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The mechanisms shaping the repertoire of $CD4^+$ $Foxp3^+$ regulatory T cells

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Summary

Regulatory T (Treg) cells expressing Foxp3 transcription factor control homeostasis of the immune system, antigenic responses to commensal and pathogenic microbiota, and immune responses to self and tumour antigens. The Treg cells differentiate in the thymus, along with conventional $CD4^+$ T cells, in processes of positive and negative selection. Another class of Treg cells is generated in peripheral tissues by inducing Foxp3 expression in conventional $CD4^+$ T cells in response to antigenic stimulation. Both thymic and peripheral generation of Treg cells depends on recognition of peptide/MHC ligands by the T-cell receptors (TCR) expressed on thymic Treg precursors or peripheral conventional $CD4^+$ T cells. This review surveys reports describing how thymus Treg cell generation depends on the selecting peptide/MHC ligands and how this process impacts the TCR repertoire expressed by Treg cells. We also describe how Treg cells depend on sustained signalling through the TCR and how they are further regulated by Foxp3 enhancer sequences. Finally, we review the impact of microbiota-derived antigens on the maintenance and functionality of the peripheral pool of Treg cells.

Keywords: regulation/suppression; T cell; T-cell receptors.

Thymic selection of the TCR repertiore of regulatory T cells

Random generation of T-cell receptors for antigens (TCRs) ensures a diverse TCR repertoire that allows recognition of a variety of pathogens. However, some TCRs generated in this process recognize self-antigens and have the potential to cause autoimmune diseases. One mechanism that evolved to protect against uncontrolled self-reactivity of developing TCR repertoire includes deletion of self-reactive T cells.[1] Another mechanism is generation of a specialized regulatory T (Treg) cell that inhibits activation of peripheral T cells specific for self-antigens and prevents autoimmune diseases.[2,3] This is particularly important because a recent report showed that a sizable proportion of $CD4^+$ T cells specific for ubiquitous self antigens are not eliminated by negative selection.[4] Regulatory T cells that sustain immune system homeostasis and control immune and inflammatory responses develop in the thymus. Their development is regulated by signals from the TCR and cytokine receptors especially interleukin-2 (IL-2) receptor.[5,6] The current paradigm postulates that interactions between TCRs expressed by double-positive thymocytes and MHC II–peptide complexes expressed by thymic epithelial cells or bone-marrow-derived thymic stromal cells induce expression of Foxp3 transcription factor and initiate the genetic programme of Treg cell differentiation. This instructive model was proposed when it was shown that expression of the self-peptide–MHC complex directed differentiation of thymocyte precursors expressing cognate transgenic TCR into Treg cell lineage.[7,8] Different sensitivity of the Treg precursors and conventional $CD4^+$ T cells to positive selection could play the role in this process.[9] A two-step model of Treg cell differentiation was later proposed where signals from the TCR induced expression of CD25 and these cells were predestined to up-regulate Foxp3 and become Treg cells under the influence of only IL-2 and without continuing signals from TCR.[5] The signals from the TCR, which lead to the up-regulation of CD25, are

Abbreviations: AIRE, autoimmune Regulator transcription factor; CNS, conserved, non-coding regulatory sequence; IL-2, interleukin-2; pTreg, peripherally derived regulatory T; TCR, T-cell receptor; Treg, regulatory T

vital for Treg cell development and highlight the importance of MHC–peptide ligand complexes in this process. This model assumes that stronger interactions of TCRs and MHC–peptide ligands induce epigenetic changes in the genetic loci encoding Foxp3 and other transcription factors critical for Treg lineage commitment and function. Hence, the strength of interactions involving TCRs on developing thymocytes with agonist, self-antigens determine not only the extent of recruitment of double-positive thymocytes into the Treg population but also diversity and abundance of their TCRs.[10] Once Treg cells mature and populate peripheral lymphoid organs, signalling through the TCR regulates gene expression, metabolism, adhesion and migration of Treg cells affecting their maintenance, survival and suppressor function.

The model that postulates that Treg development depends on increased affinity of interactions between the TCR and class II MHC bound with self, agonist peptide (s) implies that scanning for specific peptide/MHC ligands or ligands plays a critical role in the CD4$^+$ T-cells lineage decision. A recent report has shown that peptides derived from natural self antigen, myelin oligodendrocyte glycoprotein, are necessary for selection of Treg cells but not conventional CD4$^+$ T cells specific for this self antigen.[11] Random generation of TCRs for antigens expressed by selected Treg cells were protective for experimental autoimmune encephalomyelitis and had higher functional avidity for the cognate autoantigen. Moreover, ablation of myelin oligodendrocyte glycoprotein-encoding gene drastically reduced the number of Treg cells, but not conventional CD4$^+$ T cells, and rendered mice more sensitive to experimental autoimmune encephalomyelitis induction, proving that a direct link exists between the repertoire of agonist self-peptides present in the thymus and Treg cell immunoregulatory functions. The hypothesis that Treg cells express TCRs with higher affinity for self-peptides is also supported by data showing that the repertoire of Treg cells overlapped with the TCR repertoire of autoreactive T cells present in Foxp3-deficient mice.[12] Another support for the hypothesis of instructional commitment to the Treg lineage was provided by analysis of the strength of TCR signalling. TCR signalling conveyed by expression of Nur77 reporter showed that Treg cells perceive stronger TCR signals than conventional T cells during thymic development and in the periphery.[13] In addition, higher affinity of TCR is associated with more efficient recruitment into the Treg population.[14] However, other data show that TCRs expressed by Treg cells may not be more self-reactive than TCRs expressed by conventional CD4$^+$ T cells. Analysis of a panel of hybridomas derived from Treg cells failed to detect increased reactivity to self antigens while the same hybridomas responded frequently to non-self antigens.[15] By analysing a set of TCRs with different affinities to antigenic peptides it was also shown that a 1000-fold range of self-reactivity allows for Treg selection, which could explain the overlap seen between Treg cells and autoreactive T cells.[14] Despite increased self-reactivity driving Treg cell differentiation, affinities of TCRs on Treg precursors for antigens that could trigger negative selection remained considerably lower (100-fold). Extensive analyses of the TCR repertoire expressed by Treg cells and conventional CD4$^+$ T cells showed that although many TCRs are expressed predominantly on conventional or Treg cells, there is always an overlap between these repertoires.[16,17] This result shows that other factors, besides the TCR, determine thymocyte lineage commitment. Moreover, Treg cells expressing identical TCRs as naive CD4$^+$ T cells continued to develop in mice expressing single, covalently linked class II MHC/peptide complexes, demonstrating that TCRs with the same affinity for the selecting ligand can be expressed by thymocytes differentiating to both CD4$^+$ lineages.[18] The proportion of Foxp3$^+$ thymocytes in mice expressing a single class II MHC–peptide motif was smaller than in mice expressing wild-type class II MHC–peptides, but surprisingly large considering the drastic restriction in diversity of selecting ligands. When TCR repertoires expressed on Treg and conventional cells were analysed an extensive overlap between repertoires was found, suggesting that TCR affinity or limited access to the selecting self-peptide are not critical factors that guide lineage commitment of CD4 thymocytes. An additional complexity to our understanding of Treg cell differentiation was further provided by an analysis of mutant mice with reduced TCR signalling in thymocytes. Mutations of immunoreceptor tyrosine-based activation motifs of ζ chain, which attenuate TCR signalling, promoted Treg cell selection.[19] This finding also opposes the hypothesis that only high-affinity ligands, which are likely to increase TCR signalling, induce Treg cell generation. Altogether, conflicting data from studies of individual TCRs or TCR repertoire analyses need to be reconciled with signalling studies to better understand what are the ligands and signalling requirements for the Treg selection process. An alternative hypothesis, that Treg selection is not entirely instructive but depends on matching TCR-delivered signal to the pre-existing conditions in the Treg precursors should also be considered.

This problem was in part addressed by the other study that investigated conserved, non-coding regulatory sequences (CNS) in the Foxp3 locus.[20] One of these regions, CNS3, facilitates epigenetic changes in the Foxp3 locus in thymic Treg precursors before their thymic selection, and increases frequency of Treg cell formation in the thymus. Analysis of the TCR repertoires of CNS3-deficient and CNS3-sufficient Treg cells revealed that this regulatory element facilitates Treg commitment by promoting recruitment of immature thymocytes with low(er) affinity TCRs to Treg cell lineage.[21] Therefore, CNS3-deficient Treg cells had reduced TCR repertoire and upon

weakened negative selection could not control self-reactive CD4 clones, which led to a rapid development of lethal autoimmunity. These results highlighted the importance of a broad Treg repertoire as an essential feature required to sustain immune homeostasis.

To investigate the role of specific TCRs in thymocyte lineage commitment, another study used transgenic and retroviral expression of Treg-cell-derived TCRs. The results of this study showed that cloned TCRs cannot steer the majority of developing thymocytes to Treg lineage. Regulatory T cells expressing introduced TCRs were generated only at low precursor frequency, whereas most differentiating $CD4^+$ thymocytes lacked Foxp3 expression.[22,23] These findings implied that Treg precursors can be sensitive to intraclonal competition, and that only limited quantities of Treg selecting ligands are presented in 'niches' in thymic medulla. Therefore, the competition for binding to rare self MHC–peptide ligands on thymic stromal cells would limit the number of Treg cells with unique specificities, and influence the survival of these cells in the periphery.[12,24]

The nature of the rare self-antigens that induce Treg cell differentiation remains elusive. It has been proposed that Treg cells may undergo selection on peripheral self-antigens, the presentation of which in the thymus is limited to particular subsets of thymic stromal cells. Promiscuous expression of tissue-specific proteins in medullary thymic epithelial cells was found to expose developing thymocytes to a broad range of tissue-specific antigens.[25] This ectopic expression of peripheral, self-proteins that are considered the source of peptide ligands mediating Treg selection is regulated by the transcription factor, autoimmune regulator (AIRE).[26,27] Presentation of AIRE-dependent thymic ligands has been associated with clonal deletion of autoreactive T cells, but in parallel it also promotes development of Treg cells.[28] Examination of autoimmune lesions in AIRE-deficient mice revealed that TCRs expressed by pathogenic effector cells are preferentially expressed by Treg cells in wild-type mice.[28] This AIRE-dependent recruitment of potentially autoreactive T cells into the pool of Treg cells is considered an important mechanism to eliminate conventional self-reactive $CD4^+$ T cells, which enforces tissue-specific tolerance.[4] AIRE-dependent generation of Treg cells in the perinatal period is particularly important for preventing autoimmune disease and where necessary to prevent autoimmunity throughout life.[29] This latest finding indicates that the neonatal Treg population forms a distinct subset adding age as a factor that is important for the Treg cell ontogeny and maintenance of immune tolerance.

Recruitment of peripheral Treg cells

How self-antigens and TCR signals regulate the pool of peripheral Treg cells has also been extensively investigated. Mice expressing MHC II molecules only on cortical epithelial cells in the thymus showed normal proportions of $CD4^+$ $CD25^+$ T cells in the periphery.[30] However, a different study reported that deletion of MHC II on $CD11c^{high}$ dendritic cells reduces the proportions and absolute number of Treg cells.[31] Analysis of polyclonal populations of Treg cells in normal mice also found that interactions between TCRs expressed by Treg cells and heterogeneous MHC II/peptide complexes is required to maintain Treg cells that can recognize organ-specific autoantigens.[32] The ability to suppress organ-specific autoimmunity required exposure of Treg cells to natural, tissue-derived self-antigens, which sustained these cells' phenotype.[33] This was also recently confirmed for a natural antigen, myelin oligodendrocyte glycoprotein.[11] Finally, peripheral antigens were shown to not only sustain but also shape the TCR repertoire of Treg cells residing in different anatomical locations.[34] Altogether, these studies suggest that the spectrum of peptide–MHC II complexes presented in peripheral tissues controls not only Treg cell fitness but also their clonal abundance and diversity.

Interactions of peripheral T cells with self ligands have a significant impact to preserve the function and sensitivity of T cells to foreign MHC II peptide ligands.[35] Naive, peripheral T and Treg cells sustain low-level signalling through their TCRs, which varies between T-cell clones, and this basal level of recognition of self provides homeostatic cues for both subsets.[36] Similarly to thymocytes, expression of Nurr77 reporter or CD5 in T cells correlates with increased basal TCR signalling and TCR-ζ chain phosphorylation, and was used to show that mature Treg cells continue to receive stronger signals than naive $CD4^+$ T cells.[13,37,38] The Treg cells continuously scan peripheral tissues for antigens, and in this process they acquire activated phenotype and expand, particularly in conditions promoting autoimmunity.[32,39] Stronger interactions with self-antigens also shape the repertoire of the Treg population to preserve clones with higher sensitivity, potentially predisposing specific Treg cells to be more effective in their surveillance function.[37]

The impact of TCR signalling on individual Treg cells was most convincingly shown in mice where surface expression of TCR on all T cells was eliminated.[40,41] In contrast to naive $CD4^+$ or $CD8^+$ T cells, the number of peripheral Treg cells has not changed for weeks following TCR-α locus ablation; however, homeostatic expansion of Treg cells was decreased indicating that TCR signalling is needed for their clonal proliferation but not survival. Resting, TCR-deficient Treg cells had high Foxp3 expression, remained sensitive to IL-2 and sustained their epigenetic footprint and transcriptional signature; however, loss of TCR signalling inferred with these cells activation, proliferation and diminished expression of selected adhesion molecules limiting these cells' migration to

Table 1. Proposed mechanisms shaping the repertoire of CD4$^+$Foxp3$^+$ regulatory T cells

Organ	Developmental stage affected	Mechanism(s) involved	Impact on CD4$^+$ differentiation	Impact on Treg cell repertoire	Manifestations in deficient mice	Reference
Thymus	Immature TCR-negative thymocytes	CNS3, induces preconditioning by poised state to the *Foxp3* promoter	Commitment of thymocytes with low-affinity TCRs	Addition of TCRs with low-affinity for self antigens, contribute to overlap with naive CD4$^+$ TCR repertoire	None in CNS3 KO mice, lethal autoimmunity in CNS3/Aire double KO mice	Nature: 2015;528:132–6
Thymus	Immature CD4$^+$CD8$^+$ thymocytes	TGF-β, released upon enhanced apoptosis precondition thymocytes prior Foxp3 expression	Impaired Treg generation in newborns	Addition of TCRs with low-affinity for self antigens, contribute to overlap with naive CD4$^+$ TCR repertoire	Multi-organ autoimmunity	Eur J Immunol: 2015;45:958–65.
Thymus	CD4$^+$ thymocytes	AIRE, controlled selection on low-abundance, tissue-specific peptides	Commitment of thymocytes with high-affinity, self-reactive TCRs	Addition of Treg with TCRs specific for tissue antigens	Autoimmunity in AIRE KO mice, enhanced immune response to tumour antigens	Nat Immunol: 2007;8:351–8
Thymus	Immature thymocytes	Self-antigens, select Th17 in the presence of IL-6 and TGF-β	Commitment of thymocytes with high-affinity, self-reactive TCRs to Th17	Not direct, but contribute to overlap with Treg TCR repertoire	Not tested	Nat Immunol: 2009;10:1125–32
Thymus	Immature CD4$^+$ CD8$^+$ thymocytes	(A^{b}Ep) exclusively expressed self-antigens select thymocytes with identical TCRs to both lineages	Differentiation of Treg cells with low-affinity TCRs for self, but higher affinity for non-self antigens	Selection of high-affinity TCRs to non-self antigens. Contribute to overlap with naive CD4$^+$ TCR repertoire	Not tested	Nat Commun: 2014;5:5061–71
Thymus/ periphery	CD4$^+$ Foxp3$^+$ thymocytes	IL-2	Reduced absolute number of Treg cells	Reduced diversity of Treg repertoire	Multi-organ autoimmunity	Eur J Immunol: 2011;41:3467–78
Periphery	Naive CD4$^+$ T cells	CNS1, deletion results in abrogation of pTreg formation	Conversion of naive CD4$^+$ T cells to pTreg cells is abrogated	Reduced diversity of Treg peripheral repertoire	Intestinal inflammation in older mice	Nature: 2010;463:808–12
Colon	Naive CD4$^+$ T cells, Th17 effectors	pTreg cells induced by commensal antigens, SCFA, CD103$^+$ DCs, TGF-β and RA	Acquisition of Foxp3 by mature CD4$^+$ T cells	Expansion of Treg repertoire. Contribute to overlap with repertoire of naive CD4$^+$ cells	Delayed, colonic inflammation with age	Nature: 2011;478:250–4
Small intestine	CD4$^+$ CD8$\alpha\alpha^+$ intraepithelial lymphocytes, anergic CD4$^+$	Loss of ThPOK upon contact with microbial antigens	Conversion of pTreg cells to CD4$^+$CD8$\alpha\alpha^+$ intraepithelial lymphocytes	Reduction of pTreg repertoire in small intestine	Inflammation in small intestine in experimental Crohn disease	Science: 2016;352:1581–6
Periphery germinal centres	Mature CD4$^+$ Foxp3$^+$	Bcl-6, SAP, CD28 and B cells	Acquisition by CD4$^+$ Foxp3$^+$ T cells CXCR5, PD1 expression	Sustained peripheral expression of thymus selected TCRs on Treg cells	Diminished suppression of B-cell and antibodies production	Nature Med: 2011;17:975–82

Abbreviations: A^{b}Ep, class II MHC antibody covalently linked with Eα(52–68) peptide; AIRE, autoimmune regulator; CNS3, conserved, non-coding regulatory sequence 3; DC, dendritic cell; IL-6, interleukin-6; KO, knockout; pTreg, peripherally derived Treg; RA, retinoic acid; SAP, SLAM-associated protein; SCFA, short-chain fatty acid; TCR, T-cell receptor; TGF-β, transforming growth factor-β; Th17, T helper type 17; ThPOK, zinc finger transcription factor; Treg, regulatory T.

non-lymphoid tissues, ultimately abrogating their suppressor function, which led to autoimmunity. This observation was further extended by microscopic imaging of effector and Treg cell clusters in lymphatic tissues.[42] These imaging studies showed that Treg cells enforce immune homeostasis by physical association with effector T cells which, when stimulated by self antigens, locally secrete IL-2-sustaining associated clusters of Treg cells. Deletion of TCR genes in Treg lineage also disrupted anatomical organization of clusters of effector and Treg cells, allowing for uncontrolled activation of self-reactive T cells.[42] In summary, despite retaining Foxp3 expression, TCR-deficient Treg cells or Treg cells missing TCR signals were no longer able to control homeostasis of the immune system.[43] This result showed that stable Foxp3 expression does not depend on continuing TCR stimulation. This finding was further extended to show how reduction of the TCR repertoire on peripheral Treg cells will influence an onset of autoimmunity. Reduction of the Treg TCR repertoire was implemented by replacement of a polyclonal set of TCR-α chains associated with transgenic, rearranged β-chain with only one transgenic TCR-α chain early in the life time (from a polyclonal TCR-α chain repertoire to repertoire dominated by one TCR chain) made mice prone to autoimmunity, but their manifestations were less pronounced than the symptoms found in mice in which TCRs on Treg cells lacked most TCR-α chains.[44] Hence, sustained TCR signalling in Treg cells not only maintains the functional fitness of these cells but also ensures sufficient diversity of their TCR repertoire necessary to prevent autoimmunity.

In addition to thymus Treg differentiation in the thymus, suboptimal triggering of TCRs on mature, conventional $CD4^+$ T cells in the presence of transforming growth factor-β and retinoic acid induces Foxp3, which can lead to conversion of these cells to peripherally derived Treg (pTreg) cells. This route of Treg cell differentiation may also redirect pathogenic effector T helper cells into the pTreg subset, and help to restrict inflammatory responses to commensal and infectious microbiota.[45] Similarly, chronic exposure to agonist ligands under a non-immunogenic environment was found to induce pTreg cell differentiation.[46] The process of generating pTreg cells is of importance in the context of regulating host immunoresponse to antigens derived from commensal microbiota and diet in the gastrointestinal tract. Regulatory T cells constitute a key element of the immunoregulation of inflammatory responses in the gut. Local generation of Treg cells in response to oral antigens is facilitated by a a specialized population of $CD11b^+$ $CD103^+$ dendritic cells,[47,48] which support Treg cells and inhibit pro-inflammatory T helper type 17 cells.[49] Failure to properly regulate responses to commensal microorganisms disrupts homeostasis in the gut and predisposes to pathologies like inflammatory bowel disease, allergies and metabolic abnormalities.[50] Reportedly, antigens from commensal microbiota are critical for pTreg cell induction, and uncovering the relationship between the immune system and microbiota is important for our understanding of how protective adaptive immune responses and immune tolerance coexist. An association between the presence of indigenous bacterial species and Treg cell accumulation has been discovered and pTreg cells were efficiently inducted by a consortium of *Clostridium* species.[51,52] Induction of pTreg cells required an environment rich in transforming growth factor-β and converted cells secreted IL-10, which provided protection from colitis.[53] These findings underscored the fundamental role of synergy between bacteria and the immune system and initiated a search for specific commensal species, which could be used to normalize the mucosal immune system. Rational design, aimed at optimizing induction of Treg cells, and improved efficacy of probiotics can open new avenues for therapies of inflammatory colitis and allergies. Immunoregulatory networks sustained by the microbiota have the capacity to not only control local inflammation but also systemic diseases like arthritis.

Immunomodulation by the commensal microbiota depends on pleiotropic effects of microbial proteins and metabolites.[54] Both thymus-derived and locally generated Treg cells cooperate to maintain optimal homeostasis of gastrointestinal tract.[54–56] Microbial metabolites, like short-chain fatty acids, were found to enhance Treg function and promote colonic homeostasis.[57] Immunomodulatory molecules produced by commensal bacteria enhanced conversion of $CD4^+$ T cells into Treg cells, increased production of IL-10 and were not only able to prevent, but also cure experimental colitis in animals.[53] These findings underscore the importance of a mutual relationship between commensal microbiota and Treg cells in preserving mucosal immunity and general immune homeostasis (Table 1).

Conclusions

Identification of the Treg cell population marks a major milestone in our understanding of immune system functions. Unique requirements for TCR affinity for selecting peptide–MHC ligand, existence of the selecting 'niche' and requirement for IL-2 signalling were proposed to be essential for thymic Treg lineage commitment. The same factors involving TCR and IL-2 signalling are essential for sustaining Treg cellular identity and function in peripheral organs and to recruit conventional $CD4^+$ T cells to become Treg cells. Analysis of TCR signalling and diversity and specificity of TCRs expressed by Treg cells provided new insights into how self and foreign antigens shape this population. Finally, Treg cells were identified as a major component of the immune system that

facilitates tolerance to antigens derived from commensal microbiota.

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