males greater than 10 weeks of age, but not in age-matched, Aire-sufficient controls (Figure 1G). Thus, cumulative evidence demonstrates that Tcaf3 is highly susceptible to recognition by autoantibodies in settings of immune dysregulation.

Identification of Endogenous Tcaf3-specific T Cells Using pMHC-II Tetramers

Having identified the peptide ligand recognized by the MJ23 Treg cell clone, we aimed to directly identify, enumerate, and characterize endogenous, polyclonal $Tcaf3_{646-658}$-specific T cells using pMHC-II tetramers. We generated fluorescently labeled I-A^b tetramers bearing a variant of the antigenic $Tcaf3_{646-658}$ peptide (hereafter referred to as Tcaf3/I-A^b tetramers)[132]. We used a peptide variant in which serine 648, predicted to lie at an MHC-binding anchor position, was changed to tyrosine, a preferred anchor residue at this position[122,133]. This alteration enabled production of stable Tcaf3/I-A^b tetramers, and slightly enhanced the potency of peptide stimulation of MJ23tg T cells *in vitro* (Figure 3A). Tcaf3/I-A^b tetramers bearing this variant peptide stained MJ23tg T cells, but did not bind non-specifically to polyclonal cells (Figure 3B), demonstrating specificity of binding. Using this reagent, we first sought to characterize endogenous, polyclonal $Tcaf3_{646-658}$-

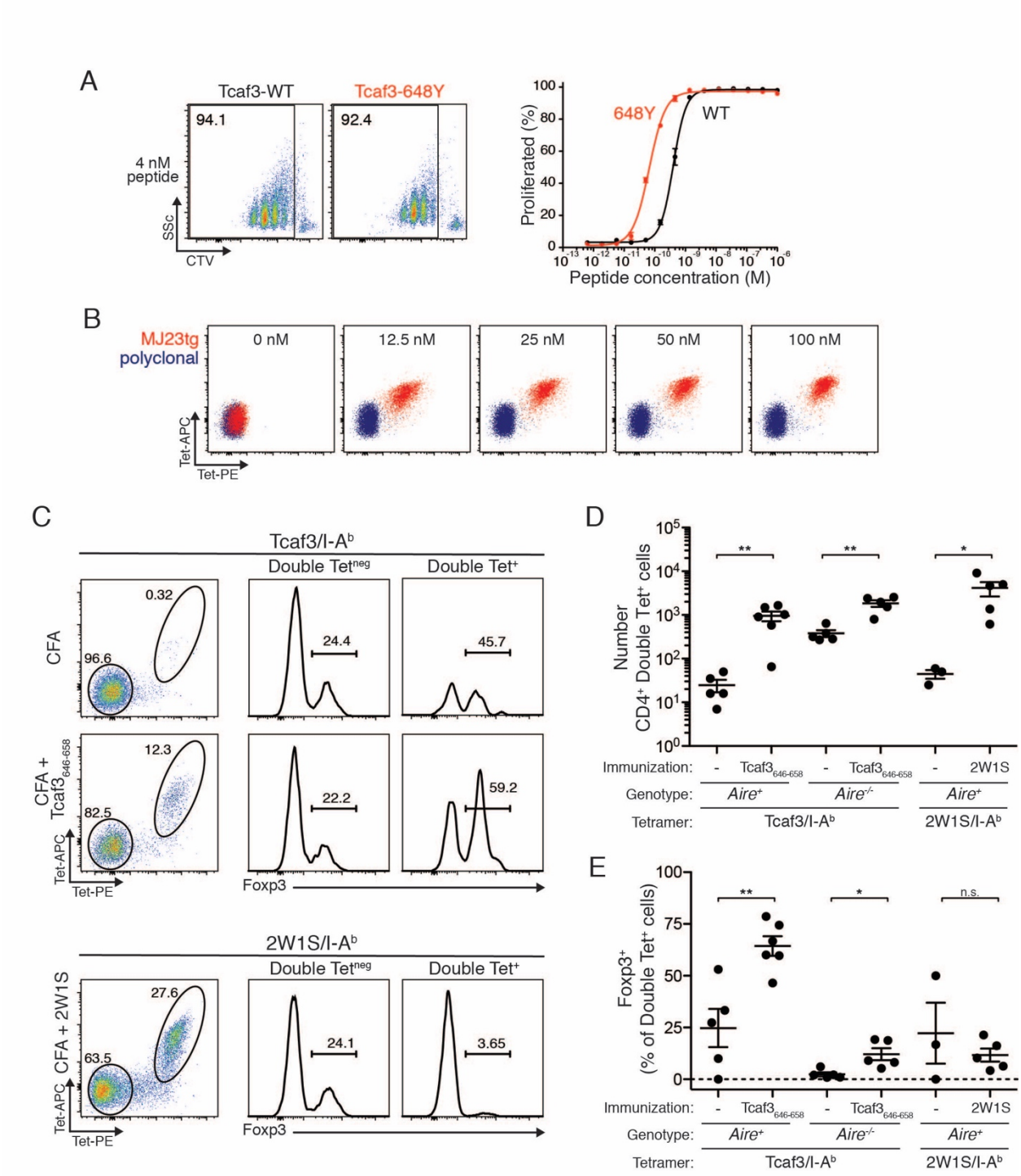

Figure 3: Analysis of Tcaf3-specific MJ23tg T cells using pMHC-II tetramers bearing a $Tcaf3_{646\text{-}658}$ variant. (A) Potency of wild-type and variant Tcaf3 peptides. MJ23tg T cells were stimulated *in vitro* as in Figure 1 with wild-type $Tcaf3_{646\text{-}658}$ peptide (Tcaf3-WT) or $Tcaf3_{646\text{-}658}$ harboring a serine to tyrosine mutation at residue 648 (Tcaf3-648Y). Left, representative flow cytometry plots of *in vitro* cultures with 4 nM peptide, analyzed at day 3. Right, dose response curves fit to a cooperative model. Points denote the mean ± SEM of three replicates. (B) Staining of MJ23tg T cells by Tcaf3/I-A^b tetramers. MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ T cells (red) were spiked into polyclonal CD45$^{.2/.2}$ B6 splenocytes (blue) and co-stained with PE- and APC-labeled tetramers of

Figure 3 continued: I-A^b bearing the Tcaf3-648Y peptide (Tcaf3/I-A^b tetramers) at the indicated concentrations. (C-E) Expansion of endogenous Tcaf3-specific Treg cells by immunization with Tcaf3 peptide plus CFA. B6 (*Aire*$^+$) or *Aire*$^{-/-}$ male mice were immunized with CFA only, $Tcaf3_{646-658}$ peptide in CFA, or 2W1S peptide in CFA, as indicated. 14 days post-challenge, lymphocytes from the pooled spleen and lymph nodes were co-stained with PE- and APC-labeled Tcaf3/I-A^b tetramers or 2W1S/I-A^b tetramers, as indicated, and tetramer-binding cells were magnetically enriched. (C) Representative flow cytometric analysis of Foxp3 expression by magnetically enriched $CD4^+$ T cells. Plots in the left column depict tetramer-PE vs. tetramer-APC staining, with double-tetramer^{neg} and double-tetramer$^+$ gates shown. The middle and right columns present histograms of Foxp3 expression by cells within the double-tetramer^{neg} and double-tetramer$^+$ gates, respectively. (D-E) Summary plots of the tetramer analysis in (C), depicting the total number of double-tetramer$^+$ cells that express Foxp3 (D), and the percentage of double-tetramer$^+$ T cells (E). Data are representative of multiple independent experiments: (A) $N = 5$, (b) $N = 5$, (C-E) $N = 2$. The mean ± SEM is indicated. Significance testing was performed using the nonparametric Mann-Whitney test. * indicates $p < 0.05$.

specific T cells in healthy mice. To do this, we employed an established approach in which plus Complete Freund's Adjuvant (CFA), and expanded T cells were analyzed 14 days later following magnetic enrichment of Tcaf3/I-A^b-tetramer$^+$ T cells from the pooled spleen and lymph nodes[123,125,132]. T cells were co-stained with both PE- and APC-labeled tetramers simultaneously to reduce false-positive staining[134,135]. As a control, we immunized mice with the I-A^b-restricted foreign peptide 2W1S and used 2W1S/I-A^b tetramers to characterize antigen-specific T cells[123]. Challenge of male mice with CFA plus $Tcaf3_{646-658}$ peptide induced expansion of Tcaf3/I-A^b-tetramer$^+$ T cells, yielding an average of ~955 (± 240 SEM) Tcaf3/I-A^b-tetramer$^+$ cells (Figure 3C, 3D). Approximately 64% (± 4.7 SEM) of Tcaf3/I-A^b-tetramer$^+$ T cells in immunized mice expressed Foxp3, a critical Treg cell transcriptional regulator and marker of the Treg cell lineage (Figure 3C, 3E), demonstrating that expanded $Tcaf3_{648-656}$-specific T cells are predominantly found in the Treg cell subset. In contrast, challenge with CFA plus 2W1S peptide yielded ~4,150 (± 1,500 SEM) 2W1S/I-A^b-tetramer$^+$ cells (Figure 3C, 3D), of which only a minor fraction (~11.7% ± 3.2 SEM) expressed Foxp3 (Figure 3C, 3E), consistent with previous studies[132]. Additionally, analysis of *Aire*$^{-/-}$ mice immunized with CFA plus $Tcaf3_{646-658}$ peptide revealed that only a minor fraction of Tcaf3/I-A^b-tetramer$^+$ cells expressed Foxp3 following expansion (Figure 3D, 3E), consistent with previous work demonstrating a role for Aire in directing MJ23 T cells into the Treg cell lineage[80,82]. Thus, our data demonstrate that polyclonal $Tcaf3_{646-658}$-specific T cells can be expanded *in vivo* by immunization with peptide plus CFA, and expanded Tcaf3/I-A^b-tetramer$^+$ T cells are skewed to the Treg cell lineage in wild-type B6 mice.

The Tcaf3-specific MJ23 TCR was originally identified based on recurrent expression of this TCR by Foxp3$^+$ Treg cells infiltrating the prostate tumors of TRAMP mice[80,136]. Given that the MJ23 TCR represents a single Tcaf3$_{646\text{-}658}$-specific TCR clone, it was not previously possible to quantify the total contribution of polyclonal Tcaf3$_{646\text{-}658}$-specific Treg cells to the T cell infiltrate of TRAMP prostate tumor lesions. To address this, we performed tetramer analysis of T cells isolated from 6-7-month-old TRAMP males bearing late-stage prostate tumors. Tcaf3/I-A^b tetramer$^+$ T cells were readily detected in all TRAMP prostate tumors examined, comprising, on average, ~2.1% (± 0.63 SEM) of all tumor-infiltrating Treg cells (Figure 4A, 4B). Consistent with the above results and previous studies of the MJ23 clone[80,82], prostate tumor-infiltrating Tcaf3/I-A^b tetramer$^+$ T cells were strongly skewed to the Foxp3$^+$ Treg cell subset (Figure 4A, 4B).

In previous studies, we demonstrated that Aire is critical for the thymic development of monoclonal MJ23 Treg cells[80], and showed that in the absence of Aire, MJ23 T cells emerge in the Foxp3^{neg} Tconv cell subset and recurrently infiltrate autoimmune prostatic lesions of *Aire*$^{-/-}$ males[82]. We therefore predicted that polyclonal Tcaf3$_{646\text{-}658}$-reactive T cells would exhibit a similar pattern. Consistent with this hypothesis, analysis of Tcaf3/I-A^b tetramer$^+$ cells from the pooled spleen and lymph nodes of *Aire*$^{-/-}$ males immunized with CFA alone or CFA plus Tcaf3$_{646\text{-}658}$ peptide revealed that only a minor fraction of Tcaf3$_{646\text{-}658}$-specific cells expressed Foxp3 (Figure 3D, 3E). Likewise, direct analysis of prostate-infiltrating T cells demonstrated that Tcaf3/I-A^b tetramer$^+$ cells were nearly undetectable in the prostates of 7-8-month-old tumor-free

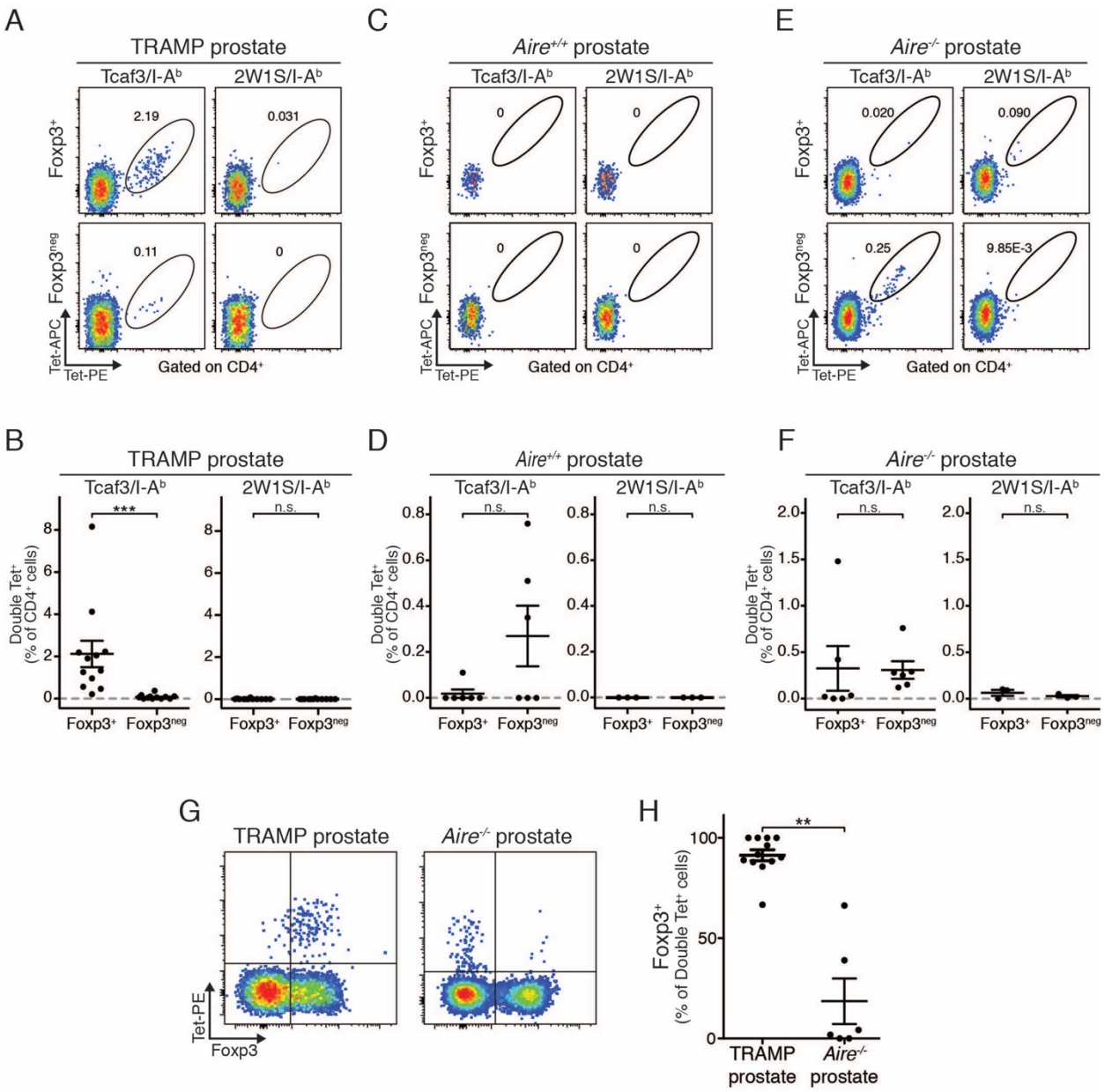

Figure 4: Detection of polyclonal Tcaf3/I-A^b-specific T cells in prostate tumors and prostatic autoimmune lesions. Tcaf3/I-A^b tetramer analysis of T cells from the prostates of tumor-bearing TRAMP males (A-B), *Aire*$^{+/+}$ males (C-D) and *Aire*$^{-/-}$ males (E-F). (A) Representative flow cytometric analysis of CD4$^+$ T cells isolated from the dorsolateral lobe of prostate tumors from 6-7-month old TRAMP$^{+/+}$ males, stained with Tcaf3/I-A^b or negative control 2W1S/I-A^b tetramers. Numbers indicate percentage of cells co-stained with PE- and APC-labeled tetramers (oval gates). (B) Summary plots of tetramer analysis in (A) for $N = 12$ mice. The mean $\pm$ SEM is shown. (C) Representative flow cytometric analysis of tetramer staining, as in (A), of CD4$^+$ cells isolated from the prostates of 7-8-month-old *Aire*$^{+/+}$ males. (D) Summary plots of analysis in (C). (E) Representative flow cytometric analysis of tetramer staining of CD4$^+$ cells isolated from the prostates of 7-8-month-old *Aire*$^{-/-}$ males. (F) Summary plots of analysis in (E).

Figure 4 continued: (G) Representative analysis of Tcaf3/I-A^b tetramer-PE vs. Foxp3 staining of $CD4^+$ T cells from the prostates of TRAMP vs. *Aire*$^{-/-}$ mice. (H) Summary plot of the proportion of Tcaf3/I-A^b double tetramer$^+$ cells (oval gates in (A) and (E)) that express Foxp3. Data are representative of multiple independent experiments: (A-B) $N = 2$, (C-D) $N = 2$, (E-F) $N = 2$. Tetramer analyses were performed with 12.5 nM (*Aire*$^{-/-}$ and *Aire*$^{+/+}$) or 20 nM (TRAMP) of each tetramer. Significance testing was performed using the nonparametric Mann-Whitney test. ** indicates $p < 0.001$; *** indicates $p < 0.0001$; n.s., not significant.

Aire$^{+/+}$ mice, which harbor very few T cells due to a lack of inflammation (Figure 4C, 4D), but were readily detected in the prostates of age-matched *Aire*$^{-/-}$ males (Figure 4E, 4F). Notably, Tcaf3$_{646\text{-}658}$-specific T cells from the prostates of *Aire*$^{-/-}$ mice were strongly skewed to the Foxp3^{neg} Tconv cell subset (Figure 4G, 4H), contrasting with polyclonal Tcaf3-specific T cells infiltrating TRAMP prostate tumors (Figure 4G, 4H). Thus, our cumulative data demonstrate that Aire plays a critical role in directing polyclonal Tcaf3$_{646\text{-}658}$-specific T cells into the Treg cell lineage.

A Second Prostate-Associated Treg Cell Clone Recognizes a Distinct Tcaf3-Derived Peptide

Having identified Tcaf3$_{646\text{-}658}$ as the self peptide recognized by MJ23 Treg cells, we reasoned that a similar approach could be used to identify antigenic peptides recognized by additional Aire-dependent, prostate-specific Treg cell clones. In previous work, we identified multiple T cell clones that are strongly skewed to the Treg cell compartment in *Aire*$^{+/+}$ mice, but are misdirected to become pathogenic, prostate-infiltrating Tconv cells in *Aire*$^{-/-}$ mice[82]. The most abundant of these Aire-dependent, prostate-associated clones, named "SP33" in the current study, expressed a TCRα chain of complementarity determining region 3 (CDR3)α sequence TRAV9D-ALSMSVNYQLI paired with the same fixed transgenic TCRβ chain as the MJ23 clone ([82] and Methods). Of note, compared to the MJ23 TCRα (TRAV14-LYYNQGKLI), the SP33 TCRα chain utilizes a different V region segment and exhibits little sequence similarity within the

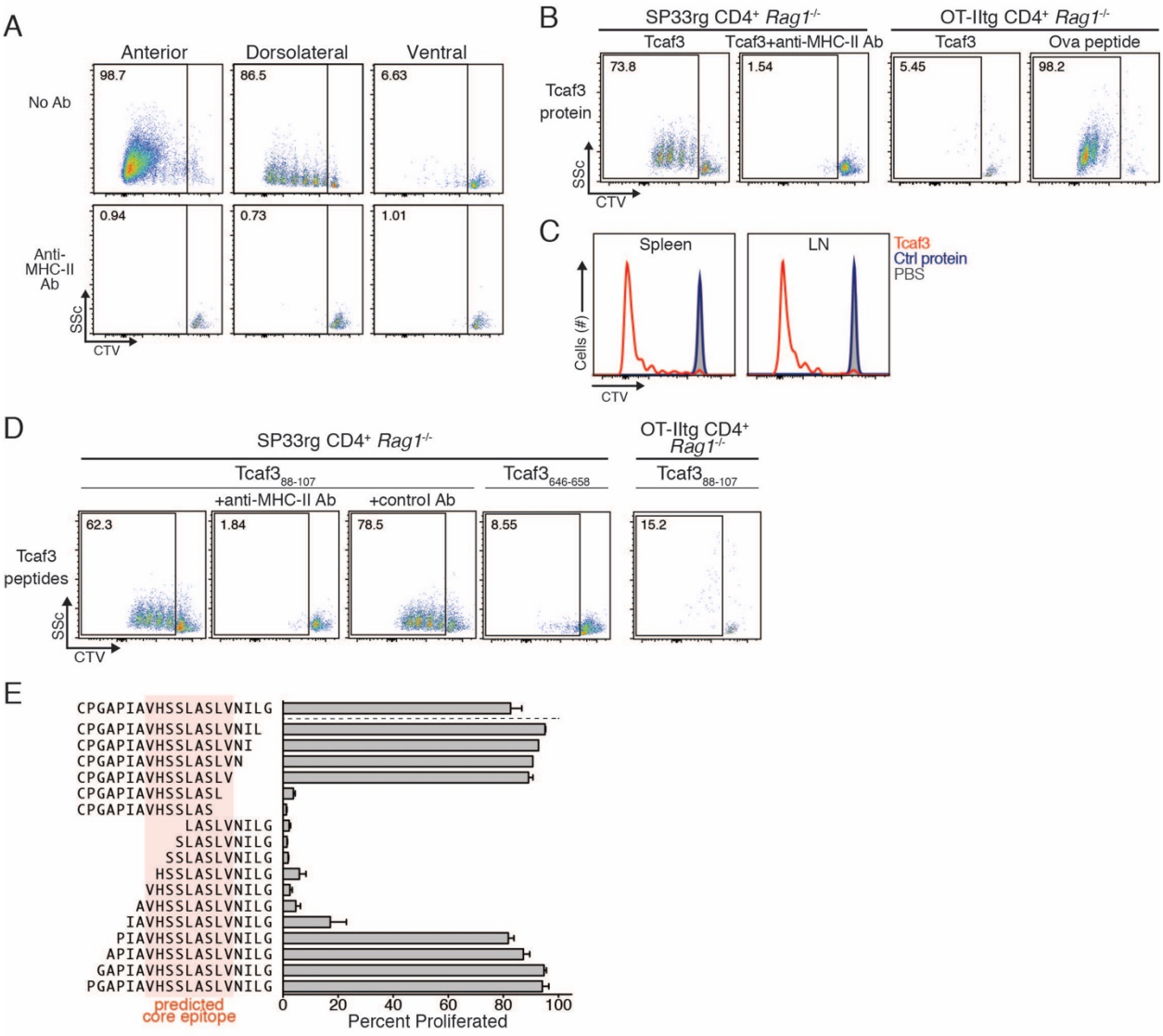

Figure 5: SP33 T cells recognize a distinct epitope derived from the prostatic protein Tcaf3. CD4$^+$ T cells were sorted from SP33rg or OT-IItg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ mice, labeled with CellTrace-Violet (CTV), and used as a probe for antigen. (A) *In vitro* stimulation of SP33rg T cells by prostatic secretory extracts. 1 x 10^4 SP33rg T cells were cultured with 5 x 10^4 CD11c$^+$ cells from B6.SJL spleen, plus secretory extracts prepared from the anterior, dorsolateral, or ventral prostate lobes of tumor-bearing TRAMP males with or without anti-MHC-II blocking antibody. Dilution of CTV was assessed by flow cytometry on day 5. (B) *In vitro* stimulation of SP33rg T cells by Tcaf3 protein. As in (A), SP33rg or OT-IItg T cells were stimulated *in vitro* with 2 µg/mL recombinant Tcaf3 protein or 1 µM Ova$_{323-339}$ peptide, and assayed on day 3. (C) *In vivo* stimulation of SP33rg T cells by Tcaf3 protein. 6.6 x 10^4 MJ23tg T cells were transferred i.v. into congenically disparate B6.SJL female hosts. 2 hours after transfer, recipients were immunized with 5 µg Tcaf3 protein, 5 µg Tgm4 protein, or PBS alone. CD4$^+$ T cells from the spleen (left panel) and pooled skin-draining lymph nodes (right panel) were analyzed for CTV dilution on day 5. (D) *In vitro* stimulation of SP33rg T cells by Tcaf3$_{88-107}$ peptide. As in (A), SP33rg or OT-IItg T cells were stimulated *in vitro* with 5 nM Tcaf3$_{88-107}$ peptide, with or without anti-MHC-II Ab or isotype control, and 5 nM

Figure 5 continued: Tcaf3$_{646-658}$ peptide. Dilution of CTV was analyzed on day 3. (E) Tcaf3$_{88-107}$ peptide truncation analysis. As in (A), SP33rg T cells were stimulated *in vitro* with 33 nM Tcaf3$_{88-107}$ peptide variants, comprising truncations from the N- and/or C-termini. Dilution of CTV was analyzed on day 3. The core nonamer epitope predicted computationally is denoted by red shading. Percent of cells proliferated is shown as the mean ± SEM of three replicates. Data are representative of multiple independent experiments: (A) $N = 3$, (B) $N = 3$, (C) $N = 3$, (D) $N = 3$, (E), $N = 3$. Significance testing was performed using the nonparametric Mann-Whitney test. * indicates $p < 0.05$. See also Figure 2.

CDR3α. To study the peptide specificity of the SP33 TCR, we generated SP33 TCR "retrogenic" (SP33rg) mice[46,126] in which bone marrow progenitors retrovirally transduced with an SP33 TCR expression construct were engrafted into host mice. Using resulting SP33rg T cells as a probe for pMHC-II ligand, we found that DCs cultured with protein extracts from the dorsolateral or anterior prostatic lobes stimulated $CD4^+$ SP33rg T cells *in vitro* (Figure 5A), mirroring the results of MJ23 T cell stimulation assays (Figure 1A). Screening of the candidate prostate-specific proteins described above revealed that Tcaf3, the same protein containing the peptide ligand recognized by MJ23 T cells, robustly stimulated SP33rg T cells *in vitro* (Figure 5B) and *in vivo* (Figure 5C).

Given that the TCRα chains of the MJ23 and SP33 TCRs exhibit little sequence similarity, we predicted that the MJ23 and SP33 TCRs may recognize distinct Tcaf3-derived peptides. Consistent with this, screening of the panel of synthetic Tcaf3 peptides demonstrated that SP33rg T cells were robustly stimulated by a peptide of sequence CPGAPIAVHSSLASLVNILG ($Tcaf3_{88\text{-}107}$) (Figures 5D and 5E), and were not stimulated by the MJ23 agonist peptide THSKAPWGELATD ($Tcaf3_{646\text{-}658}$) (Figure 5D). Taken together, our data demonstrate that two recurrent Treg cell clones, the first identified in mouse prostate tumors (MJ23) and the second identified in prostatic autoimmune lesions in *Aire*$^{-/-}$ mice (SP33), recognize two distinct peptides derived from a single prostate-specific protein, Tcaf3.

DISCUSSION

Due to the technical challenges associated with identifying MHC-II-restricted self peptides, the natural antigens recognized by recurrent thymus-derived Treg cell populations had thus far remained elusive. In this study, we identified endogenous Treg cell ligands in mice, demonstrating that two recurrent Treg cell clones, the first identified in mouse prostate tumors (MJ23) and the second associated with prostatic autoimmune lesions (SP33), recognize distinct non-overlapping peptides derived from a single prostate-specific protein, Tcaf3. By focusing our efforts on Aire-dependent Treg specificities reactive to tissue-specific antigens, we generated a tractable list of candidates using available transcriptional profiling data, which enabled screening for antigenic activity using standard immunological assays. As discussed below, the findings and experimental approach described here have implications for basic Treg cell biology and for the discovery of additional Treg cell ligands in mice and humans.

Previous reports demonstrate that a minor fraction of Foxp3$^+$ Treg cells can be identified within antigen-specific T cell populations reactive to both self and foreign pMHC-II[49,132,137]. However, these populations do not reflect naturally occurring antigen specificities that drive the robust selection of Treg cells in the thymus. For example, immunization with self peptide plus adjuvant is commonly used to induce tissue-specific autoimmunity in animal models such as experimental autoimmune uveitis and experimental autoimmune encephalomyelitis[138,139]. In such models, immunization induces the emergence of both Tconv and Treg cells specific for the antigenic self peptide[86,87,140]. However, in these settings, antigen-specific Treg cells are outnumbered

by pathogenic Tconv cells of the same specificity and fail to prevent autoimmune pathology[86,87,140]. Furthermore, it is unclear whether Foxp3$^+$ Treg cells specific for these self peptides are present in the endogenous repertoire in the absence of peptide immunization. These findings suggest that these self peptides do not efficiently direct T cells into the Treg cell lineage. In contrast to these studies, our previous work using TCR repertoire profiling has demonstrated that at steady state, in the absence of peptide immunization, the Tcaf3-specific T cell clones MJ23 and SP33 are strongly skewed to the Foxp3$^+$ Treg cell lineage[80,82]. The importance of directing these clonotypes into the Treg cell lineage is revealed when this process is dysregulated in *Aire*$^{-/-}$ mice, in which the MJ23 and SP33 clones are misdirected into the Tconv subset and infiltrate autoimmune lesions of the prostate[80]. Our current data using pMHC-II tetramers corroborate and extend these findings, demonstrating that endogenous polyclonal Tcaf3$_{648\text{-}656}$/I-A^b-specific T cells isolated from prostate tumors are strongly biased to the Treg cell subset, and that Tcaf3$_{648\text{-}656}$/I-A^b-specific T cells expanded by immunization with peptide plus CFA are skewed to the Foxp3$^+$ subset. Overall, our cumulative data demonstrate that Tcaf3-derived peptides efficiently direct T cells into the Treg cell lineage, and serve as the antigenic targets of naturally occurring prostate-associated Treg cell populations.

Because little is known about the identity of the self antigens that direct Treg cell development in the thymus, it has not been possible to determine whether the differentiation of a given Treg cell clone relies on the specific recognition of a single agonist ligand, or can be conferred by TCR cross-reactivity to an array of self ligands. By generating *Tcaf3*^{tm1} mice harboring a targeted deletion of the Tcaf3$_{646\text{-}658}$ 13-mer peptide,

we demonstrated that MJ23 Treg cell development was abolished in *Tcaf3*$^{tm1/tm1}$ mice. Thus, the thymic development of this tissue-specific Treg cell clone requires thymic expression of a single self peptide, and cannot be mediated by reactivity to other self ligands presented in the thymus.

The data presented here, combined with evidence from previous studies[80,82], support a model in which the Aire-dependent presentation of Tcaf3-derived peptides directs Tcaf3-specific thymocytes into the Treg cell lineage. In the absence of Aire, Tcaf3-specific T cells, including the MJ23 and SP33 clonotypes, become skewed to the Tconv subset and infiltrate the prostate. Informally, we refer to these cells as "T-rogue cells" – Treg cell-biased clonotypes that "go rogue" in a setting of Aire deficiency[82]. Of note, *Aire*$^{-/-}$ mice also develop autoantibodies against Tcaf3, likely aided by the provision of T cell help by T-rogue cells. Consistent with this notion, a recent study demonstrated that Aire-deficient human subjects harbor highly mutated, high-affinity autoantibodies[141], indicative of T helper cell-dependent affinity maturation. Thus, our data reveal a link between the pMHC-II antigens recognized by Aire-dependent, tissue-specific Treg cells and the protein antigens recognized by autoantibodies in settings of Aire deficiency. Based on this conceptual link, we hypothesize that the specificities of autoantibodies that arise in human subjects with loss-of-function AIRE mutations[141,142] may reveal the specificities of recurrent Aire-dependent Treg cell populations in humans. One such candidate is the prostatic protein Tgm4, which is recurrently targeted by autoantibodies in AIRE-deficient men and *Aire*$^{-/-}$ male mice[141,143]. Broadly speaking, we suggest that this approach may serve as a general strategy to identify the constellation of tissue-specific

self peptides that are targeted by recurrent Treg cell populations relevant to human autoimmune diseases and cancer.

The concept of immunodominance, in which the immune response is focused on a limited set of antigenic determinants, has been demonstrated extensively for effector T cell responses directed at foreign antigens[144,145]. Our finding that two recurrent prostate-associated Treg cell clones recognize distinct peptides derived from a single self protein raises the possibility that the concept of immunodominance may extend to Treg cell antigen recognition at regional sites, and that Treg cell populations at a given site may be largely focused on those autoantigens that are most susceptible to autoimmune attack. Early studies of TCR repertoire complexity in the secondary lymphoid organs have demonstrated that the diversity of the bulk Treg cell repertoire in naive mice is comparable to that of the Tconv cell repertoire[34,128,146], suggesting that the peripheral Treg cell repertoire is not focused on a limited array of antigens. However, a subsequent survey revealed that Treg cell-expressed TCRs are asymmetrically distributed in lymph nodes throughout the body, suggestive of antigen-driven enrichment of distinct Treg cell clones in regional lymph nodes[109]. More recently, Treg cell repertoire analysis in mouse prostate tumors[80] and non-lymphoid organs such as muscle[147] and visceral adipose tissue[107,108] have revealed the enrichment of oligoclonal Treg cell populations at these sites, some of which were recurrent. Integrating this evidence with our current findings, we envision a model of “regional Treg cell immunodominance”, in which select Treg cell specificities are drawn from the diverse peripheral Treg cell pool and enriched and/or expanded in response to inflammatory reactions at different non-lymphoid sites.

In addition to Tcaf3, there are other studies in the literature describing autoimmune responses converging on a single tissue-specific autoantigen. For example, it has been shown that Aire deficiency in mice on the B6 background leads to the development of effector T cell and autoantibody responses directed at unique structures of the eye[68,75]. Intriguingly, the development of uvea-specific autoimmunity in *Aire*$^{-/-}$ mice is dependent on the peripheral expression of a single target antigen, retinol binding protein 3 interstitial (Rbp3, also known as interphotoreceptor retinoid binding protein [IRBP][77]). Moreover, in an *Aire*$^{+/+}$ setting, deficiency of Rbp3 specifically in the thymus is sufficient to induce uveitis[77]. Although it remains unclear whether T-rogue cells specific for Rbp3 are implicated in this system, the results support the idea that provocation of autoimmune responses to the eye in mice may be uniquely focused on a limited number of autoantigens such as Rbp3.

Finally, the identification of endogenous self peptides recognized by Aire-dependent tTreg populations will enable the interrogation of fundamental questions in Treg cell biology which have thus far been experimentally intractable. First, the role of cognate antigen in driving the thymic development and peripheral homeostasis of Tcaf3-specific Treg cells, and the role of TCR-pMHC affinity in coordinating these processes can be addressed in future studies. Second, the establishment of Tcaf3/I-A^{b} tetramers will permit the enumeration and phenotypic analysis of endogenous Tcaf3-specific T cells at different stages of ontogeny and in various settings of health and disease. Third, our findings will enable studies to determine the structural and biochemical basis of antigen recognition by the MJ23 and SP33 TCRs, to determine whether tTreg TCRs recognize

self antigen with unique binding characteristics. Lastly, the Tcaf3 system may provide a model for the development of additional approaches for the treatment of autoimmune or inflammatory disorders based on the selective *in vivo* expansion of organ-specific Treg cells, and the recruitment of such cells to inflamed sites.

Chapter IV: Role of Antigen in the Development and Homeostasis of Antigen-Specific Regulatory T Cells

ABSTRACT

The display of agonist peptide ligands in the thymus, driven in part by Aire, can promote both the clonal deletion of antigen-specific T cells and the differentiation of such cells into the Treg cell lineage. Here, through the study of T cells reactive to a natural Aire-dependent peptide ligand in wild-type mice and gene-targeted mice lacking this epitope, we define the role of cognate antigen expression in directing these alternate cell fates and preventing prostate infiltration by antigen-specific T cells. We show that expression of this peptide ligand in the thymus does not drive clonal deletion of monoclonal T cells. Instead, our data demonstrate that peptide antigen is required to direct antigen-specific cells into the Treg cell lineage, and that skewing of this single specificity to the Treg compartment is critical for the prevention of prostate-specific T cell infiltration.

INTRODUCTION

During T cell development in the thymus, each T cell precursor generates a unique T cell antigen receptor (TCR). TCR gene segments randomly recombine to allow for recognition of a wide array of potential pathogens. However, due to the stochastic recombination and insertion of random nucleotides, many T cell precursors express TCRs conferring overt reactivity to self peptide-MHC (pMHC) ligands. The immune system, therefore, must have robust mechanisms to keep these potentially harmful cells in check.

Deletional tolerance is one form of recessive tolerance by which self-reactive T cells undergo deletion in the thymus, thereby eliminating these potentially harmful cells from the repertoire. However, the process of clonal deletion is imperfect, necessitating additional mechanisms of tolerance to restrict the activation of autoreactive T cells. Dominant tolerance is enforced in part by Foxp3^{+} regulatory T cells (Tregs), which act *in trans* to suppress autoreactive T cells that escape clonal deletion. Treg-mediated suppression is required for the prevention of spontaneous autoimmunity, as illustrated by the development of fatal systemic in human subjects and mutant mice harboring loss-of-function mutations in *FOXP3*/*Foxp3* as well as following sustained Treg cell ablation[17] in mice. Loss of tolerance is associated with a variety of autoimmune diseases such as lupus, rheumatoid arthritis, and diabetes. Therefore, there is a critical need to understand the mechanisms that drive both recessive and dominant T cell tolerance.

The transcription factor Autoimmune regulator (Aire) drives the ectopic expression of peripheral tissue-restricted antigens (TRAs) in medullary thymic epithelial cells (mTECs)[66,68,74]. The presentation of TRAs is necessary to prevent organ-specific autoimmunity at distinct peripheral sites. Available evidence suggests that Aire can promote both the clonal deletion of TRA-specific T cells[76,148] and the differentiation of antigen-specific T cells into the Treg cell lineage[70,80]. However, the factors that induce Treg cell differentiation opposed to clonal deletion in the thymus remain undefined.

Early studies using double transgenic mice expressing transgenic TCRs reactive to model antigens demonstrated that agonist ligand expression in the thymus was sufficient to drive both clonal deletion, and the emergence of a minor fraction of Foxp3^{+}

cells that evade deletion[56]. However, the use of high affinity transgenic TCRs and model antigens in these studies may not faithfully recapitulate the natural process of antigen expression with respect to ligand density, the nature of the antigen presenting cells displaying antigenic ligands, and the affinity of TCR-pMHC interactions.

The role antigen plays in maintenance of endogenous Treg populations is not well established. In the periphery, many Treg cells perceive strong TCR signals[44] and proliferate[104,105] at steady state, suggesting that a large fraction of peripheral Tregs recognize agonist ligands in the absence of inflammation. Additionally, Lathrop et al.[109] demonstrated an asymmetric distribution of Tregs throughout the body, providing further support for antigen driven enrichment. We have previously shown enrichment of a naturally occurring prostate-specific Treg clone, MJ23, in the prostate draining lymph nodes, which is abolished following castration and prostate involution[71]. This is consistent with previous reports that Tregs from the prostate draining lymph nodes are more efficient at suppressing prostatitis than Tregs from distal lymph nodes[112,149].

Recently, we reported the identification of a natural Treg cell ligand recognized by the recurrent prostate-specific Treg clone, MJ23[119]. MJ23 Tregs recognize an unmodified peptide derived from the prostate-specific protein, Tcaf3, for which thymic expression is Aire-dependent[74,119]. In the previous study, we showed that the $Tcaf3_{646\text{-}658}$ peptide was required for the development of monoclonal MJ23 Treg cells in the thymus, and that Tcaf3/I-A^b tetramers could be used to detect polyclonal T cells reactive to this peptide in multiple mouse models of prostatic inflammation[119]. Here, using Tcaf3/I-A^b tetramers and mice harboring a targeted deletion of the $Tcaf3_{646\text{-}658}$ epitope, termed *Tcaf3*$^{tm1/tm1}$ mice,

we examine the impact of self-ligand recognition on clonal deletion and differentiation into the Treg lineage, and the impact of these processes on prostate-specific autoimmunity.

RESULTS

Tcaf3$_{646-658}$ Peptide is Required for Thymic Development of Polyclonal Antigen-Specific Tregs

In previous work, we demonstrated that the Tcaf3$_{646-658}$ peptide is required for the thymic development of monoclonal MJ23tg T cells[119]. Here, we sought to extend these findings by determining if this requirement is also applicable for diverse polyclonal specificities. To do this, we used a previously developed Tcaf3$_{646-658}$/I-A^b tetrameric staining reagent (hereafter referred to as Tcaf3/I-A^b tetramers)[119] to analyze thymocytes from 4-6 week old naïve mice. To maximize the sensitivity of detection, we used a dual-staining approach in which cell suspensions were stained concurrently with both PE- and APC-labeled Tcaf3/I-A^b tetramers[134,135].

We analyzed the thymi of Tcaf3$_{646-658}$ sufficient (*Tcaf3*$^{+/+}$ and *Tcaf3*$^{+/tm1}$, hereafter called *Tcaf3*$^+$ mice collectively) and Tcaf3$_{646-658}$ deficient (*Tcaf3*$^{tm1/tm1}$) mice. First, we analyzed the thymi of *Tcaf3*$^+$ male and female mice to determine if Tcaf3/I-A^b tetramer$^+$ cells could be detected. Using these tetramers, we found a rare population of Tcaf3$_{646-658}$-specific cells within the thymi of both male and female *Tcaf3*$^+$ mice (Figure 6A-B), a fraction of which expressed Foxp3 (Figure 6A and C). In contrast, Tcaf3/I-A^b tetramer$^+$ cells expressing Foxp3 were not detected in the thymi of *Tcaf3*$^{tm1/tm1}$ mice (Figure 6A and C), indicating that peptide antigen is broadly required for the thymic development of

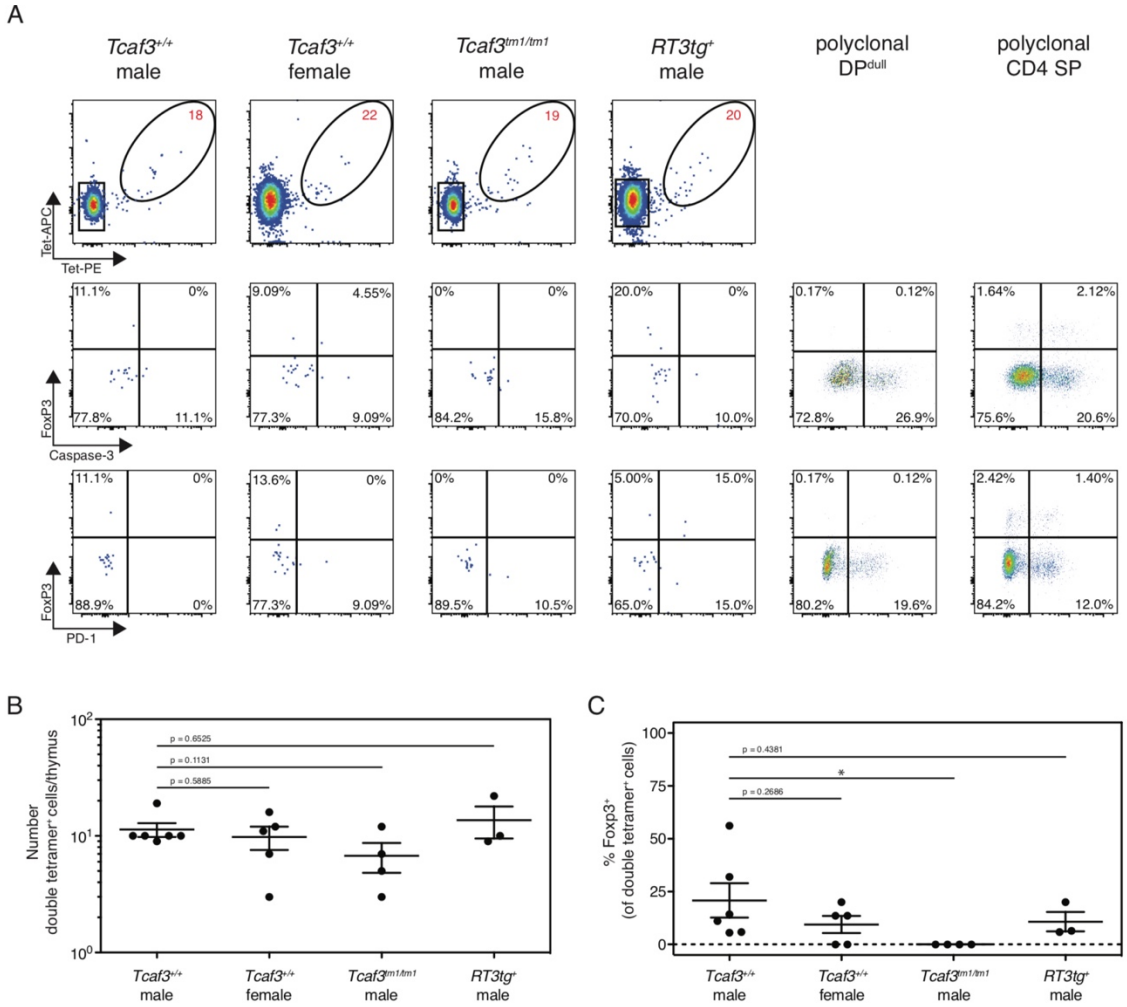

Figure 6: Tcaf3$_{646-658}$ peptide directs polyclonal antigen-specific T cells into the Treg cell lineage with negligible clonal deletion. Thymi from 4-6 week old naïve mice of denoted genotypes were pooled and stained with APC- and PE-labeled Tcaf3/I-A^b tetramer followed by magnetic enrichment using AutoMACS. (A) Representative flow-cytometric analysis of Foxp3 development and clonal deletion markers of enriched CD4$^+$ T cells. Plots in the top row depict tetramer-APC versus tetramer-PE staining and show double-tetramer$^+$ and double-tetramer^{neg} gates. The number of cells within the double-tetramer$^+$ gate is indicated. The second and third row depict Foxp3 versus Caspase-3 and Foxp3 versus PD-1 expression, respectively, in double-tetramer$^+$ or polyclonal cells. The percentage of cells falling within the denoted gate is indicated. (B and C) Summary plots of (B) the number of double-tetramer$^+$ cells per thymi and (C) the percentage of Foxp3$^+$ cells among the double-tetramer$^+$ cells. Data are representative of multiple independent experiments; N = 6. The mean ± SEM is indicated. Significance testing was performed using the nonparametric Mann-Whitney test. * indicates $p < 0.05$

of tetramer^{+} cells in the thymus and the fact that each analysis represents a snapshot in time, it remains unclear from these analyses whether Tcaf3 peptide is absolutely required for the development of all Tcaf3$_{646\text{-}658}$-specific Treg cells, or whether there are rare Tcaf3$_{646\text{-}658}$-specific Tregs that can develop in the absence of cognate antigen due to cross-reactivity with other self-ligands.

Additionally, we analyzed the thymus of RT3tg mice, which express the fixed TCRβ chain used by MJ23tg cells[80], to determine if these mice had a higher frequency of Tcaf3/I-A^{b} thymocytes[80]. Notably, the number of Tcaf3/I-A^{b} tetramer^{+} cells was similar in all the mice analyzed (Figure 6B) regardless of antigen expression in the thymus.

The Thymic Development of Monoclonal MJ23 Tcaf3$_{646\text{-}658}$-specific Cells is not Impacted by Clonal Deletion

The Tcaf3$_{646\text{-}658}$ peptide was previously shown to be the natural peptide epitope recognized by MJ23 T cells *in vivo*[119]. Since Aire has been proposed to promote both negative selection and Treg cell generation, we first sought to determine if thymic expression of Tcaf3$_{646\text{-}658}$ peptide drives both of these alternate cell fates concurrently. To do this, we analyzed thymic development of monoclonal MJ23tg T cells in *Tcaf3*$^{+}$ and *Tcaf3*$^{tm1/tm1}$ mice. We isolated thymocytes from MJ23tg *Rag1*$^{-/-}$ CD45.1^{+} females, which harbor no Foxp3^{+} cells due to intraclonal competition[80], and injected them intrathymically into *Tcaf3*$^{+}$ or *Tcaf3*$^{tm1/tm1}$ CD45.2^{+} hosts (Figure 7A-B). As previously shown[119], MJ23tg cells readily developed into Treg cells in the thymus of *Tcaf3*$^{+}$ hosts but not *Tcaf3*$^{tm1/tm1}$ hosts. To assess clonal deletion, we also stained MJ23tg thymocytes with antibodies

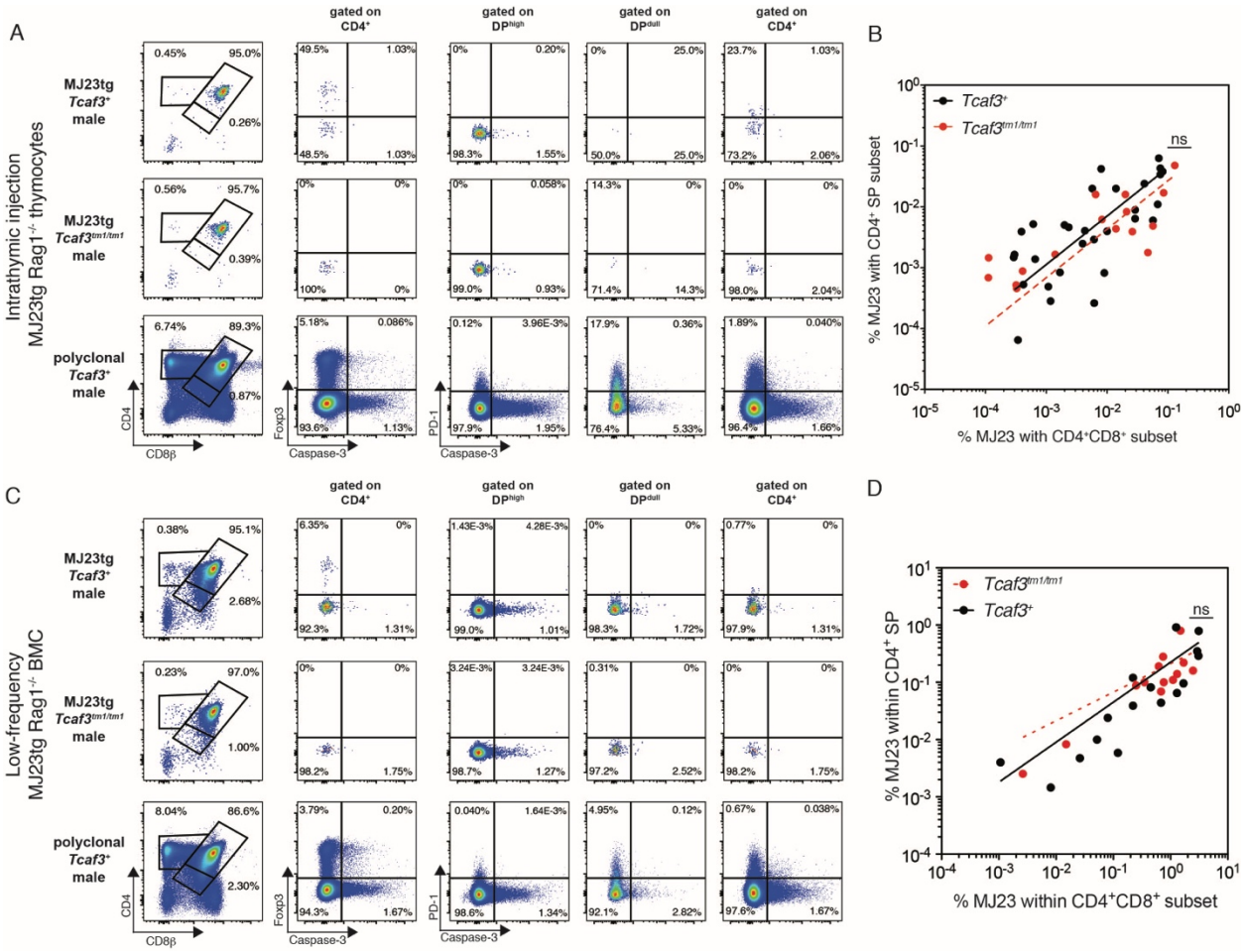

Figure 7: Clonal deletion does not impact the thymic development of monoclonal Tcaf3$_{646-658}$ specific MJ23 Treg cells. Analysis of thymi of intrathymic injection (A and B) or low frequency bone marrow chimera (C and D) in *Tcaf3*$^{+/+}$, *Tcaf3*$^{+/tm1}$, or *Tcaf3*$^{tm1/tm1}$ CD45$^{.2/.2}$ hosts. (A and B) Bulk MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ thymocytes from females were intrathymically injected into 4-8 week old *Tcaf3*$^{+/+}$, *Tcaf3*$^{+/tm1}$, or *Tcaf3*$^{tm1/tm1}$ CD45$^{.2/.2}$ hosts. Mice were analyzed 7 days later for expression of Foxp3, active-caspase-3, and PD-1 as makers of Treg development and clonal deletion. (A) Representative flow-cytometric plots of male *Tcaf3*$^{+}$ or *Tcaf3*$^{tm1/tm1}$ hosts of intrathymic injections. The left column depicts CD4 versus CD8 expression of MJ23tg cells or polyclonal cells respectively. The percentage of cells falling within the CD4 SP, DP^{high}, and DP^{dull} gate is indicated. The second column depicts Foxp3 expression versus active-caspase-3 expression of CD4^{+} thymocytes after CD8α depletion. The remaining columns depict PD-1 and active-caspase-3 expression of DP^{high}, DP^{dull}, and CD4 SP cells falling within the gates on the left. (B) Clonal deletion driven by antigen expression was assessed in *Tcaf3*$^{+/+}$, *Tcaf3*$^{+/tm1}$, or *Tcaf3*$^{tm1/tm1}$ hosts that received intrathymically injected MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ thymocytes. The frequency of MJ23tg cells present within the CD4^{+}CD8^{+} DP subset versus the frequency of MJ23tg cells present within the CD4^{+} SP subset. A lower frequency of MJ23tg cells within the CD4^{+} SP subset of *Tcaf3*$^{+/+}$ or *Tcaf3*$^{+/tm1}$ would indicate clonal deletion driven by expression of cognate antigen. Black circles – *Tcaf3*$^{+}$ hosts; red circles – *Tcaf3*$^{tm1/tm1}$ hosts. (C and D) Bone marrow chimeras were generated by reconstitution of sub-lethally irradiated 4-6 week old *Tcaf3*$^{+/+}$, *Tcaf3*$^{+/tm1}$, or *Tcaf3*$^{tm1/tm1}$ CD45$^{.2/.2}$ hosts with *Tcaf3*$^{+/+}$ bone marrow and a low frequency of MJ23tg

Figure 7 continued: *Rag1*$^{-/-}$ CD45$^{.1/.1}$ bone marrow. Mice were analyzed 6 weeks after bone marrow chimera generation. (C) Representative flow-cytometric plots of male *Tcaf3*$^{+}$ or *Tcaf3*$^{tm1/tm1}$ hosts. Columns represent populations described above (A). (D) As in (B) clonal deletion was assessed in *Tcaf3*$^{+/+}$, *Tcaf3*$^{+/tm1}$, or *Tcaf3*$^{tm1/tm1}$ hosts that received MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ bone marrow. Black circles – *Tcaf3*$^{+}$ hosts; red circles – *Tcaf3*$^{tm1/tm1}$ hosts. Data are representative of multiple independent experiments. (A-B) N = 6. (C-D) N = 2. Nonlinear regression analysis. Significance testing was performed using the Extra sum-of-squares F test; ns indicates not significant.

specific for active-caspase-3, a hallmark of T cell precursors undergoing apoptosis[45]. Notably, the fraction of MJ23tg thymocytes that stained positive for active-caspase-3 was minimal, and was comparable in *Tcaf3*$^{+}$ hosts and *Tcaf3*$^{tm1/tm1}$ hosts lacking the antigen, indicating that clonal deletion plays a negligible role in driving thymic deletion of this monoclonal T cell specificity.

In the same intrathymic injection experiments, we quantified the fraction of MJ23tg thymocytes making the transition from the CD4^{+}CD8^{+} double-positive (DP) stage to the CD4 single-positive (SP) stage of thymic development (Figure 7B), a transition that reflects the migration of maturing thymocytes into the medulla, where Aire is expressed. To do this, for each mouse we plotted the prevalence of MJ23tg cells as a percentage of all CD4 SP thymocytes vs. the percentage of MJ23tg cells amongst DP thymocytes. If MJ23tg T cells were impacted by deletion, the data in such a plot would be expected to exhibit a downward shift in the presence of antigen in *Tcaf3*$^{+}$ mice (when compared to similar plots in *Tcaf3*$^{tm1/tm1}$ mice), reflecting the deletion of cells between the DP and CD4 SP stages. However, scatter plots revealed that the transition of MJ23tg precursors from the DP to CD4 SP was not impacted by the presence of antigen in *Tcaf3*$^{+}$ hosts, suggesting that clonal deletion plays a negligible role in shaping the thymic development of MJ23tg T cells.

In addition, we performed complementary experiments involving bone marrow chimeric mice (BMC) in which sublethally irradiated *Tcaf3*$^{+}$ or *Tcaf3*$^{tm1/tm1}$ CD45.2^{+} mice were seeded with MJ23tg *Rag1*$^{-/-}$ CD45.1^{+} bone marrow cells at a low frequency, and MJ23tg T cell development was assessed 6-8 weeks later. As previously shown[80], MJ23tg

cells developed into Tregs in thymus of *Tcaf3*$^{+}$ hosts but not *Tcaf3*$^{tm1/tm1}$ hosts (Figure 7C-D). As in the intrathymic injection experiments, active-capase-3 expression on MJ23tg cells is not elevated compared to polyclonal cells (Figure 7C). Additionally, the frequency of MJ23tg thymocytes making the transition from the $CD4^{+}CD8^{+}$ DP stage to the CD4 SP stage is not impacted by the presence of the $Tcaf3_{646\text{-}658}$ peptide (Fig 7D).

Together, these results demonstrate that thymic expression of peptide drives development of monoclonal MJ23tg Tregs, with little evidence of concurrent clonal deletion.

Impact of Global $Tcaf3_{646\text{-}658}$ Deficiency on Antigen-Specific T Cells in the Periphery

Having analyzed the role of $Tcaf3_{646\text{-}658}$ antigen in the thymus, we next assessed the impact of $Tcaf3_{646\text{-}658}$ deficiency on the skewing of antigen-specific T cells to the Treg lineage in the periphery and the distribution of such cells in the secondary lymphoid organs. Previously, we utilized low-frequency MJ23tg BMCs to demonstrate that Aire is required to direct MJ23tg T cells into the Treg cell lineage[80]. However, given that Aire deficiency is associated with organ-specific inflammatory reactions in the periphery, and various thymic defects including alterations of thymic architecture and reduced density of distinct DC populations[150,151], we aimed to repeat these experiments in healthy *Tcaf3*$^{tm1/tm1}$ mice, which lack only a single peptide antigen and are devoid of the confounding conditions observed in *Aire*$^{-/-}$ mice.

To determine the role of antigen in the periphery, we analyzed the periphery of low frequency MJ23tg BMCs. MJ23tg cells were found in the periphery of both *Tcaf3*$^{+}$ and *Tcaf3*$^{tm1/tm1}$ mice (Figure 8A), however, they only express Foxp3 in *Tcaf3*$^{+}$ mice (Figure

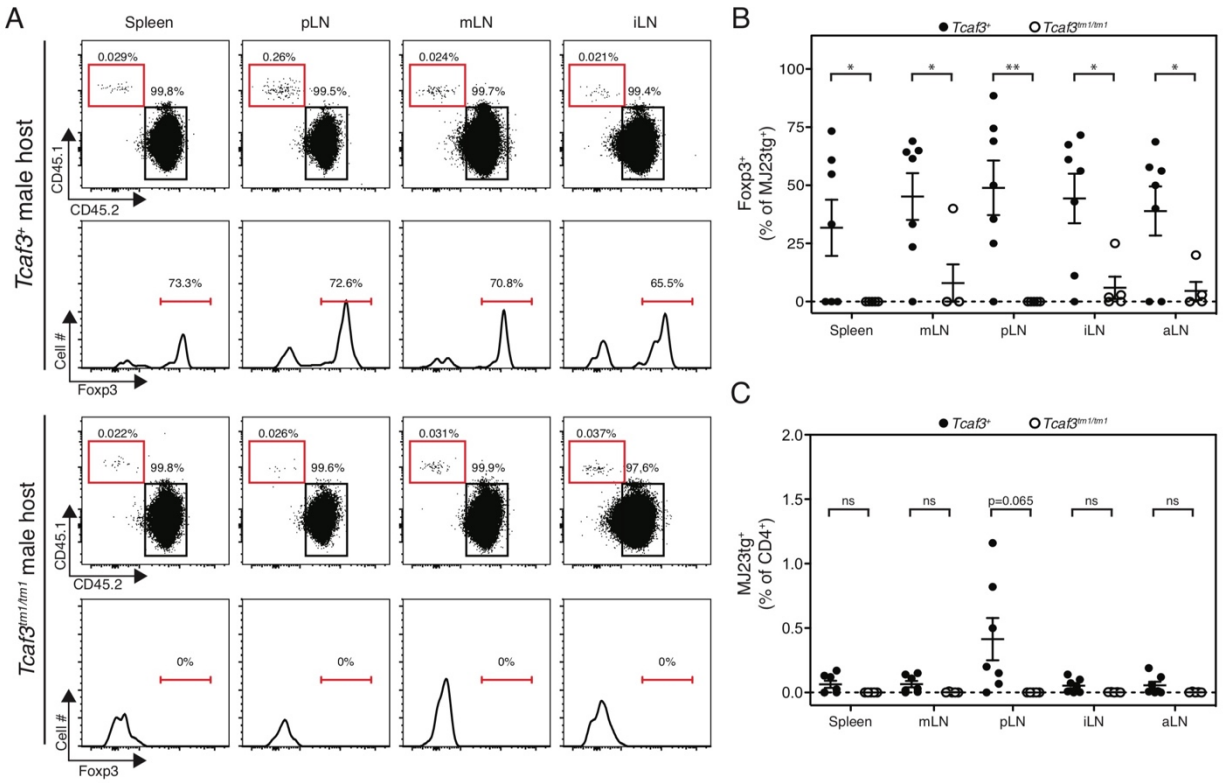

Figure 8: Global deficiency of the Tcaf3$_{646-658}$ peptide abrogates enrichment of MJ23 Tregs in the pLN of male mice. (A) Representative flow-cytometric analysis of *Tcaf3*$^{+}$ or *Tcaf3*$^{tm1/tm1}$ mice 6 weeks after bone marrow engraftment. The top row shows the expression of CD45.1 (red gate) and CD45.2 (black gate) by CD4^{+} T cells from denoted secondary organs within a *Tcaf3*$^{+}$ male host. The second row shows expression of Foxp3 by CD45.1^{+} MJ23tg cells falling within the red gate. Plots are repeated below within a *Tcaf3*$^{tm1/tm1}$ male host. (B and C) Summary plots of (B) the percentage of Foxp3^{+} cells within the MJ23tg^{+} subset of each site and (C) the percentage of MJ23tg^{+} cells within the CD4^{+} population found in peripheral lymphoid sites in male hosts. Closed circles – *Tcaf3*$^{+}$ hosts; open circles *Tcaf3*$^{tm1/tm1}$ hosts. Organ sites: spleen, mLN – mesenteric lymph node, pLN – periaortic lymph node, iLN – inguinal lymph node, aLN – axillary lymph node. Data are representative of multiple independent experiments; N = 3. The mean ± SEM is indicated. Significance testing was performed using the nonparametric Mann-Whitney test. * indicates $p < 0.05$, ** indicates $p < 0.01$, ns indicates p is not significant.

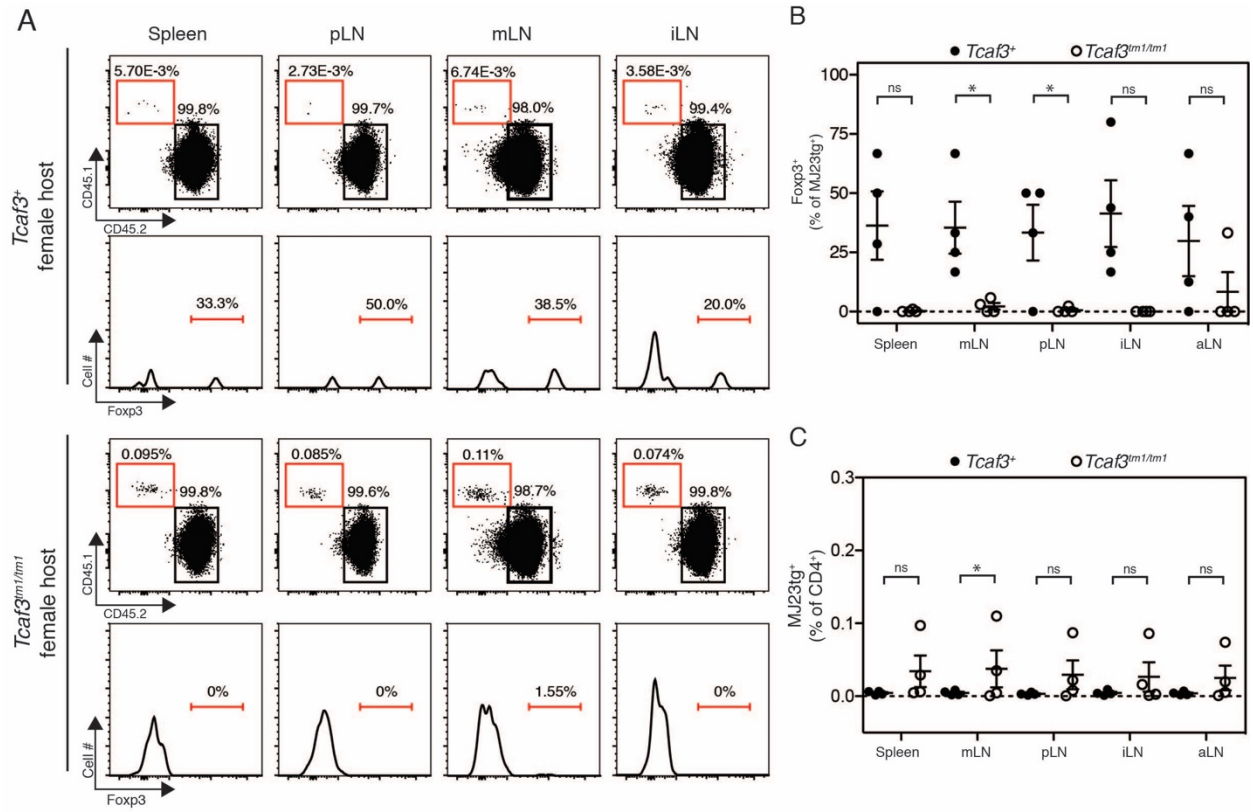

Figure 9: Global deficiency of the Tcaf3$_{646-658}$ peptide leads to skewing of monoclonal Tcaf3$_{646-658}$-specific T cells in female mice. Peripheral analysis of low frequency MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$ bone marrow chimeras in *Tcaf3*$^{+/+}$, *Tcaf3*$^{+/tm1}$, or *Tcaf3*$^{tm1/tm1}$ CD45$^{.2/.2}$ female mice. (A) Representative flow-cytometric analysis of *Tcaf3*$^{+}$ or *Tcaf3*$^{tm1/tm1}$ female mice 6 weeks after bone marrow engraftment. The top row shows the expression of CD45.1 (red gate) and CD45.2 (black gate) by CD4^{+} T cells from denoted secondary lymphoid organs within a *Tcaf3*$^{+}$ female host. The second row shows expression of Foxp3 by CD45.1^{+} MJ23tg cells falling within the red gate. Plots are repeated below within a *Tcaf3*$^{tm1/tm1}$ female host. (B and C) Summary plots of (B) the percentage of Foxp3^{+} cells within the MJ23tg^{+} subset of each site and (C) the percentage of MJ23tg^{+} cells within the CD4^{+} population found in peripheral lymphoid sites in male hosts. Closed circles – *Tcaf3*$^{+}$ hosts; open circles *Tcaf3*$^{tm1/tm1}$ hosts. Organ sites: spleen, mLN – mesenteric lymph node, pLN – periaortic lymph node, iLN – inguinal lymph node, aLN – axillary lymph node. Data are representative of multiple independent experiments; N = 3. The mean ± SEM is indicated. Significance testing was performed using the nonparametric Mann-Whitney test. * indicates $p < 0.05$, ns indicates p is not significant.

8B) in agreement with the thymic data presented in Figure 6. MJ23tg cells were found throughout the body of *Tcaf3*$^{+}$ and *Tcaf3*$^{tm1/tm1}$ mice, and Foxp3 expression was observed in both male and female *Tcaf3*$^{+}$ hosts (Figure 9).

MJ23 Tregs were enriched in the prostate-draining lymph node (pLN) of *Tcaf3*$^{+}$ males. In contrast, enrichment in the pLN of *Tcaf3*$^{tm1/tm1}$ males and female mice (Figure 9A-C) was not observed due to the absence of antigen in the periphery of these mice. Together these data demonstrate that expression of antigen is necessary for proper localization and enrichment of monoclonal MJ23 Treg cells.

Global Deficiency of the Tcaf3$_{646-658}$ Peptide Abolishes Skewing of Polyclonal Antigen-Specific T Cells to the Treg Cell Lineage

In order to characterize endogenous Tcaf3$_{646-658}$-specific cells in the periphery, we employed a strategy in which antigen-specific cells are expanded by immunization with CFA plus Tcaf3$_{646-658}$ peptide, and Tcaf3/I-A^{b} tetramer^{+} cells are analyzed 14 days later[123,125,132]. Using this approach, we assessed the impact of Tcaf3 peptide deficiency by comparing the response to immunization in wild-type *Tcaf3*$^{+}$ males to the response in *Tcaf3*$^{tm1/tm1}$ males. Our data revealed two findings of note. First, in *Tcaf3*$^{+}$ males, Tcaf3/I-A^{b} tetramer^{+} cells were consistently skewed to the Foxp3^{+} subset, with Foxp3^{+} Treg cells predominating in most mice (Figure 10A and C). In contrast, global deficiency of the Tcaf3$_{646-658}$ peptide abolished this skewing, resulting in tetramer^{+} populations in which Foxp3-negative cells predominated (Figure 10A and C). It is not clear whether the Foxp3^{+} cells observed following peptide immunization of *Tcaf3*$^{tm1/tm1}$ mice represent rare thymus-

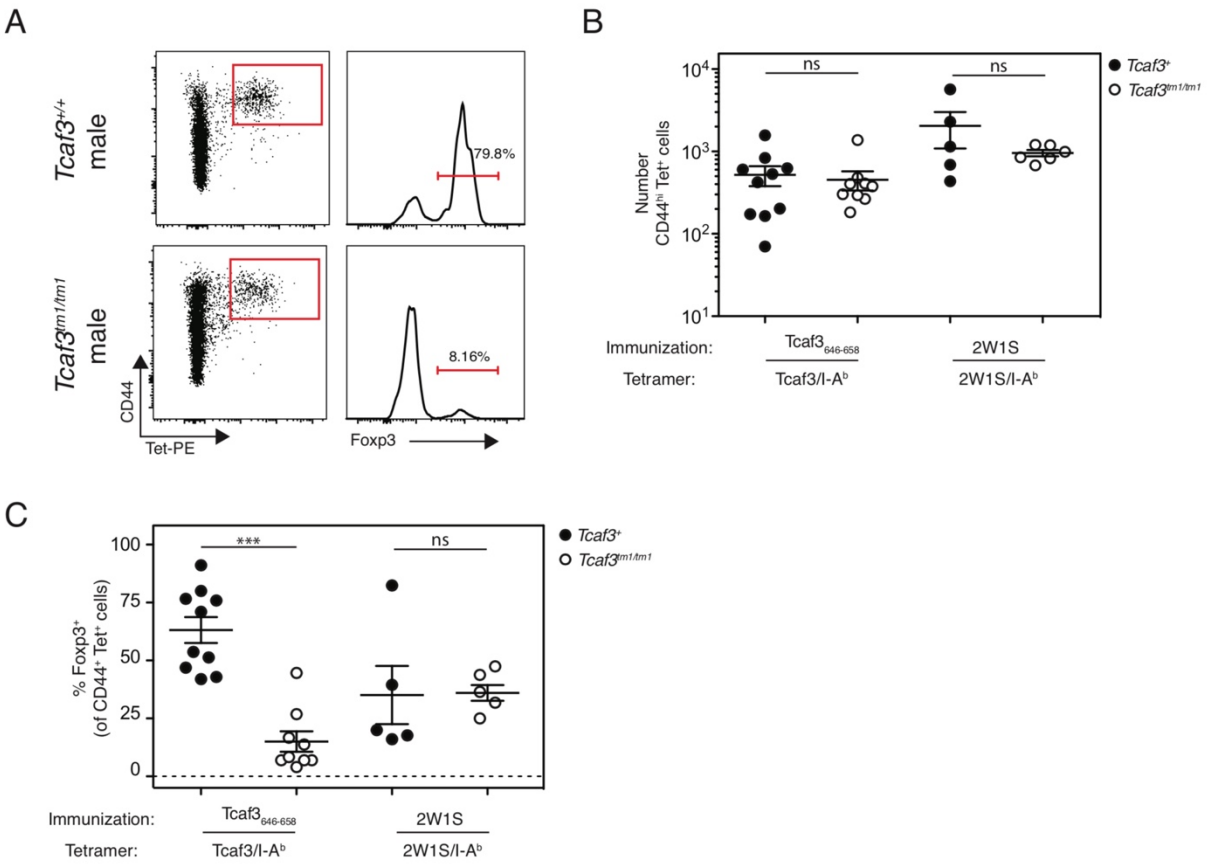

Figure 10: Global deficiency of the Tcaf3$_{646-658}$ peptide abolishes skewing of polyclonal antigen-specific T cells to the Treg cell lineage after immunization. (A-C) Expansion of Tcaf3$_{646-658}$-specific cells in *Tcaf3*$^{+/+}$, *Tcaf3*$^{+/tm1}$, or *Tcaf3*$^{tm1/tm1}$ male mice. Mice were immunized with Tcaf3$_{646-658}$ peptide emulsified in CFA. 14 days after immunization, lymphocytes from secondary lymphoid organs were pooled and tetramer enriched using Tcaf3/I-A^{b}. (A) Representative flow-cytometric plots of *Tcaf3*$^{+/+}$ or *Tcaf3*$^{tm1/tm1}$ mice after immunization. Plots on the left show Tcaf3/I-A^{b}-PE versus CD44 expression. Histograms are based on cells falling within the CD44^{hi} tetramer-PE^{+} gate (red gate) showing Foxp3 expression within the tetramer-PE^{+} population. (B and C) Summary plots of (B) the number of CD44^{hi} tetramer-PE^{+} cells and (C) the percentage of Foxp3^{+} CD44^{hi} tetramer-PE^{+} cells. Closed circles = *Tcaf3*$^{+}$ males; open circles = *Tcaf3*$^{tm1/tm1}$ males. Data are representative of multiple independent experiments; N = 5. The mean ± SEM is indicated. Significance testing was performed using the nonparametric Mann-Whitney test. *** indicates $p < 0.0001$, ns indicates not significant.

derived Tregs that expand following immunization, or peripherally induced Tregs that are generated in response to CFA plus peptide challenge. Second, on average, the number of tetramer$^+$ cells recovered following immunization of *Tcaf3*$^{tm1/tm1}$ males was comparable to that following immunization of *Tcaf3*$^+$ males (Figure 10B). Thus, formation of the Tcaf3$_{646-658}$-specific repertoire in the absence of Tcaf3$_{646-658}$ peptide did not alter the number of antigen-specific cells that are recovered following peptide immunization, but instead altered the ratio of Foxp3$^+$ Treg cells and Foxp3$^-$ T conventional cells (Tconv) at the peak of the response, abolishing skewing to the Treg cell lineage.

Tcaf3$_{646-658}$-specific Tconv Cells Can Drive Prostatic infiltration in the Absence of Inflammation

To determine whether the loss of skewing of Tcaf3$_{646-658}$-specific cells to the Treg cell lineage observed in *Tcaf3*$^{tm1/tm1}$ mice (Figure 10) has functional implications for prostate-specific autoimmunity, we performed cell transfer experiments in which bulk CD4$^+$ T cells were isolated from *Tcaf3*$^+$ or *Tcaf3*$^{tm1/tm1}$ males, and transferred into T cell-deficient *Tcr*$\beta^{-/-}$ male recipients that were wild-type with respect to the Tcaf3$_{646-658}$ antigen (*Tcr*$\beta^{-/-}$ *Tcaf3*$^+$ hosts). In this way, CD4$^+$ T cells that developed in the presence or absence of Tcaf3$_{646-658}$ peptide (from *Tcaf3*$^+$ or *Tcaf3*$^{tm1/tm1}$ donor males, respectively), were engrafted into male mice that express the Tcaf3$_{646-658}$ peptide. The effects on T cell infiltration of the prostate were assessed 9 weeks post-transfer. Strikingly, our data revealed that in most recipient mice, the transfer of donor CD4$^+$ T cells from *Tcaf3*$^{tm1/tm1}$

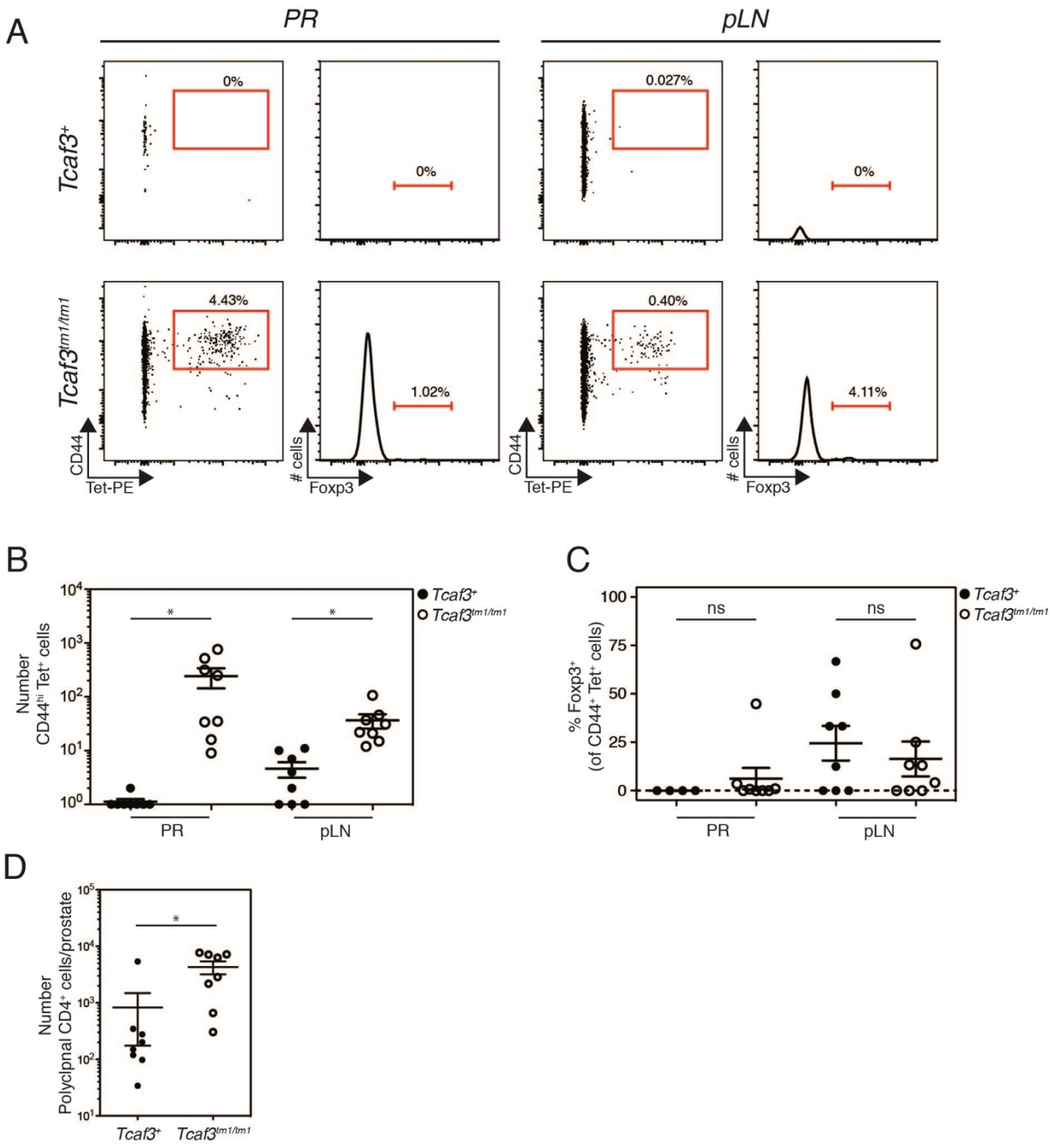

Figure 11: Exposure to Tcaf3$_{646-658}$ peptide during development prevents prostatic infiltration of antigen-specific T cells. *Tcrb*$^{-/-}$ male mice were reconstituted with CD4^{+} T cells pooled from the spleen and lymph nodes of *Tcaf3*$^{+/+}$ or *Tcaf3*$^{tm1/tm1}$ male mice 6-8 weeks old. 9 weeks flowing CD4^{+} T cell transfer, prostates and pLNs were analyzed (A) Representative flow-cytometric analysis of Tcaf3$_{646-658}$ cells within the prostate of *Tcrb*$^{-/-}$ hosts receiving either *Tcaf3*$^{+/+}$ CD4^{+} T cells (top) or *Tcaf3*$^{tm1/tm1}$ CD4^{+} T cells (bottom). The two left plots represent prostate analyses, the two right plots represent pLN analyses. CD44 expression by tetramer-PE is depicted in the first and third column. Cells within the CD44^{hi} tetramer^{+} population (red gate) are plotted in the second and fourth columns showing the Foxp3 expression. The percentage of cells

Figure 11 continued: falling within denoted gates is indicated. (B-D) Summary plots of (B) the number of $CD44^{hi}$ $tetramer^{+}$ cells, (C) the percentage of $Foxp3^{+}$ cells within the $CD44^{hi}$ $tetramer^{+}$ cells, and (D) the number of polyclonal $CD4^{+}$ cells within the prostate of mice receiving $CD4^{+}$ T cells from either *Tcaf3*$^{+/+}$ or *Tcaf3*$^{tm1/tm1}$ mice. Closed circles – *Tcaf3*$^{+}$ hosts; open circles *Tcaf3*$^{tm1/tm1}$ hosts. Data are representative of multiple independent experiments; N = 3. The mean ± SEM is indicated. Significance testing was performed using the nonparametric Mann-Whitney test. * indicates $p < 0.05$, ns indicates p is not significant.

males, resulted in spontaneous prostatic infiltration by Tcaf3/I-A^b tetramer$^+$ T cells as well as additional polyclonal CD4$^+$ T cells (Figure 11A-D). This finding demonstrates that exposure to a single peptide, Tcaf3$_{646\text{-}658}$, during formation of the T cell repertoire is required to prevent antigen-specific T cell infiltration of the prostate in recipient males expressing this antigen.

Furthermore, infiltration of the prostate by Tcaf3$_{646\text{-}658}$-specific Tconv cells is sufficient to drive the infiltration of other T cell specificities as demonstrated by massive increase in polyclonal CD4$^+$ cells within the prostate of recipient mice (Figure 11D). It is currently unknown if these cells may be reactive to Tcaf3-specific epitopes, other prostatic proteins, or ubiquitous antigens. Importantly, these results indicate that loss of one tissue specific Treg specificity during development is sufficient to drive prostatic infiltration upon transfer of polyclonal cells. These Tcaf3$_{646\text{-}658}$-specific Tconv cells may represent cells normally found in the Treg subset, but due to their development in *Tcaf3$^{tm1/tm1}$* mice prior to transfer, they are skewed to the Tconv lineage, so called "Trogue" cells. As previously observed in *Aire$^{-/-}$* mice[82], these Trogue cells are capable of driving prostatitis upon encounter with antigen in the periphery.

Given our cumulative results demonstrating that Tcaf3$_{646\text{-}658}$ peptide is required to skew polyclonal antigen-specific cells to the Foxp3$^+$ subset (Figure 10), coupled with data suggesting that clonal deletion has negligible impact on Tcaf3$_{646\text{-}658}$-specific T cells (Figure 7), our collective findings suggest that the Aire-dependent skewing of Tcaf3$_{646\text{-}658}$-specific precursors into the Treg cell lineage is essential for the prevention of prostate-specific T cell infiltration.

DISCUSSION

Since Aire-dependent ligands that are important in directing Treg differentiation were not defined until recently, the role of Aire in directing T cell fate has remained undefined. In this study, we investigated the role of an Aire-dependent antigen in promoting either clonal deletion or Treg development. Using Tcaf3/I-A^b tetramers and mice harboring a targeted deletion of the $Tcaf3_{646\text{-}658}$ epitope, we show that thymic expression of a natural Treg antigen does not drive clonal deletion of antigen-specific cells, but instead is required to direct these cells into the Treg lineage to prevent organ infiltration. This work together with previous studies on the role of Aire in promoting Treg development[70,80,82,119] indicate the primary role of Aire is to promote promiscuous gene expression that is necessary for tTreg development.

The recognition of agonist self ligands by developing thymocytes is critical for the generation of tolerance and prevention of autoimmunity. In the data presented here, we demonstrate that thymic expression of antigen is required for the development of polyclonal antigen-specific Treg cells. Additionally, we find little evidence of antigen expression driving deletion of the monoclonal MJ23 Treg cell of thymocytes in contrast to previous reports that Aire expression is required to drive deletion of TCR transgenic T cells reactive to a natural self antigen[152]. Our data does not exclude a role of Aire in driving deletion of some harmful clonotypes, but it does corroborate previous findings[80,82] that Treg differentiation is a primary mechanism by which Aire enforces tolerance to TRAs.

Using *Tcaf3*$^{tmt/tm1}$ mice we were able to evaluate the role of antigen in driving enrichment of antigen-specific Tregs. We demonstrate that global deficiency results in the

loss of enrichment of MJ23tg Tregs in the prostate draining lymph node in the absence of inflammation. Further, we demonstrate that antigen-specific cells that develop in the absence of antigen are heavily skewed to the Tconv lineage and are able to infiltrate the prostate after transfer into *Tcrβ*$^{-/-}$ hosts that express the antigen. Altogether these data indicate that antigen-specific Tregs are enriched at organ-draining lymph nodes in response to encounter with antigen, and this enrichment is essential for the prevention of prostate-specific T cell infiltration.

Notably, a small fraction of Tcaf3$_{646\text{-}658}$-specific Tregs are found in *Tcrβ*$^{-/-}$ hosts after transfer, as has previously been observed with MJ23tg cells in *Aire*$^{-/-}$ mice[80], which could be due to encounter with antigen in the periphery, or due to expansion of rare Tcaf3-specific Treg clones normally present in the repertoire of *Tcaf3*$^{tm1/tm1}$ mice. Importantly, these Tcaf3$_{646\text{-}658}$-specific Tregs are not sufficient to protect the prostate from infiltration indicating that organ-specific autoimmunity may represent a “numbers game” in which sufficient numbers of Tregs are required for protection. These data indicate that Tregs may directly suppress Tconv cells of the same specificity. Further investigation of the peptide epitopes recognized by the polyclonal cells that infiltrate the prostate after transfer of *Tcaf3*$^{tm1/tm1}$ cells is needed to determine if tissue-specific Tregs directly suppress Tconv cells of same specificity, or if they are able to broadly suppress cells of other specificities.

Taken together, our results demonstrate that Aire-dependent antigen expression is required for proper skewing of antigen-specific cells into the Treg lineage as a mechanism of protection from organ-specific autoimmunity.

Chapter V: Future Directions

Overall this work has described the identification of natural ligands recognized by endogenous Tregs. We demonstrate that two recurrent Treg clones, identified in different sequencing studies, MJ23 and SP33, are reactive to distinct non-overlapping peptides derived from a single prostate-specific protein, Tcaf3. Further, this work reveals the convergence of natural Tregs on epitopes derived from one prostate protein, suggesting that protection against autoimmune infiltration may require recognition of a limited set of autoantigens that are susceptible to autoimmune attack.

Defining Treg epitopes enabled the generation of new tools, such as self-peptide tetramers and peptide-deficient transgenic mice, to study the biology of natural Treg cells for the first time. The development of tetramers specific for the MJ23 T cell epitope, $Tcaf3_{646\text{-}658}$, facilitated the direct visualization of polyclonal T cells reactive to a natural antigen for the first time. Furthermore, since the expression of Tcaf3 in the thymus is Aire-dependent, the generation of mice harboring a targeted deletion of the $Tcaf3_{646\text{-}658}$ epitope, termed *Tcaf3*$^{tm1/tm1}$, allowed us to study the role of Aire in driving Treg development or clonal deletion in wildtype mice, leading to advances in our understanding of thymic Treg development.

Beyond the results presented here, these tools can be used to answer many of the lingering questions in the Treg field. First, the role of antigen expression in the thymus versus the periphery in driving Treg development can be teased apart, revealing any redundancies in Treg-mediated immunity. Second, the role of antigen in the maintenance

of organ-specific Tregs can be assessed to determine the processes that coordinate peripheral homeostasis of these cells, and how antigen encounter in the periphery shapes the repertoire. Third, the affinity of regulatory T cells can be studied for the first time, illuminating the role of TCR signal strength in Treg development. Finally, defining natural Treg ligands will allow for the study of the biochemical nature of self-antigen recognition.

In all, the identification of natural Treg epitopes enables a more comprehensive understanding of the factors that are required to maintain lifelong Treg-mediated immunity. Further, with a better understanding of the molecular mechanisms of Treg development, new therapies can be developed to manipulate Tregs in settings of autoimmunity and cancer.

The Role of Antigen Expression in the Thymus and Periphery

Our work has demonstrated that antigen expression in the thymus is required for the generation of monoclonal MJ23 Tregs (Figure 1) as well as polyclonal $Tcaf3_{646\text{-}658}$-specific cells (Figure 6). Further, we have shown that thymic expression of this antigen does not induce measurable deletion of MJ23 T cells (Figure 7), but we have not yet determined if thymic expression of $Tcaf3_{646\text{-}658}$ drives deletion of polyclonal antigen-specific T cells, or if thymic expression of antigen is required to prevent prostatic infiltration.

The analysis of mice that lack the proapoptoic molecule Bim (*Bim*$^{-/-}$ mice) will help reveal if antigen expression plays a role in deletion. If similar numbers of Tcaf3/I-A^b tetramer$^+$ cells are found in the thymi of *Bim*$^{-/-}$ mice compared to *Tcaf3*$^+$ mice, this would

reveal that expression of this Aire-dependent antigen does not drive thymic deletion of polyclonal Tcaf3-specific cells. However, if elevated numbers of Tcaf3/I-A^b tetramer$^+$ cells are found in the thymi of *Bim*$^{-/-}$ mice, this would indicate that other factors play a critical role in T cell fate in the thymus. These factors could include TCR signal strength consistent with the affinity hypothesis[53], cytokine availability[59], or non-canonical TCR-pMHC engagement as has been shown for ex-vivo induced Tregs[153].

To determine the role of thymic versus peripheral expression of antigen in preventing prostatic infiltration, reciprocal thymic grafts of *Tcaf3*$^+$ or *Tcaf3*$^{tm1/tm1}$ thymi into athymic *Foxn1*$^{nu/nu}$ mice have been generated. Mice will be analyzed 12 weeks after thymic lobe engraftment. As demonstrated in Figure 10, polyclonal $Tcaf3_{646\text{-}658}$-specific cells that expand in Tcaf3$^{tm1/tm1}$ mice after immunization are skewed to the Tconv lineage. Further, the Tconv skewing results in prostatic infiltration when transferred to mice expressing $Tcaf3_{646\text{-}658}$ (Figure 11) even in the absence of inflammation. Thus, if thymic development of antigen-specific Tregs is required to prevent prostatic infiltration, then infiltration of Tcaf3/I-A^b Tconv cells would be found in the prostate of athymic *Foxn1*$^{nu/nu}$ mice that receive *Tcaf3*$^{tmt/tm1}$ thymi. However, it is possible that $Tcaf3_{646\text{-}658}$-specific Tregs could develop extrathymically upon encounter with antigen in the periphery, in which case we would not find prostatic infiltration. Based on the observations of CD4$^+$ T cell transfer studies described in *Tcr*$\beta^{-/-}$ mice (Figure 11), I would expect that any peripherally induced $Tcaf3_{646\text{-}658}$-specific Tregs that do develop in mice that receive *Tcaf3*$^{tm1/tm1}$ thymi will not be sufficient to prevent prostatic infiltration. If this is not observed, this would indicate that tTreg and pTreg play redundant roles in tolerance. This would also support an earlier

study that showed that Tregs generated during the perinatal period of life are required for protection from Aire-mediated diseases[84]. Since the engrafted thymic lobes are derived from 3d old neonatal mice, the Tregs that develop in the thymic graft should resemble those generated in a wild-type setting.

Impact of Antigen Expression in the Periphery on Antigen-specific Treg Repertoire

The role of antigen expression in the periphery on shaping the Treg repertoire has not been evaluated. We have clearly demonstrated that expression of antigen in the thymus is required for the development of polyclonal Tcaf3-specific Tregs. However, it remains to be determined if the peripheral repertoire of antigen-specific Tregs is selected over time. Transfer experiments using Tregs isolated from organ-draining lymph nodes demonstrated that these were more efficient than Tregs isolated from non-draining lymph nodes at suppressing organ-specific autoimmunity[110-112], suggesting that the asymmetric distribution of Tregs observed[71,107,108] has functional implications.

In order to determine the early peripheral repertoire of antigen-specific Tregs, we analyzed the periphery of naïve *Tcaf3*$^{+}$ and *Tcaf3*$^{tm1/tm1}$ mice for the presence of Tcaf3/I-A^{b} tetramer^{+} cells (Figure 12A-C). As in the analysis of naïve thymi, similar numbers of Tcaf3/I-A^{b} tetramer^{+} cells were found in the periphery of *Tcaf3*$^{+}$ and *Tcaf3*$^{tm1/tm1}$ mice (Figure 12B). Surprisingly, however, Tcaf3/I-A^{b} specific cells in naïve *Tcaf3*$^{+}$ males are not heavily skewed to the Treg lineage (Figure 12A-B) in contrast to the skewing observed after immunization with peptide in CFA (Figure 3 & Figure 10). Additionally, a small fraction of Tcaf3/I-A^{b} specific cells in the periphery of naïve *Tcaf3*$^{tm1/tm1}$ mice express

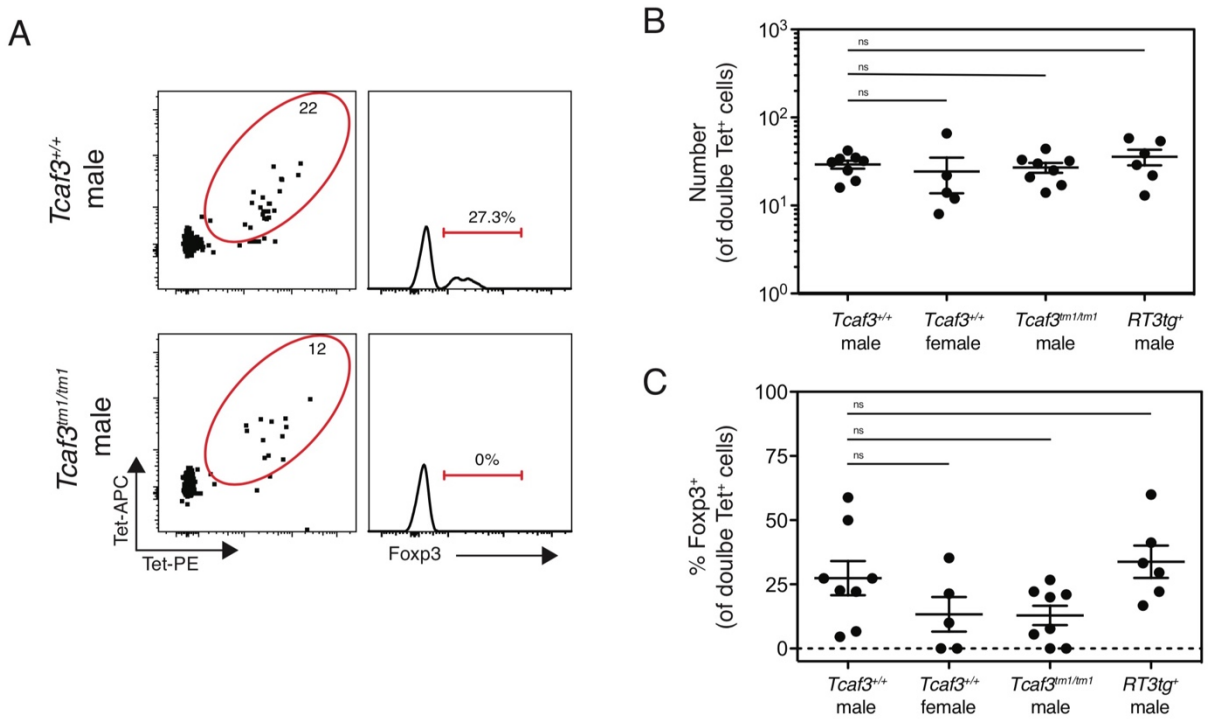

Figure 12: Tcaf3$_{646-658}$ peptide specific Tregs are present in periphery of *Tcaf3$^{tm1/tm1}$* mice. Pooled secondary lymphoid organs from 4-6 week old naïve mice of denoted genotypes were stained with APC- and PE-labeled Tcaf3/I-A^{b} tetramer followed by magnetic enrichment using AutoMACS. (A) Representative flow-cytometric plots of *Tcaf3$^{+/+}$* or *Tcaf3$^{tm1/tm1}$* male mice. Plots depict tetramer-APC versus tetramer-PE staining and show the double-tetramer^{+} gate. The number of cells within the double-tetramer^{+} gate is indicated. Histograms are based on cells falling within the double-tetramer^{+} gate (red gate) showing Foxp3 expression within the double-tetramer^{+} population. (B and C) Summary plots of (B) the number of double-tetramer^{+} cells and (C) the percentage of Foxp3^{+} double-tetramer^{+} cells. Data are representative of multiple independent experiments; N = 4. The mean ± SEM is indicated. Significance testing was performed using the nonparametric Mann-Whitney test. *** indicates $p < 0.0001$, ns indicates not significant.

Foxp3 in the periphery (Figure 12C). These data indicate that antigen expression is not absolutely required for the development of antigen-specific Tregs in the periphery. These Foxp3-expressing cells may represent rare Tcaf3/I-A^b tetramer$^+$ Tregs that emerged from the thymus of *Tcaf3$^{tm1/tm1}$* mice due to cross-reactivity with other ligands, or they may develop in the periphery due to cross-reactivity. The best way to determine if these cells are specific to $Tcaf3_{646-658}$ or cross-reactive to other antigens would be to generate retrogenic mice using TCR sequences that differ between naïve *Tcaf3$^+$* and *Tcaf3$^{tm1/tm1}$* mice and assaying their reactivity *in vivo* in the primary retrogenic hosts as well as secondary hosts after intrathymic transfer of the retrogenic thymocytes. Importantly, the mice analyzed in Figure 12 were all 4-6 weeks old. Thus, further studies of naïve mice at different ages will be needed to determine if antigen availability in the periphery plays a role in shaping the naïve repertoire, since prostate develop is not complete until ~6 weeks of age.

To evaluate how expression of antigen in the periphery plays a role on shaping the repertoire after immunization, we analyzed *Rag2^{GFP} Foxp3^{RFP}* male mice 14 days after immunization of $Tcaf3_{646-658}$ peptide plus CFA at 4, 8, or 12 weeks of age (Figure 13). The expression of $Rag2^{GFP}$ identifies cells that have recently recombined their receptors, allowing us to determine if Tcaf3/I-A^b tetramer$^+$ cells are expanding in response to immunization or are recent thymic emigrants. Interestingly, similar numbers of Tcaf3/I-A^b tetramer$^+$ cells were observed regardless of the age at which mice were immunized (Figure 13B), indicating the absolute number of $Tcaf3_{646-658}$-specific cells in the periphery does not expand significantly in the periphery in the absence of inflammation. However,

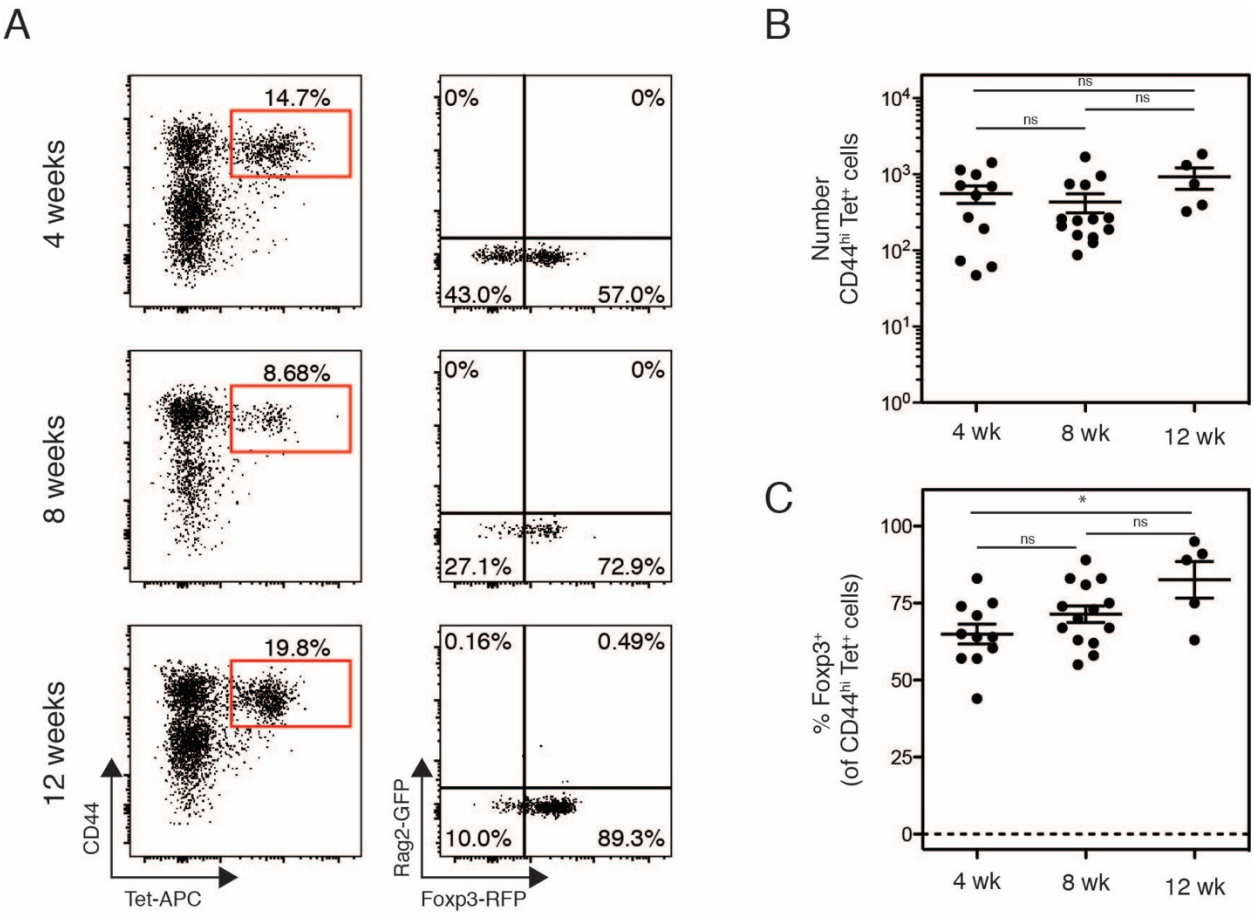

Figure 13: Foxp3 expression increases in polyclonal Tcaf3$_{646-658}$-specific cells with age of immunization. *Rag2^{GFP}Foxp3^{RFP}* male mice were immunized at different ages with CFA plus 100 mg Tcaf3$_{646-658}$ peptide and analyzed 14 days later. (A) Representative flow-cytometric plots of mice immunized at the age indicated on the left. Plots on the left show Tcaf3/I-A^{b}-APC versus CD44 expression. Plots on the right show Foxp3^{RFP} expression by Rag2^{GFP} expression of cells falling within the CD44^{hi} tetramer-APC^{+} gate (red box). (B and C) Summary plots of (B) the number of CD44^{hi} tetramer^{+} cells and (C) the percentage of Foxp3^{+} CD44^{hi} tetramer^{+} cells. Data are representative of multiple independent experiments. (A-C) N = 5. The mean ± SEM is indicated. Significance testing was performed using the nonparametric Mann-Whitney test. * indicates $p < 0.05$, ns indicates p is not significant.

the frequency of Foxp3^{+} Tcaf3/I-A^{b} tetramer^{+} cells increased with age (Figure 13C). Preliminary analysis of female mice immunized at 4, 8, or 12 weeks of age shows similar expansion of Tcaf3$_{646\text{-}658}$-specific cells at all ages, but does not show the same increase in Foxp3 expression over time (data not shown). The presence of antigen in the periphery in the male mice may result in the selective maintenance of Tcaf3$_{646\text{-}658}$-specific Tregs compared to Tconv cells in the periphery. This hypothesis is supported by previous reports that have demonstrated strong TCR signaling of Tregs in the periphery[44]. The strong signaling may be indicative of constant pMHC engagement in the organ draining lymph node. Alternatively, the increase in frequency of Foxp3^{+} Tcaf3$_{646\text{-}658}$-specific cells could result from increased antigen availability in older mice, resulting in the generation of pTregs.

Follow up studies will be necessary to determine the requirement for antigen expression in driving this enrichment of Tcaf3$_{646\text{-}658}$-specific Tregs in older males. The thymic graft of Tcaf3$^{tm1/tm1}$ thymic into wild-type Foxn1$^{nu/nu}$ mice will demonstrate if pTreg generation plays a significant role in development of Tcaf3$_{646\text{-}658}$-specific cells, however it is unlikely based on the results of our CD4^{+} transfers into *Rag1*$^{-/-}$ or *Tcrβ*$^{-/-}$ mice. TCR sequencing of Tcaf3/I-A^{b} tetramer^{+} Tregs cells of mice of varying ages will be needed to determine if there is expansion of specific clonotype overtime or if all Tcaf3$_{646\text{-}658}$-specific Tregs are maintained throughout life.

The Role of Peripheral Antigen Expression on Maintenance of Antigen-specific Tregs

The role of antigen expression in the periphery is not well understood. Previous studies have revealed asymmetric distribution of Tregs in the periphery[80,106,107,109], indicating antigen driven enrichment, however, it is possible that other factors, such as chemokine receptors or trafficking molecules direct Treg trafficking, as observed for some Treg specificities[108,114]. Our low frequency bone marrow chimeras of MJ23tg in *Tcaf3*$^{+}$ or *Tcaf3*$^{tm1/tm1}$ mice (Figures 8 and 9) revealed that expression of antigen is necessary and sufficient for proper localization and enrichment of antigen-specific T cells in the periphery. However, it is unclear if antigen expression in the periphery is required for this enrichment of polyclonal Tcaf3$_{646\text{-}658}$ Tregs. To determine the role of antigen expression in the periphery in driving localization, a second set of thymic graft experiments will be performed. *Tcaf3*$^{tm1/tm1}$ mice will be crossed to the *Foxn1*$^{nu/nu}$ mice to produce mice that lack expression of the antigen in the periphery. In this setting, grafting of *Tcaf3*$^{+}$ thymi is expected to result in the development of Tcaf3$_{646\text{-}658}$-specific tTregs, as in wild-type thymi. If antigen is required for their enrichment in the pLN, this would be abolished. However, if other signaling molecules are sufficient to drive enrichment we would find Tcaf3$_{646\text{-}658}$ cells enrichment in the pLN. Furthermore, maintenance of Tcaf3$_{646\text{-}658}$-specific Treg cells will be evaluated in this setting to determine if cognate antigen expression is required for their maintenance, or if tonic TCR signaling is sufficient similar to naïve Tconv cells.

Additionally, a series of thymectomy studies should be conducted to determine the maintenance of Tcaf3$_{646\text{-}658}$-specific T cells throughout life. A study by Yang et al.[84]

demonstrated that Treg cells that emerge early in life have a distinct repertoire and are required for protection against Aire-dependent autoimmunity. Based on this observation, it is likely that thymectomy of mice after ~ 4 weeks of age would not impact the ability of $Tcaf3_{646\text{-}658}$-specific cells to respond to immunization, as these would be maintained in the periphery in response to antigen. However, since the prostate is not fully developed by 4 weeks of age, there might not be sufficient antigen at the time to maintain a protective pool of $Tcaf3_{646\text{-}658}$-specific Tregs. However, the lack of $Rag2^{GFP}$ expression in Tcaf3/I-A^b tetramer$^+$ cells after immunization at 4 weeks of age indicates that recent thymic emigrants do not contribute significantly to the $Tcaf3_{646\text{-}658}$-specific T cell pool after 4 weeks of age.

Affinity of Treg TCRs

Regulatory T cells have been shown to recognize self antigens with high affinity[154]. However, these studies have used downstream targets of TCR signaling as a proxy for signaling[44,45] or double transgenic mouse models using foreign peptide antigens[54-58]. The analysis of bulk Treg cells has limited the ability to detect small differences in repertoire and affinity at the individual cell level. Defining the peptide ligand recognized by a monoclonal Treg population, MJ23, as well as polyclonal cells from wildtype C57BL/6 (*Tcaf3*$^+$) mice allows us to test the affinity of endogenously expressed Treg TCRs for the first time.

The MJ23 Treg TCR has been well characterized *in vitro* and *in vivo*, however the affinity of this TCR has not been measured. *In vitro* cultures of MJ23 cells with cognate

antigen, Tcaf3$_{646-658}$, showed robust proliferation even when cultured with low concentrations of the peptide (half-max stimulation at ~330 pM) (Figure 14A-B). This stimulation was more potent than OT-IItg cells cultured with their cognate peptide, OVA$_{323-339}$ (half-max stimulation at ~730 pM) (Figure 14A) as wells a SP33rg cells cultured with their cognate peptide, Tcaf3$_{88-107}$ (half-max stimulation at ~2160 pM) (Figure 13B). Importantly, this indicates that natural Tregs sense antigen over a wide range of affinities, supporting previous conclusions using model antigens[57]. While these data imply that the MJ23 TCR senses antigen with high affinity, it does not directly identify the affinity of the MJ23 TCR. The TCR affinity of the MJ23 TCR can be biochemically measured using surface plasmon resonance (SPR) to measure the interaction of the MJ23 TCR and pMHC molecules. The development of Tcaf3/I-A^b tetramers for the studies described earlier can be modified slightly and used in these studies as the pMHC molecules. Additionally, work expressing MJ23 TCRs in insect cells is underway in the Adams lab. The use of SPR to biochemically characterize the MJ23 TCR will identify the affinity and kinetics of this TCR as well as others identified in subsequent studies.

Preliminary work identifying Tcaf3$_{646-658}$-specific TCRs of different affinities has been done. We performed initial sequencing studies on Tcaf3/I-A^b tetramer$^+$ cells isolated from Aire$^{-/-}$ mice with the fixed TCRβ that was previously used to identify the MJ23 TCR[80]. These mice were immunized with CFA and Tcaf3$_{646-658}$ peptide and Tcaf3/I-A^b tetramer$^+$ cells were isolated 14 days for sequencing analysis. MJ23tg *Rag1*$^{-/-}$ Tcaf3/I-A^b staining was used as a control for tetramer staining. To identify cells of high and low affinity, the MJ23tg gate was bisected and polyclonal cells from the secondary lymphoid organs or

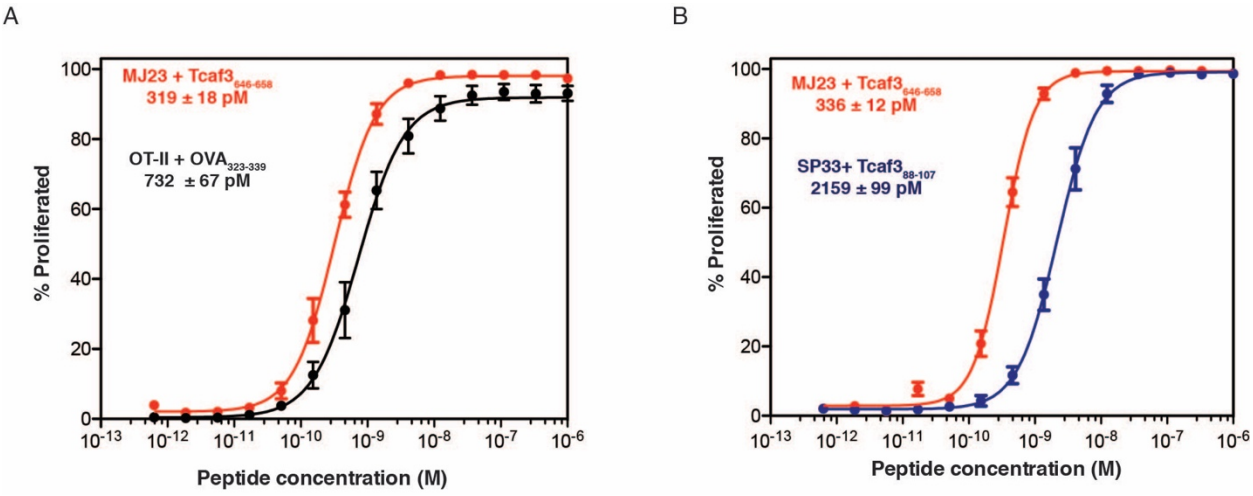

Figure 14: Potency of TCRtg cells with their cognate antigens. As in Figure 1, $CD4^+$ T cells were isolated from MJ23tg *Rag1*$^{-/-}$ CD45$^{.1/.1}$, OT-IItg *Rag1*$^{-/-}$ CD45$^{.1/.1}$, or SP33rg mice, labeled with CellTrace-Violet (CTV) and cultured with peptide. (A) MJ23tg T cells were cultured with $Tcaf3_{646\text{-}658}$ (red circles and line); OT-IItg T cells were cultured with $OVA_{332\text{-}339}$ (black circles and line). (B) MJ23tg T cells were cultured with $Tcaf3_{646\text{-}658}$ (red circles and line); SP33rg cells were cultured with $Tcaf3_{88\text{-}107}$ (blue circles and line). (A-B) Dose response curves fit to a cooperative model. Points denote mean ± SEM of three replicates.

prostate were sorted accordingly. Due to the low cell numbers in the prostate, only cells from the secondary lymphoid organs were sequenced using iRepertoire. However, the sorting process revealed a surprising finding. Tcaf3/I-A^b tetramer staining of cells from the prostate of these mice was consistently stronger than that of MJ23tg *Rag1*$^{-/-}$ cells, with the majority of Tcaf3/I-A^b tetramer$^+$ cells from the prostate falling within the tetramer^{hi} gate. This result suggests that the cells that infiltrate the prostate in this setting may be of higher affinity than the MJ23 Tregs. Thus, further work to identify prostate infiltrating $Tcaf3_{646\text{-}658}$ cells is ongoing using a single cell sequencing of Tcaf3/I-A^b tetramer$^+$ cells as a complementary approach. Initial data from these sequencing results indicate that there is a greater diversity of TCRβ usage among Tcaf3/I-A^b tetramer$^+$ cells in the prostate compared to the secondary lymphoid organs, however this is based on a small sample size and will need to be further interrogated.

TCRs identified though iRepertoire or single cell sequencing can be used to study the affinity of $Tcaf3_{646\text{-}658}$ specific Tregs. In addition to SPR affinity measurements, the generation of retrogenic mice expressing these TCRs can be used study the role of affinity *in vivo*. Thymocytes from the retrogenic mice transferred intrathymically into wildtype mice can be assessed for Treg development or clonal deletion similar to studies of MJ23 intrathymic injections (Figure 7). It is expected that these studies together will reveal the nature of TCR affinity in directing antigen-specific cells into the Treg lineage or clonal deletion.

Summary & Model

Taken together, the elucidation of natural ligands recognized by endogenous Tregs has yielded new insights into Treg cell biology and the mechanism by which tissue-specific Tregs protect against autoimmunity. Altogether, our data lead to a new model for the development of Aire-dependent Tregs (Figure 15). The data presented here demonstrate that under wild-type settings (Figure 15A), tTreg precursors have two fates upon encounter with cognate antigen: tTreg development or Tconv development. We find no evidence for deletion of antigen-specific Treg cells within the thymus as demonstrated above (Figure 6). Further, we find that encounter with antigen within the thymus leads to a skewing of cells to the Treg lineage within the thymus, with a minor population of Tconv cells to the same specificity. Upon encounter with antigen in the periphery, the skewing of antigen-specific cells to the Treg lineage prevents infiltration of any Tconv cells of the same specificity, therefore leading to tolerance. In the absence of antigen expression in the thymus (i.e. Aire-deficiency), antigen-specific tTreg precursors do not develop into Tregs, but instead are shuttled into the Tconv subset (Figure 15B). Upon subsequent encounter with antigen in the periphery, these Tconv cells proliferate and can infiltrate the tissue, resulting in autoimmunity. Additionally, any antigen-specific Tregs that develop in the absence of antigen are unable to prevent these Tconv cells from infiltrating the tissue.

The identification of endogenous Aire-dependent epitopes has also allowed the study of the role of Aire-dependent antigens in a wild type setting. Our results show that antigen plays a critical role in directing self-reactive cells into the Treg lineage in the thymus. Further we show that in the absence of antigen expression, Tconv cells develop

and can infiltrate the prostate in the absence of inflammation, indicating that antigen-specific Tregs are present to restrain Tconv cells of the same specificity from causing organ-specific autoimmunity. These results coupled with the future studies presented here expand our understanding of the role of self-antigen specific Tregs play in health and disease.

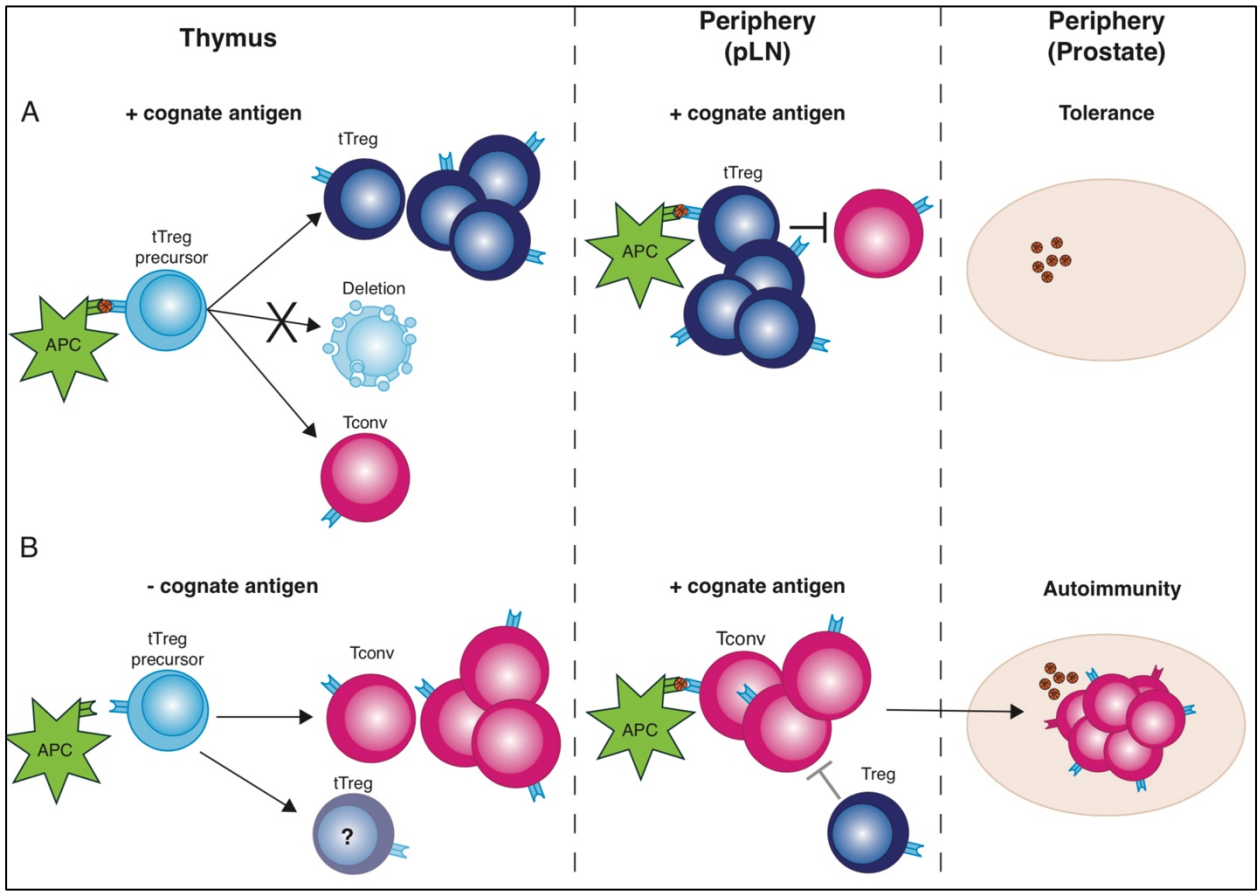

Figure 15: Model of self antigen specific Treg development. Depiction of Aire-dependent Treg development and maintenance within the thymus (left), peripheral lymph node (middle) and target organ (right). (A) Wild-type setting in which antigen is expressed in the thymus. (B) Auto-immune prone setting in which antigen is not expressed in the thymus. Green stars are antigen presenting cells; light blue cells are tTreg precursors; dark blue cells are tTregs; pink cells are Tconv cells.

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