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Becoming self-aware: the thymic education of regulatory T cells

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Summary

The generation of Foxp3⁺ regulatory T (Treg) cells in the thymus is essential for immune homeostasis. In the past several years, substantial progress has been made in understanding the mechanisms by which a minor portion of developing thymocytes are selected to become Treg cells. Although previously controversial, recent data support the importance of TCR specificity as a primary determinant for selecting self-reactive thymocytes to become Treg cells in a multi-step process involving cytokines, co-stimulatory molecules, and a variety of antigen-presenting cells. Importantly, the antigenic niche for Treg cell development appears to be typically quite small, implying the recognition of tissue-specific, rather than ubiquitous, self-antigens. Finally, it appears that an NF- κ B transcription factor, c-Rel, may be the link between TCR recognition and the induction of Foxp3 expression, which is required for the function and stability of the natural Treg cell population.

Introduction

An adaptive immune system based on somatic gene rearrangement to generate a vast array of antigenic receptors is crucial for protection against foreign pathogens. However, some receptors in this broad repertoire will inevitably recognize self tissues with the potential for causing autoimmunity. To mitigate this problem, immature self-reactive T cells are purged during their development in the thymus via negative selection [1]. However, this process is not foolproof and some self-reactive T cells escape into the periphery. It is now well established that these self-reactive cells are controlled in periphery by natural CD4⁺ Foxp3⁺ regulatory T (Treg) cells [2–4]. The importance of Treg cell-mediated tolerance is illustrated by the dramatic observation that the acute elimination of Foxp3⁺ Treg cells in normal healthy animals can lead to death due to multi-organ autoimmunity within 10 days [5,6].

Early studies of Treg cells pointed to the thymus as a crucial site for their development. A classic observation was that day 3, but not day 7, thymectomy resulted in multiple autoimmune diseases due to the delayed generation and export of thymic Treg cells relative to conventional T cells [7,8]. Based on studies suggesting that the presence of a tissue was required for maintaining a protective suppressor cell population to that tissue, it was theorized that thymic Treg cell development was related to self-antigen recognition [9,10]. Studies of transgenic mice with TCRs specific to foreign antigens offered direct support for

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this model, as Treg cell development was observed only when the antigen was also transgenically expressed in the thymus [11,12].

However, follow-up studies on the role of TCR specificity for thymic Treg cell selection reached conflicting conclusions. For example, some studies of the Treg TCR repertoire suggested that Treg cells used TCRs that were mostly different from those TCRs found on non-Treg CD4 T cells, supportive of the original hypothesis [13–15]. Although the degree of self-reactivity of these Treg TCRs could not be determined as the self-antigens were unknown, recognition of self was suggested by the observation that many of these Treg TCRs could enhance the proliferation of T cells transferred into either normal or lymphopenic hosts [13,16]. However, it has also been argued that TCR specificity plays a limited role in Treg cell development based on the large degree of overlap in TCR usage between the Treg and non-Treg cell subsets observed in their system [17]. Additionally, it was reported that very early thymic developmental events prior to TCR rearrangement may play an important role in Treg cell selection [18], implying that the induction of Foxp3 itself was not dependent on TCR recognition of antigen. Thus, the role of TCR specificity in thymic Treg cell development was unclear.

There has been considerable progress in addressing these controversies in the past several years. Here, we will discuss the recent advances in understanding the cellular and molecular mechanisms of thymic Treg cell development, starting with experiments affirming the important role of TCR specificity, the relevance of small antigen-specific niches for Treg TCRs, the identification of a potential Treg cell precursor, and culminating with TCR-dependent signals that may directly facilitate Foxp3 gene expression.

Natural Treg TCRs and their developmental niches

The debate appears to have shifted in favor of TCR specificity playing a dominant role in thymic Treg cell development based on reports by two independent groups using TCR transgenic mice expressing natural Treg TCRs [19,20]. The TCRs were selected from the Treg cell subset based on TCR repertoire studies of mice expressing TCR β transgenes that limit the repertoires to an experimentally manageable level. Surprisingly, none of these Treg TCR transgenic lines showed appreciable frequencies of thymic Foxp3⁺ cells, in contrast to previous studies using foreign antigen-specific TCR and antigen transgenic mice [21]. Although the transgenic lines were generated years ago, both groups went through considerable efforts to exclude technical issues that may hinder thymic Treg cell generation in these lines. For example, the possibility that an altered developmental sequence at the DN stage prevented subsequent Treg cell development [18] due to early transgene-driven TCR expression was ruled out using CD4-promoter driven expression of TCR α at the DP stage (unpublished data and [20]). Eventually, serendipitous observations from mixed bone marrow chimeras or intrathymic injection of TCR transgenic thymocytes suggested that developing T cells with the same antigen specificity compete for a limited niche for Treg cell development, based on the observation of an inverse relationship between the clonal frequencies of Treg TCR transgenic thymocytes and frequency of Foxp3⁺ cells. For the TCRs tested, the clonal frequency needed to be below 1% to observe efficient generation of Treg cells (e.g. 20–80%). Moreover, the number of Treg cells plateaus at higher clonal frequency, suggesting the existence of a limited niche for a given Treg TCR specificity [19,20]. This niche was much smaller than that for positive selection, resulting in the perceived inability of these TCRs to facilitate thymic Treg cell development in TCR transgenic lines. By contrast, no Foxp3⁺ cells were observed under the same conditions using transgenic thymocytes expressing naïve TCRs. Thus, these data using natural Treg TCR transgenic lines demonstrated the importance of antigen specific niches for thymic

Treg cell development, and offered strong support for the original notion that TCR specificity plays an important role in directing Treg cell development.

These data also revealed that TCRs should not simply be classified as yes/no in terms of their ability to induce Treg cell development. Rather, individual TCRs fall along a broad spectrum of efficiencies in their ability to facilitate Treg cell development. This is illustrated by the plot of the frequency of T cells expressing the same TCR in the CD4SP subset by the percentage of those cells that are Foxp3⁺ (Fig. 1, right [19]). For the TCRs tested thus far, the slopes appear similar, suggesting that similar mechanisms regulate the ability of these TCRs to facilitate Treg cell development. However, the intercepts vary considerably. Since the absolute number of CD4SP thymocytes with a unique TCR rearrangement at any given time is likely to be < 10 cells, the efficiency of Treg cell generation should ideally be assessed at those low clonal frequencies. Even if TCRs with equivalent specificity but different rearrangements are included, the aggregate number of cells is still likely to be less than 100 [22]. In lieu of direct measurements at these normal, but very low, clonal frequencies, we can extrapolate the data to estimate the efficiency of TCR-dependent Treg cell development (Fig. 1, left [23]). If the TCR repertoire contains a normal distribution of efficiencies, the boundary effects at high and low efficiencies may appear as if Treg and non-Treg TCRs is a binary yes/no phenomenon [23], when in actuality, the ability of TCRs to facilitate Treg cell development is a quantitative, and not qualitative, property.

While it has been suggested for some time that TCR avidity for self antigens may represent this quantitative property, experimental evidence has not been conclusive. First, the use of a low affinity peptide antigen in the aforementioned TCR transgenic model did not result in Treg cell development, even though deletion could be achieved via high level expression [12]. Second, the expression of graduated doses of cognate antigen in a different TCR transgenic model failed to increase the number of Treg cells generated [21]. However, it was recently shown that decreased presentation of cognate antigen on medullary thymic epithelial cells (mTECs) or dendritic cells (DCs) could tip the balance in favor of Treg development rather than negative selection [24,25]. Thus, an unanswered question in Treg cell development is whether TCR avidity for self-antigens is a primary determinant of thymic Treg cell selection efficiency, or whether other TCR:ligand properties are also relevant [26].

One implication of the small niche size for Treg cell development is that these TCRs likely recognize rare tissue-specific rather than ubiquitously presented antigens [27]. This is consistent with a number of studies suggesting the existence of tissue-specific peripheral Treg cells [9,10], as well as demonstrating that the Treg TCR repertoire varies by anatomic location [28]. One possible reason that the Treg cell population preferentially recognizes uncommon self-antigens is that ubiquitous antigens may favor negative selection. Another reason may be that uncommon self-antigens might be more suitable for dynamic, rather than static, Treg cell responses to preserve immune homeostasis. For example, Treg cells reactive to albumin would be expected to show little variability in its activation status regardless of injury or infection, whereas Treg cells specific to myosin may become fully activated only after muscle injury. Dynamic responses to uncommon self-antigens would be consistent with the evolution of a Treg cell population with a diverse TCR repertoire [13–16,29–31] to provide complex antigen-specific immune regulation, and not be a simple counterweight to T cell activation.

APC subsets and Treg cell development

Although it was initially reported that Treg cell development starts at the CD4⁺CD8⁺ (DP) stage [32,33], more recent studies suggest that this occurs rarely [34,35]. It therefore appears

that most Treg cells develop after positively selected CD4 thymocytes migrate from the cortex to hundreds of medullary compartments comprised of mTEC and hematopoietic APCs, predominantly DCs (Fig. 2) [36,37]. Previous studies have examined these medullary APCs for their ability to select Treg cells. In TCR transgenic systems, the expression of cognate antigens on either cell type seems to be able to induce Treg cell development [12,38]. However, chimeras in which bone marrow derived APCs are deficient in MHC class II expression showed normal numbers of thymic Treg cells [32,39], suggesting that mTECs may be the major APC for Treg cell selection.

Recent evidence suggests that all of these original observations are likely to be correct: both DCs and mTECs can facilitate Treg cell development. Although the mutation of co-stimulatory molecules CD80/86 or CD40 results in a dramatic decrease in Treg cell numbers, expression of these co-stimulatory molecules on only one APC subset is sufficient to generate normal percentages of Treg cells [40–42] (and unpublished data). Moreover, normal or even elevated percentages of Treg cells were observed when MHC II presentation is decreased on Aire⁺ mTEC cells by microRNA-mediated knockdown of CIITA [24], supporting the notion that either APC subset is sufficient for Treg cell generation.

Although the number of Treg cells selected by each APC subset may be equivalent when the other is incapacitated, it is quite possible that different TCR specificities are selected by each APC subset. For example, AIRE expression on mTEC cells might favor tissue-specific antigens in the thymus. Some peripheral DCs can migrate into the thymus and present peripheral antigens to developing thymocytes (Fig. 2) [43]. Beyond the antigenic repertoire displayed by different APC subsets, their ability to mediate negative selection versus Treg cell development may differ [44]. An important future question will be to understand why there are multiple thymic APC subsets involved in generating Treg cell mediated tolerance, and whether these subsets play unique roles in generating the Treg cell repertoire.

A multi-step process for thymic Treg cell development

Although TCR recognition of self-antigen appears to be important for Treg cell selection, additional events beyond TCR signaling appear to be required for the induction of Foxp3 [45–47]. It has been proposed that TCR signaling results in the development of Foxp3⁺ Treg cell precursors which can then utilize interleukin-2 (IL-2), or to a lesser extent IL-15 or IL-7, to induce Foxp3 expression without the need for additional TCR signals. An attractive, but unproven, idea is that these cytokines may be preferentially expressed within the Treg cell developmental niches. The process may also apply to peripheral Treg cell development [35]. Thus, there is accumulating evidence that multiple steps beyond TCR:antigen engagement are required to induce Foxp3 gene-expression, which appears to represent a late event in Treg cell development [48,49]

However, it is not entirely clear why a multi-step process is required. One interesting possibility stems from the observation that early withdrawal of TCR stimulation can promote the expression of Foxp3 by peripheral naïve T cells [50]. If this is applicable to thymic development, it could be hypothesized that T cells which continually encounter antigen (e.g. ubiquitous) would be deleted, whereas antigens present only on a fraction of APCs (e.g. tissue specific antigens) may favor Treg cell development as a result of a more limited duration of TCR signaling.

Molecular mechanisms of thymic Treg cell development

TCR specificity may be the most important factor determining Treg cell fate, but other factors are clearly involved. It has previously been shown that CD28 plays an important cell-intrinsic role in thymic Treg cell generation [51]. Recent studies suggest that CD28

primarily improves the efficiency of Treg cell development rather than enhancing the number of TCRs that can facilitate Treg cell development [52]. CD28 signaling is important for the generation of cytokine-responsive Treg cell precursors, and is partially dependent on the cytoplasmic Lck-binding motif PYAP, which can induce the activation of PKC θ and the NF- κ B pathway [51–54].

Another downstream signal of CD28 is the PI3K/Akt pathway important for cell survival and proliferation (reviewed by [55]). Paradoxically, the over-expression of Akt inhibits thymic Treg cell development at least partly via mTOR and the subsequent inhibition of Foxo1/Foxo3a, transcription factors which can bind to Foxp3 promoter and at conserved nucleotide sequences (CNS2, between exon -2 and exon -1) and activate Foxp3 expression [50,56–58]. However, mice deficient in CNS2 have normal thymic Treg cell numbers, suggesting that the promoter or other Foxo binding sites may be more important. An important future question is whether TCR/CD28 signaling in the thymocytes, but not peripheral T cells, is dominated by NF- κ B but not AKT, consistent with a simple avidity model for Treg cell development, or whether TCR specificities or other molecular events can selectively modulate positive and negative differentiation signals to determine thymic Treg cell-fate.

c-Rel, a molecular mechanism linking TCR and Foxp3

The NF- κ B pathway has been recognized for some time to be required for Treg cell differentiation as mutations of PKC θ , CARMA1, Bcl-10, TAK1, and IKK β greatly reduce thymic Treg cell numbers (reviewed by [59]). Recently, several independent groups have reported that c-Rel is the NF- κ B family member that binds to the Foxp3 locus and facilitates its expression [60–63]. Importantly, expression of a constitutively active IKK β is sufficient to restore normal thymic Treg cell numbers in Tak1- and Carma1-deficient mice and can override the requirement for TCR-signals to induce Foxp3 expression in OTII and P14 *Rag1*^{-/-} transgenic thymocytes in the absence of cognate antigen [61]. c-Rel, potentially as a homo-dimer, can bind to CNS3 of Foxp3 [63], which contains largely permissive chromatin (H3K4me1) in DP and CD4SP cells, in contrast with other CNS regions. Together with the reports that NF- κ B activation is sufficient for Foxp3 expression, this suggests that c-Rel may be a pioneer transcription which opens the Foxp3 locus to other transcription factors [63].

The NF- κ B pathway is widely used by many immune cells. How this common pathway facilitates the specific induction of Foxp3 primarily in CD4SP cells, and not other cells, is unclear. One possibility is that different NF- κ B subunits play cell-type specific roles. For example, natural killer T cells require RelA, but not c-Rel, for their development [64]. Additionally, NF- κ B may provide a threshold of TCR signaling for Treg cell development [65]. It has been recently shown that anti-CD3-mediated activation of RelA is digital in nature and is regulated prior to the translocation of PKC θ to immunological synapse [66], suggesting the conversion of analog signal generated by TCR to digital output happens at the TCR proximal signaling machinery. The relevance of this mechanism to thymic Treg cell development remains to be determined.

Conclusions

The field of thymic Treg cell development has advanced considerably. New data support the role of TCR specificity in thymic Treg cell development, and also revealed the existence of limited antigen-specific niches. What defines these niches? Is it antigens, APCs, cytokines? Why are larger niches not found? The role of TCR specificity in thymic Treg cell development has also been supported by the identification of Treg cell precursors as well as

new data on molecular signals such as NF- κ B/c-Rel and Akt/mTOR which can initiate or inhibit Treg cell development, respectively. Are there other transcription factors beyond c-Rel that initiate the expression of Foxp3? What is the interplay between NF- κ B and Akt? What are the molecular features of the immediate Treg cell precursor? Are thymic and peripheral Treg cell development mechanistically related? It is clear that there are still many unanswered questions regarding thymic Treg cell development.

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Figure 1.

A probabilistic model for TCR specificity and Treg cell development. For the TCRs reported so far, the clonal frequency of cells expressing a specific TCR (x-axis, plot on right) is inversely related with the percentage of the cells that are Foxp3⁺ (y-axis). These data illustrate that there are quantitative differences between TCRs, primarily at the y-intercept. Three general classes of TCRs are shown (primarily Treg, incomplete Treg, and naïve). It is expected that TCR affinity, abundance of antigen, and APC costimulatory molecules, contribute to these quantitative differences. Note that using the entire CD4SP subset includes thymocytes that have not yet encountered antigen, potentially resulting in an under estimation of the efficiency of Treg cell development. Future analyses may be improved by restricting the analyses to the CD4SP subset ready to exit the thymus, perhaps by using molecular markers such as KLF2. A hypothetical extrapolation of the experimental data to the low clonal frequencies in the normal polyclonal setting (dashed lines) is shown on the left. Green area depicts the Treg cell subset.

Figure 2.

Summary of thymic Treg cell development. After positive selection, immature CD4SP cells migrate from thymic cortex to the medulla. The CD4SP cells then interact extensively with APCs, such as mTECs, as well as DCs that originated from the thymus (Sirp α^+ cDC) or periphery (Sirp α^- cDC and pDC), to screen for potential self-reactivity before emigrating to the periphery. All of these APC subsets are capable of directing Treg cell development, but probably present differing antigenic repertoires (depicted), of which some can be peripherally derived. Aire-dependent antigens can be transferred from mTECs to DCs. Chronic TCR/CD28 engagement with ubiquitous antigens is hypothesized to favor negative selection. Cells that express TCRs recognizing uncommon antigens may become Foxp3 $^+$ cytokine-responsive Treg cell precursors, in which IL-2 subsequently induces Foxp3. Cells without overt self-reactivity will be exported as naïve T cells. Quantitative thresholds for these different cell fates are currently not available.