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The role of T-cell receptor recognition of peptide:MHC complexes in the formation and activity of Foxp3$^+$ regulatory T cells

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Summary

Foxp3$^+$ regulatory T (Treg) cells are required to prevent the immune system from spontaneously mounting a severe autoaggressive lymphoproliferative disease and can modulate immune responses in a variety of settings, including infections. In this review, we describe studies that use transgenic mice to determine how signals through the T-cell receptor (TCR) contribute to the development, differentiation and activity of Treg cells in *in vivo* settings. By varying the amount and quality of the self-peptide recognized by an autoreactive TCR, we have shown that the interplay between autoreactive thymocyte deletion and Treg cell formation leads to a Treg cell repertoire that is biased toward low abundance agonist self-peptides. In an autoimmune disease setting, we have demonstrated that diverse TCR specificities can be required in order for Treg cells to prevent disease in a mouse model of autoimmune inflammatory arthritis. Lastly, we have shown that Treg cells initially selected based on specificity for a self-peptide can be activated by TCR recognition of a viral peptide, and that they can acquire a specialized phenotype and suppress anti-viral effector cell activity at the site of infection. These studies provide insights into the pivotal role that TCR specificity plays in the formation and activity of Treg cells.

Keywords

autoimmunity; tolerance; transgenic mice; thymus; viral

Introduction

Regulatory T (Treg) cells are a subset of CD4$^+$ T cells that are crucial for the maintenance of self-tolerance and immune homeostasis. Treg cells are defined by expression of the transcription factor Forkhead box protein 3 (Foxp3), which is necessary for their development and persistence, and loss of Foxp3 function through either mutation or deletion leads to rapid onset of an autoaggressive lymphoproliferative disease in both mice and humans (1–5). There is increasing evidence that Treg cells also function in other settings in which immune activation occurs, such as during the immune response to a pathogen (6–8). In this setting, the ability of Treg cells to limit effector cell activity may aid in preventing

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the excessive immunopathology that can accompany a prolonged or severe immune response to a pathogen (9, 10). Indeed, one could imagine that during any immune response, a fine balance must be maintained between the generation of an effective response that eliminates invading microorganisms and the control of immune-mediated tissue damage and loss of self-tolerance (11).

T cells are formed in the thymus, where developing thymocytes use somatic gene rearrangement to create a unique T-cell receptor (TCR). This process allows the immune system to generate a vast variety of TCR specificities with the potential to recognize an unknown universe of foreign antigens in populations that contain diverse major histocompatibility complex (MHC) alleles for peptide presentation. As a result of this process, however, many of the thymocytes generate TCRs that are unable to recognize the host's own MHC molecules, while other TCRs can bind strongly with peptides derived from self-antigens, and T cells bearing these TCRs have the potential to react with the host's own tissues if they enter the periphery. It is broadly accepted that thymocytes therefore undergo selection processes that are governed by the reactivity of the TCR towards peptide:MHC complexes, such that weak reactions with peptide:MHC complexes are necessary to rescue thymocytes from apoptosis (positive selection), while stronger signals can induce their deletion (negative selection). For conventional $CD4^+$ and $CD8^+$ T cells, this avidity model has provided an excellent framework for understanding how a TCR repertoire that is generated by random gene rearrangement can be shaped to ultimately include predominantly those specificities that could potentially react to the host's own MHC alleles when they are presenting pathogen-derived peptides (positive selection), while eliminating those that might be overtly reactive with host-derived peptides (negative selection). However, it is less clear how to fit Treg cells into this model, particularly in light of the apparent role that Treg cells play in regulating immune responses to self-antigens. It is also unclear how TCR specificity can shape the activity of Treg cells; there is convincing evidence that TCR signaling is necessary to activate Treg cell-mediated suppression in *in vitro* cultures (12), but how TCR specificity can direct Treg cell activity in response to either self or foreign antigens *in vivo* remains poorly understood. This review describes studies examining how signals transmitted through the TCR can govern both the development and activity of Treg cells in a transgenic mouse model system in which the specificity of the TCR for foreign- and/or self-peptide:MHC complexes can be defined.

Regulatory T cells form in the thymus upon TCR-mediated recognition of self-peptide

Our studies concerning the role of TCR specificity in directing Treg cell formation and *in vivo* effector activity have derived from an initial observation that was made while using transgenic mice to analyze how TCR reactivity with self-peptides could shape $CD4^+$ T-cell development in the thymus. To define the specificity of $CD4^+$ T cells, we used TS1 mice, which express a transgenic TCR that recognizes the Site 1 (S1) epitope of PR8 influenza virus hemagglutinin (HA) presented I-Ed (13). The TS1 TCR is recognized by the anti-clonotypic mAb 6.5, which can be used to track its expression in flow cytometry, and was originally obtained from a $CD4^+$ T-cell clone isolated from a BALB/c mouse that had been

infected with influenza virus strain PR8. When we crossed TS1 mice to a lineage of transgenic mice that express the PR8 HA as a neo-self antigen (termed HA28 mice), the resultant TS1xHA28 mice contained significantly higher percentages and numbers of both 6.5^+CD4SP thymocytes and 6.5^+CD4^+ lymph node cells that expressed CD25 than were found in TS1 mice that did not express the HA as a self-peptide (14, 15). These 6.5^+CD25^+ T cells also expressed low levels of CD45RB, which, like high levels of CD25, had been associated with regulatory T-cell activity, and could exert potent suppressor function *in vitro*. Subsequent studies showed that these 6.5^+CD4SP$CD25^+$ thymocytes and $6.5^+CD4^+CD25^+$ T cells also expressed Foxp3, firmly establishing their identity as Treg cells (16, 17). Importantly, 6.5^+ Treg cells were also found to form in mice in which the use of endogenous TCR α-chains was prevented, either by mating TS1xHA28 mice into a $TCR\alpha^{-/-}$ background or by generating radiation chimeras in which bone marrow from TS1. $Rag^{-/-}$ mice was introduced into HA28 mice (14, 16). Collectively, these studies showed that TCR recognition of a self-peptide could induce thymocytes bearing an autoreactive TCR to selectively become $Foxp3^+$ Treg cells. Similar conclusions were reached by others studying the fate of 6.5^+ CD4SP thymocytes in TS1xHA transgenic mice using alternative promoters to drive HA expression, and in studies in which ovalbumin-specific DO11.1 TCR transgenic mice were mated to mice expressing ovalbumin as a neo-self antigen (18–21).

More recently, other investigators have analyzed the role of TCR specificity in directing $Foxp3^+$ Treg cell formation through a related approach, in which they assessed the ability of TCRs derived from $Foxp3^+$ Treg cells to instruct thymocytes to develop along the Treg cell differentiation pathway. TCRs derived from $Foxp3^+$ T cells were able to direct Treg differentiation, but only when present at low clonal frequencies, leading to the conclusion that thymic Treg cell formation is limited by a selection niche (22,23). We similarly observed limited thymic $Foxp3^+$ Treg cell formation in studies using a different TCR transgenic mouse lineage [termed TS1(SW)] in which reducing the clonal precursor frequency enhanced neo-self-peptide-induced $Foxp3^+$ Treg cell formation (24). The factors that might govern the size of this apparent selection niche are unknown, and it is currently unclear why the frequencies of $Foxp3^+$ Treg cells that are generated in the TS1 and OVA systems can be substantially higher than those found in studies where a selection niche has been observed. In additional studies analyzing the role of self-reactivity in Treg cell formation, $CD4^+$ T cells transduced with TCRs taken from Treg cells proliferated to a greater degree when transferred into WT host mice than did T cells transduced with TCRs acquired from non-Treg cells, and $CD4^+$ T cells expressing a Treg cell-derived TCR caused more rapid wasting disease in $Rag^{-/-}$ hosts (25, 26). Collectively, these studies all support the conclusion that TCR specificity for self-peptides can play a critical role in the formation of $Foxp3^+$ Treg cells.

Thymocyte deletion biases Foxp3⁺ Treg cell formation toward low abundance self-peptides

The conclusion that Treg cell formation is promoted by interactions with self-peptides provided a basis for understanding how Treg cells might be able to regulate immune responses to the host's own cells and tissues but was difficult to reconcile with the avidity

model of thymocyte formation in which strong TCR reactivity with a self-peptide mediates thymocyte deletion. It was also unclear how variations in the amount of a self-peptide present and/or the cell types by which it is expressed might affect the extent to which autoreactive thymocytes undergo deletion versus Treg cell formation, as well as whether deletion and Treg cell formation represented distinct or inter-related processes governing autoreactive thymocyte development. We examined these questions by developing multiple lineages of HA transgenic mice in which there were variations in the amount of HA mRNA that was expressed in the thymus. In some cases, these variations were the result of differences in the promoters being used to drive transgene expression, while in other cases, they were caused by the distinct sites in which the transgenes had become integrated in the genome. We found that 6.5^+CD4SP thymocytes underwent varying degrees of deletion that directly correlated with the amount of HA mRNA that was expressed in the thymus; the least deletion was observed in mice with the lowest amount of HA mRNA, while there was substantial deletion of 6.5^+CD4SP thymocytes in mice with high levels of HA mRNA expression (17). Strikingly, among the 6.5^+CD4SP thymocytes that evaded deletion, the proportions that acquired Foxp3 expression were very similar, averaging between 20 and 35% of the 6.5^+CD4SP thymocytes. Moreover, in all cases, the numbers of 6.5^+CD4SPFoxp3$^+$ thymocytes that were present in mice expressing the HA transgenes were significantly higher than were present in TS1 mice (which do not express the S1 self-peptide). This observation indicates that even in the context of thymocyte deletion, the formation of 6.5^+CD4SPFoxp3$^+$ Treg cells is a selective process that is induced by the presence of the neo-self HA, rather than the selective survival of thymocytes that had acquired Foxp3 expression in response to other cues, as has been proposed based on studies in another system (22, 27, 28). We also used radiation bone marrow chimeras created with TS1xHA transgenic mice to examine a possible role for specialized cell types in either deletion or Foxp3$^+$ Treg cell formation. However, we found that both radioresistant and radiosensitive cell types could induce each of these processes (14, 15, 17), suggesting that the amount of self-antigen, rather than the cell type in which it is expressed, is the critical factor in determining the developmental fate of autoreactive thymocytes.

The findings in the different lineages of TS1xHA transgenic mice provide evidence that thymocyte deletion and Treg cell formation can be intertwined rather than distinct processes, at least in the context of 6.5^+CD4SP thymocytes developing in the presence of the S1 self-peptide. Moreover, since the variations that we observed in the expression of the HA transgene are likely to correspond to variations that can exist among *bona fide* self-peptides (i.e. some self-peptides are expressed in low amounts, while others are more abundantly expressed), our studies suggest that the Treg cell repertoire may be biased toward low abundance self-peptides, because these peptides induce less effective deletion. This conclusion may explain why one study concluded that self-peptides are not the cognate antigens for Treg cells, after hybridomas generated from Treg cells were found not to display detectable activity toward self-antigens *in vitro* (29). However, if the self-peptides that mediate Treg cell formation are of low abundance, it is possible that these studies failed to detect reactivity because the levels of cognate peptides that are recognized by the Treg-derived TCRs were insufficient to activate hybridomas to an extent that would permit detection in an *in vitro* assay. Indeed, we cannot detect activation of 6.5^+CD4$^+$Foxp3$^+$ T

cells obtained from TS1xHA28 mice in *in vitro* assays when we use APCs from HA28 mice as stimulators, even though we know that the S1 self-peptide can induce abundant formation of these cells in TS1xHA28 mice (authors' unpublished observations). Further experiments in the above-mentioned study showed that mice in which all MHC class II molecules express the same self-antigen do not form Treg cells against that self-antigen (29), and this outcome could again be explained by our conclusion that a self-antigen expressed at relatively higher levels is likely to result in very little Treg cell formation.

A notable finding in the different lineages of TS1xHA28 mice is that the size of the deletional niche can be a critical parameter in determining the overall efficiency of Treg cell formation since the degree of deletion increased in relation to the amount of self-antigen, while among the 6.5^{+}CD4SP thymocytes that evaded deletion, the rate of $Foxp3^{+}$ Treg cell formation remained relatively constant. Based on the studies suggesting that precursor frequency and intraclonal competition determine the rate of Treg cell formation (22), we might have expected that increased thymocyte deletion would lead to increased rates of Treg cell formation due to decreases in precursor frequency, but this was not observed. The studies in the different lineages of TS1xHA transgenic mice raise an additional question in this respect; why do only a subset of the 6.5^{+}CD4SP thymocytes in the different TS1xHA transgenic mice become $Foxp3^{+}$, and why does this remain relatively constant in the different mice? One possibility is that a selection niche exists that limits the total number of Treg cells but is insensitive to the frequency of precursors of any one specificity, perhaps because of interclonal competition for resources, as opposed to the intraclonal competition that was observed in prior studies. Another possibility is that there are cell intrinsic processes that limit the ability of individual precursors to become $Foxp3^{+}$ in response to TCR stimulation. For example, there might be stochastic differences in the distribution of factors (such as those mediating epigenetic modification) that are necessary to allow precursor cells to undergo selection, and this disparity may limit the ability of individual precursors to acquire Foxp3 expression in response to self-peptide. Alternatively, the high levels of TCR signaling that promote deletion may also limit the probability that an individual precursor acquires Foxp3 expression by dampening the corresponding pathways.

Agonist self-peptide promotes Treg cell formation

The preceding studies in TS1xHA transgenic mice provided evidence that reactivity with self-peptides can promote both the deletion of autoreactive thymocytes and the induction of Foxp3 expression among a subset of CD4SP thymocytes that evade deletion. A notable feature of these studies is that these processes were induced by a self-peptide that is recognized by the TCR as an 'agonist'. The term agonist was developed to describe TCR reactivity with a peptide following the discovery by several groups (30, 31) that an individual TCR can exhibit a range of reactivities with peptide ligands that can lead to both quantitative and qualitative differences in the responses induced in T cells. In this nomenclature, agonist peptides were those that could induce peripheral T cells to execute diverse effector functions when present at relatively low concentrations. In many cases, these agonist peptides corresponded to the peptide against which a responding T cell had initially been elicited (and are sometimes termed "cognate" peptides); in the case of TS1 mice, the 6.5 TCR was isolated from a $CD4^{+}$ T-cell clone that had been obtained from a

mouse infected with an influenza virus strain containing the S1 peptide. The term agonist peptide is useful inasmuch it can distinguish such peptides from those with which a TCR is more weakly reactive; such 'partial agonist' peptides typically require higher concentrations to induce T-cell activation, and a partial agonist might prompt only a subset of the effector functions induced by an agonist. The finding that Treg cell formation is induced by an agonist self-peptide in TS1xHA transgenic mice (and also in the OVA system) seemed counterintuitive in light of the avidity model of thymocyte development, in which thymocytes expressing a TCR that has a strong intrinsic reactivity with self-peptide were thought to be subjected to deletion. We therefore sought to analyze how modulating TCR reactivity towards the S1 peptide could affect the extent of deletion and/or Foxp3 induction among 6.5^+CD4SP thymocytes.

As a first approach to modifying the signal being transmitted through the 6.5 TCR, we generated an additional lineage of transgenic mice [termed Pev(SW)HA mice] expressing an HA molecule derived from the SW strain of influenza virus, which contains an analog of the S1 peptide that differs from S1 by two amino acid substitutions. As a result of these substitutions, the 6.5 TCR is only weakly cross reactive with the S1(SW) HA molecule (i.e. it recognizes this peptide as a partial agonist). When we mated these mice with TS1 mice to generate TS1xPev(SW)HA mice, we found that interactions with the S1(SW) peptide induced significant deletion of 6.5^+CD4SP thymocytes but that no formation of 6.5^+CD4SP thymocytes occurred (24). Importantly, the S1(SW) self peptide in Pev(SW)HA mice did induce the formation of $CD4^+Foxp3^+$ T cells when we mated them with another TCR transgenic lineage [termed TS1(SW)] whose TCR recognizes the S1(SW) peptide as an agonist, and conversely, no such formation occurred when TS1(SW) mice were mated with PevHA transgenic mice expressing the S1 peptide [which is recognized by the TS1(SW) TCR as a partial agonist]. In addition, the deletion of 6.5^+CD4SP thymocytes by the S1(SW) partial agonist in TS1xPev(SW)HA mice was accompanied by significant upregulation of Nur77, a transcription factor that is associated with thymocyte deletion, although the levels of Nur77 induced in 6.5^+CD4SP thymocytes in response to the partial agonist S1(SW) were lower than was the case in TS1xPevHA mice expressing the S1 agonist self-peptide, consistent with lower levels of TCR signaling. These findings suggest that lower signaling thresholds are necessary for the induction of deletion than are required to initiate Foxp3 expression, consistent with recent studies using a Nur77 reporter mouse that concluded that $Foxp3^+$CD4SP thymocytes have perceived stronger TCR signals than is the case for conventional CD4SP thymocytes (32).

To further examine how decreasing the intensity of signaling through the TCR could affect autoreactive thymocyte deletion and/or Treg cell formation, we used mutant mice (termed Y145 mice) in which one of three critical N-terminal tyrosine phosphorylation sites on the adapter molecule SLP76 (Src homology 2 domain-containing leukocyte protein of 76 kDa) has been mutated to phenylalanine in order to prevent phosphorylation (33). Previous studies had shown that the Y145 mutation decreased TCR signal strength when the TCR was cross-linked with anti-CD3 (33, 34), and we found that this mutation also led to a modest 2–4-fold decrease in the activation of 6.5^+CD4^+ T cells from TS1 mice (as assessed by upregulation of CD69 and CD25) in response to S1 peptide *in vitro* (35). Nur77 levels were also

significantly lower among 6.5^+CD4SP thymocytes from TS1xPevHA. Y145 mice than those from TS1xPevHA mice with wildtype SLP-76 alleles, indicating reduced TCR signaling from the agonist S1 self-peptide (35). Strikingly, this mutation completely abrogated the deletion of 6.5^+CD4SP thymocytes in response to the agonist S1 peptide in TS1xPevHA. Y145 mice. However, 6.5^+CD4SP$Foxp3^+$ thymocyte formation still occurred in TS1xPevHA. Y145 mice, albeit with reduced efficiency relative to mice expressing wild-type SLP76 alleles. Interestingly, in BALB/c. Y145 mice containing polyclonal TCR repertoires, the presence of the Y145 mutation led to increased formation of $CD4^+Foxp3^+$ Treg cells and was also associated with increased representations of $CD4^+$ T cells expressing TCR Vβ regions that are sensitive to deletion by endogenous *Mls* superantigens, especially among the $CD4^+Foxp3^+$ Treg cells (34–36). In this case, then, the increased representation of Treg cells appears to be a reflection of a decrease in the stringency of thymocyte deletion, which in turn allowed greater numbers of autoreactive thymocytes to enter the Treg selection pathway, even though the efficiency of Foxp3 induction in individual Treg cell precursors was also lowered by the effects of the SLP-76 mutation.

These studies show that even though autoreactive thymocytes undergo both deletion and Foxp3 induction in TS1xHA transgenic mice, the two processes can be at least partially segregated by modifying the nature of the signal transmitted by the 6.5 TCR. Most notably, they reinforce the notion that agonist self-peptides might be uniquely able to promote Treg cell formation, because Foxp3 induction was lost in TS1xPev(SW)HA mice where a partial agonist caused reduced signaling. By contrast, in TS1x(Pev)HA. Y145 mice where agonist-induced TCR signaling still occurs but signaling intensity is diminished by a SLP-76 mutation, Foxp3 was induced among a subset of 6.5+CD4SP thymocytes, despite abrogation of thymocyte deletion.

TCR recognition of self-peptides drives peripheral Treg cell expansion

The preceding studies focused on the role of TCR recognition of self-peptides in the formation of Treg cells during thymocyte development, and we have also used this system to examine the role that such interactions might play in shaping the peripheral Treg cell repertoire. 6.5^+ Treg cells from TS1xHA28 mice underwent little or no proliferation when transferred into BALB/c mice, but underwent several rounds of division following transfer into HA28 mice in which the cognate S1 peptide is expressed (37). In addition, sublethal irradiation of mice to produce lymphopenic conditions caused conventional effector $CD4^+$ T cells to expand following transfer, but 6.5^+ Treg cells only expanded in lymphopenic HA28 mice and did not expand in lymphopenic BALB/c mice (37). These studies show that TCR interactions with self-peptides can play a critical role in the expansion and/or maintenance of Treg cells in the periphery, as has also been observed in other systems (21, 38, 39). This antigen-driven expansion may provide a mechanism by which Treg cells that are generated based on intrathymic expression of tissue-specific self-peptides (for example due to AIRE-mediated expression) can accumulate selectively at sites in the periphery where the tissue-specific antigen is expressed.

A diverse Treg cell TCR repertoire can be crucial for prevention of autoimmune arthritis

Many studies have established that the presence of $Foxp3^+$ T cells is necessary to prevent the immune system from mounting a spontaneous autoaggressive inflammatory disorder characterized by the uncontrolled expansion of lymphocytes, including effector $CD4^+$ T cells. Although this syndrome is often referred to as autoimmunity, it is different from the syndromes that are found in many autoimmune diseases (such as type II diabetes or rheumatoid arthritis), which tend to show strong genetic linkages with MHC class II alleles (rather than with Foxp3) and are generally thought to be the result of a sustained and uncontrolled immune response to one or more self antigens. Moreover, in many cases these autoimmune diseases can develop despite the presence of Treg cells (40, 41). Whether the Treg cells that are present in these circumstances are unable to suppress disease because of functional defects, a lack of correct TCR specificity, or some other reason is still not known. Indeed, the role that TCR specificity for self-peptides can play in determining the ability of Treg cells to prevent autoimmune disease remains uncertain. Signals through the TCR are required to activate Treg cell suppressive function *in vitro*, which allows them to directly suppress effector T cells in the same culture, but once activated, Treg cells can also mediate 'bystander suppression' by modulation of antigen presenting cell function and/or secretion of anti-inflammatory cytokines (42). Whether Treg cells achieve suppression *in vivo* via direct recognition of the same target peptides recognized by autoaggressive effector $CD4^+$ T cells or through bystander effects, perhaps by manipulation of antigen presenting cells, is still unclear.

We have been investigating the requirements of TCR specificity for Treg activation and suppression *in vivo* in a mouse model of autoimmune arthritis that is also based on the TS1xHA transgenic mouse system. To focus expression of the HA toward antigen presenting cells, we had generated HACII mice, which express the PR8 HA molecule under the control of an MHC class II promoter. Our goal was to use these mice to examine how $CD4^+$ T-cell tolerance is established to abundantly expressed self-peptides, and when we mated these mice with TS1 mice, we unexpectedly found that the resulting TS1xHACII mice developed inflammatory arthritis, identified by swelling of the ankle and/or wrist joints, with approximately 80% penetrance by the age of 12 weeks (43, 44, reviewed in 45). The swollen joints in adult TS1xHACII mice contained infiltrates of immune cells and underwent substantial cartilage loss and bone erosion. Additional manifestations of inflammatory autoimmune disease have been observed in the lungs and occasionally in heart tissues, but no evidence of pathology has been found in kidney, liver, thyroid, salivary glands, pancreatic islets, or intestinal tissues (43). Disease development appears to depend on T-cell recognition of a self-antigen expressed on MHC class II antigen-presenting cells (APCs) since no signs of inflammatory arthritis have been observed in our other TS1xHA transgenic lineages.

Autoimmune arthritis develops in TS1xHACII mice despite extensive deletion of 6.5^+ thymocytes and despite the presence of $CD25^+Foxp3^+$ Treg cells, some of which express the 6.5 TCR. To investigate whether the presence of additional Treg cells could prevent disease

development in this model, we transferred 1×10^6 polyclonal Treg cells from BALB/c or HACII mice into 5–6-week-old pre-arthritic TS1xHACII mice and found that arthritis did not occur in the recipient mice (44). While the total number of lymphocytes and $CD4^+$ T cells within the draining lymph node remained similar between polyclonal Treg cell recipients and unmanipulated TS1xHACII mice, the percentage of CD4+ cells that could produce IL-17 decreased in Treg cell recipients. Treatment of TS1xHACII mice with anti-IL-17 antibody was also able to prevent arthritis development, showing that IL-17 is necessary for disease. Interestingly, TS1xHACII mice that received Treg cells that were purified from TS1xHA28 mice and enriched for S1-specificity developed arthritis to a similar extent as unmanipulated animals, suggesting that some diversity of TCR specificities on Treg cells is required to suppress disease (44). Importantly, the Treg cells from TS1xHA28 mice were able to suppress the proliferation and interferon-γ (IFN-γ) production of 6.5^+ conventional T cells in HACII. TCR$\alpha^{-/-}$ animals, showing that the TCR signal received from HA expression on $MHCII^+$ cells is sufficient to activate *in vivo* suppressive function in the 6.5^+ Treg cells from TS1xHA28 mice. Furthermore, Treg cells from TS1xHA28 mice were able to limit IL-17 production by endogenous 6.5^+CD4^+ T cells within the draining lymph node of TS1xHACII mice, but not cytokine production by 6.5^-CD4^+ T cells. We also failed to obtain evidence that the 6.5^+ Treg cells had themselves acquired effector cell phenotypes (such as IL-17 secretion). Thus, the 6.5^+ Treg cells from TS1xHA28 mice can and do mediate suppression of immune responses in the TS1xHACII environment, but they failed to prevent the development of inflammatory arthritis.

One explanation for these findings is that 6.5^+CD4^+ T cells initiate autoimmunity, but that $CD4^+$ T cells that utilize endogenously rearranged TCRs are involved in the inflammatory arthritis phase of the disease. This hypothesis is supported at least in part by the observation that TS1xHACII mice on a $Rag^{-/-}$ background develop less severe pathology than do TS1xHACII mice that are Rag-sufficient and thus can generate TCRs with alternative specificities based on endogenous TCR gene rearrangement. In support of a two-phase model of disease development, we recently showed that the autoimmune process in TS1xHACII mice is characterized by the early production of high levels of IFN-γ by the autoreactive 6.5^+CD4^+ T cells, and that this in turn leads to the mobilization of TH17-trophic inflammatory monocytes that promote joint swelling (46). It is possible that this latter phase depends on the recruitment of Th17 cells utilizing diverse TCR specificities and is sensitive to the activity of polyclonal Treg cells. However, the mechanism of this suppression is unclear, and why Treg cells that can recognize the S1 peptide (that is expressed by the MHC class II^+ inflammatory monocytes) fail to mediate this suppression is also not yet understood. The fact that polyclonal Treg cells can suppress arthritis development could imply that they express some of the same TCR specificities as are expressed by the effector T cells, and that Treg cell expression of these shared specificities is required to mediate antigen-specific suppression, but this remains a matter of speculation. It is also unclear why the Treg cells that are quite abundant in TS1xHACII mice fail to prevent the disease; there are studies in human RA patients that suggest that factors such as TNF can prevent Treg cell function (47, 48), although this explanation would require that the Treg cells from BALB/c mice are insensitive to such effects. Additional studies in this

system will aim to determine how TCR specificity for self-antigens can contribute to the ability of Treg cells to prevent the development of autoimmune arthritis.

Thymically generated Treg cells respond to viral antigen during infection

In a landmark study establishing the involvement of Treg cells in an infection, $CD4^+CD25^+$ cells were shown to constitute nearly 50% of $CD4^+$ T cells within the skin at sites of chronic *Leishmania major* infection in mice (49). At the time of this study, Foxp3 had not yet been identified as a marker for Treg cells, but the $CD4^+CD25^+$ cells from the sites of infection displayed many of other the hallmarks of a Treg cell, including high expression of CTLA4 and low expression of CD45RB, a hypoproliferative phenotype *in vitro*, and strong suppression of $CD4^+CD25^-$ cell proliferation following anti-CD3 stimulation. Subsequent studies showed that these Treg cells were responsive to microbial antigens (50), and additional studies by multiple groups have shown that Treg cells can be active participants in the immune responses to a variety of pathogens (7, 8, 51, 52). However, in many cases the extent to which these Treg cells are derived from thymically generated Treg cells (which as outlined above are generated based on specificity for self-peptides) as opposed to being generated from conventional $CD4^+$ T cells in response to signals such as TGF-β and retinoic acid (53,54) [termed peripherally derived $Foxp3^+$ Treg (pTreg) cells] has not been established. Indeed, because $CD4^+$ T cells with identical TCR specificity can be induced to become either pTreg cells or differentiated cytokine-secreting effector cells (e.g. Th1 cells) in response to different cytokines (e.g. TGF-β *vs.* IL-12) *in vitro*, it has been thought that the formation of pTreg cells from conventional $CD4^+$ T cells may be a typical source of $Foxp3^+$ T cells during immune responses (55). However, the extent to which this process actually occurs during infections remains poorly understood.

We have been using the TS1xHA system to examine how TCR specificity for self or viral peptides can contribute to the accumulation and activity of Treg cells during an infection by exploiting the fact that the antigen that we have used as a neo-self antigen in the HA transgenic mice is also expressed as a viral antigen in the PR8 strain of influenza virus. As a preliminary to these experiments we analyzed the lungs and the lung-draining mediastinal lymph nodes (medLN) of PR8-infected BALB/c mice and found significant increases in the frequencies of $CD4^+CD25^+Foxp3^+$ cells in both locations during the acute phase of influenza virus infection (days 5 to 8 post-infection) (6). To assess how peripherally derived and/or thymically generated Treg cells could contribute to the accumulation of Treg cells during influenza virus infection, we performed adoptive transfer studies to determine the extent of Treg cell formation and/or expansion using donor T cells derived from two different sources. In one set of experiments, TS1 mice were mated with mice expressing a $Foxp3^{eGFP}$ reporter allele to produce TS1. $Foxp3^{eGFP}$ mice, and $CD4^+CD25^-Foxp3^-$ T cells were isolated by cell sorting. When these cells were transferred into BALB/c mice, PR8 infection led to significant expansion and accumulation of 6.5^+CD4^+ T cells in the lungs and medLNs, but little or no conversion of these cells to become $Foxp3^+$ was found to occur. Conversely, when similar experiments were carried out using PR8-infected BALB/c mice that had been seeded with $CD4^+Foxp3^+$ T cells obtained from TS1xHA28. $Foxp3^{eGFP}$ mice, these thymically generated Treg cells were found to divide and accumulate in the lungs and medLNs, and little or no loss of Foxp3 expression occurred (6). Thus, when $CD4^+$ T cells

with identical specificity for a viral antigen were analyzed in PR8-infected mice, the only Treg cells that accumulated in sites of infection were thymically derived Treg cells that had been generated based on their specificity for a self-peptide, and *de novo* formation of pTreg cells did not occur. In several other infectious settings, including *L. major* and *Mycobacterium tuberculosis* (Mtb) infection, no conversion of conventional $CD4^+$ T cells into $Foxp3^+$ Treg cells has been observed, but rather the Treg cells found at the site of infection expanded from pre-existing thymically generated Treg cells (50,56). Using a tetramer to identify Mtb-specific cells, Shafiani *et al.* (56) were able to show that $tetramer^+$ $CD4^+Foxp3^+$ cells expanded from circulating Treg cells present in a naïve mouse, confirming that pathogen-specific Treg cells exist in the steady state and expand after encountering cognate antigen (57). In addition to there being very little evidence to support the theory that Treg cells involved in an infection may convert from circulating conventional T cells, some studies have shown that an ongoing inflammatory response may actually be detrimental to peripheral Treg cell induction. T cells whose cognate antigen was fed to mice were significantly less likely to initiate Foxp3 expression when a lethal *T. gondii* infection was present in the gut, and the presence of the inflammatory cytokine IFN-γ was found to inhibit peripheral Treg cell development (58, 59). Thus, it appears that thymically derived Treg cells may in many cases be the dominant source of Treg cells that accumulate during an infection.

Functional specialization of Treg cells following recognition of viral antigen

For many years, a puzzling aspect of Treg cell biology was an apparent inconsistency in the phenotypes of Treg cells that were examined in different settings. For example, early studies describing $CD4^+CD25^+$ Treg cells obtained from naive mice showed that Treg cells mediated suppression *in vitro* by contact dependent mechanisms that were unlikely to involve soluble factors such as cytokines, and that Treg cells themselves were not strong producers of anti-inflammatory cytokines such as interleukin-10 (IL-10). However, studies showing the ability of Treg cells to modulate immune response in *in vivo* settings such as colitis and in the *Leishmania* model described above found that Treg cells obtained from IL-10-deficient mice were ineffective (49, 60). This dichotomy is likely to at least partly reflect a diversity of mechanisms by which Treg cells can exert regulatory functions, but additional insights into this area have been obtained from a variety of studies showing that Treg cells can themselves differentiate and acquire novel characteristics (including cytokine production) in response to stimuli that can be encountered in *in vivo* settings, in a process termed functional specialization (61). Notably, these studies have also suggested that Treg cells might respond to signals and utilize pathways involved in effector $CD4^+$ T-cell differentiation to acquire properties that allow them to selectively modulate these same pathways. For example, when Foxp3-driven cre expression is used to selectively ablate transcription factors such as IRF-4 or STAT3 from Treg cells, the depleted mice spontaneously develop pathologies with characteristics that are typical of the phenotypes that these molecules promote in effector $CD4^+$ T cells (e.g. Th2 responses for IRF4, Th17 responses for STAT3) (62, 63). Similarly, a subset a Treg cells (termed Th-1 or $T\text{-}bet^+$ Treg cells) uses the transcription factor T-bet to acquire expression of CXCR3 and IL-10 and specializes in trafficking to sites of Th1 cell-mediated immunopathology and limiting IFN-γ

production (64). The transcription factor T-bet is necessary for CXCR3 expression, and IFN-γ is thought to be at least partially responsible for induction of T-bet expression, as IFN-γR$^{-/-}$ Treg cells express much lower levels of both T-bet and CXCR3 (64). The diversity of effector phenotypes into which Treg cells can differentiate was demonstrated by another study showing that IRF4 and Blimp1 can act in concert to promote the development of a population of Treg cells that, like T-bet^{+} Treg cells, produce IL-10 and are found selectively in the lungs, but in this case, T-bet is not required for their formation (65).

In light of these studies demonstrating the differentiation of Treg cells in inflammatory environments, we analyzed the phenotype of the 6.5^{+}CD4^{+}Foxp3^{+} T cells that accumulated in the lungs and medLNs of PR8-infected BALB/c mice that had been seeded with Treg cells from TS1xHA28 mice. Indeed, the Treg cells isolated from the lungs 8 days post-infection closely resembled the T-bet^{+} Treg cells initially characterized by the Campbell group (64); they expressed elevated levels of T-bet, CXCR3, IL-10, and Prdm1 (Blimp1) compared to the 6.5^{+}CD4^{+}Foxp3^{+} T cells from donor TS1xHA28 mice (6). Interestingly, the 6.5^{+} Treg cells in the medLNs also had higher levels of these molecules relative to the input Treg cells, but their levels were significantly lower than was observed in the lungs. Thus, in the steady state of an infected mouse, the thymically derived Treg cells that accumulated at the site of infection versus the draining lymph node appear to have each undergone differentiation, but to have adopted distinct phenotypes. One explanation for this phenomenon could be that these phenotypes arise progressively, and the virus-reactive Treg cells initially become activated in the medLN where they upregulate T-bet, Blimp-1, and CXCR3 and produce low levels of IL-10. They may then migrate to the lungs, where they undergo further differentiation. It is also possible that the extent of differentiation might be linked to the Treg cells in the lungs having undergone more extensive division, consistent with studies showing that regional proliferation in the lungs makes a substantial contribution to the number of CD8^{+} T cells that are generated during influenza virus infection (66). An alternative and not mutually exclusive model is that cytokines and/or specialized cell types that are present at different amounts in the lungs versus the medLNs support differentiation of T-bet^{+} Foxp3^{+} Treg cells that express high versus low amounts of IL10. For example, neutrophil-derived IL-27, acting in concert with IL-2, can induce IL-10 production in CD8^{+} T cells in the lungs of influenza virus-infected mice by a Blimp 1-dependent mechanism (67). Ongoing studies are evaluating how these mechanisms could lead to the accumulation of virus-reactive T-bet^{+} Treg cells with significantly different phenotypes in the lungs and the medLNs during influenza virus infection.

Virus-reactive Treg cells suppress immune responses at the site of influenza virus infection

In addition to analyzing the ability of Treg cells to differentiate following activation, the studies in influenza virus-infected mice that had been seeded with Foxp3^{+} Treg cells from TS1xHA28 mice also allowed us to examine how Treg cells can modify an immune response *in vivo*. Despite the many studies documenting the requirement for Foxp3^{+} Treg cells in maintaining normal immune system homeostasis and in modulating immune responses in settings such as infections, the mechanisms by which these cells exert their

regulatory effects remain quite poorly understood. One approach to this question has been to examine immune responses in settings where Treg cells have been depleted either by treatment of mice with anti-CD25 monoclonal antibodies or in mice that express diphtheria toxin receptor on $Foxp3^+$ Treg cells and to which diphtheria toxin has been administered to selectively eliminate Treg cells. When this latter approach was used to analyze immune responses in mice following acute herpes virus infection, the depletion of Treg cells was found to modify the rate of egress of effector T cells from the LNs draining the site of infection, while many other aspects of the antiviral immune response appeared to be relatively unaffected by this depletion (8).

One caveat to the use of depletion strategies for understanding how Treg cells modulate infections is that effective Treg cell depletion also causes severe disruption of immune system homeostasis, such that uninfected mice can begin to die from the effects of Treg cell depletion as early as two weeks after beginning the depletion treatment (68), and it is possible that effects that are observed in infected Treg cell-depleted mice are secondary to this disruption of immune homeostasis, rather than a direct effect of Treg cells on anti-viral effector cells that would occur in Treg cell-replete mice. In our studies using influenza virus-infected mice that had been seeded with Treg cells from TS1xHA28 mice, we could examine the consequences of enhancing the representation of Treg cells that could react with a viral antigen; the caveat to this approach is that the frequencies of these cells are likely to be substantially higher than typically arise in a natural infection, but the advantage is that the immune cell homeostasis that is normally maintained by Treg cells has not been disrupted. The introduction of Treg cells from TS1xHA28 mice led to significant decreases in the numbers of IFN-γ-secreting effector $CD4^+$ and $CD8^+$ T cells in both the lungs and the medLN of infected mice, although notably, the basis for these differences was distinct in the two sites. Thus, the total number of $CD4^+$ and $CD8^+$ T cells that accumulated, as well as the percentages of these cells that expressed IFN-γ were lower in the lungs of infected mice that had received Treg cells. By contrast, in the medLN, adoptive transfer of 6.5^+ Treg cells resulted in decreased percentages and numbers of IFN-γ-secreting effector $CD4^+$ and $CD8^+$ T cells, but did not significantly alter the total number of $CD4^+$ and $CD8^+$ T cells that accumulated (6). The frequencies and numbers of viral antigen-specific $CD8^+$ T cells detected with a $H2\text{-}K^d$:$NP147^+$ tetramer were similarly decreased in the lungs but not in the medLN. The decreased accumulation of effector $CD4^+$ and $CD8^+$ T cells had an apparent effect on virus replication, since virus titers in the lungs were significantly increased at 5 days post infection in the presence of virus-reactive Treg cells.

To examine how the presence of T-bet^+ Treg cells led to reduced effector T-cell accumulation in the lungs of infected mice, we assessed expression of the proliferation marker Ki-67 that identifies cells that have entered active phases of the cell cycle. In the lungs of PR8-infected mice that did not receive additional Treg cells, approximately 40% to 80% of the $CD8^+$ and $CD4^+Foxp3^-$ effector cells were Ki-67^+, consistent with previous studies showing that effector cells undergo active proliferation in the lungs of influenza virus-infected mice (66, 69). Notably, adoptive transfer of Treg cells from TS1xHA28 mice significantly reduced the percentages of $CD8^+$ and $CD4^+Foxp3^-$ cells in the lungs that were Ki-67^+. It was also interesting that the majority of $Foxp3^+$ Treg cells (both the $6.5^+CD4^+Foxp3^+$ cells derived from TS1xHA28 mice and the endogenous $6.5^-CD4^+Foxp3^+$

cells) in the lungs were actively proliferating, and that the presence of Treg cells from TS1xHA28 mice did not result in decreased Ki-67 expression by the endogenous Treg cells. Compared with the abundant Ki-67 expression that was evident in the lungs of infected mice, smaller percentages (~10%) of the $CD8^+$ and $CD4^+$ T cells in the medLN of infected mice were Ki-67^+. Moreover, there was no difference in the percentage of Ki-67^+ cells in the medLN of mice that had or had not received virus-reactive Treg cells. The presence of 6.5^+ Treg cells also did not affect expression of CXCR3 by $CD4^+$ and $CD8^+$ T cells in the medLN; since CXCR3 plays a major role in directing the migration of T cells from the medLN to the lungs during respiratory infection (70), this observation argues against the possibility that Treg cells affect the accumulation of effector cells in the lungs by blocking their migration from the medLN. As previously mentioned, 6.5^+ Treg cells that were present in the lungs, but not those present in the medLN, were capable of producing large amounts of IL-10, and it is conceivable that the resulting high local concentration of IL-10 in the lungs was responsible for suppression of effector T-cell proliferation and accumulation, either by acting directly on effector cells or by modulating APC activity. In support of this possibility, IL-10 is known to be important in limiting excessive inflammatory responses in the lungs during an anti-influenza virus response, since treatment with IL-10R blocking antibody resulted in lethal pulmonary inflammation in infected mice (69). Collectively, then, these studies of the effects of Treg cells in influenza virus-infected mice contrasted and extended previous studies indicating that Treg cells modify the immune response to pathogens by controlling the homing of effector T cells from the draining LN to the infected site (7,8,56). Instead, the studies in this system provide evidence that Treg cells can exert regulatory effects primarily through their ability to suppress the differentiation and proliferation of $CD4^+$ and $CD8^+$ effector T cells in the infected tissues.

Conclusions and future perspectives

In mice with unmanipulated immune repertories, the vast diversity of TCR specificities expressed by Treg cells makes it difficult or impossible to know to what exactly each Treg cell is responding. The transgenic approaches we have adopted have allowed us to study Treg cells of a known specificity and to manipulate the expression of a target peptide presented both as a self-peptide and as a viral antigen. Although these approaches have caveats related to the relatively high frequencies of Treg cells with the same specificity, they have emphasized the role of TCR signaling as a crucial mediator of Treg development, persistence and activity, and there remain additional questions that this system should allow us to address. Firstly, the factors that govern the decision of autoreactive thymocytes to undergo deletion versus development along the Treg cell pathway remain to be understood, and the approaches here have begun to determine how these different fates are specified. In particular, a greater understanding of the roles that different signaling pathways can play and of the epigenetic changes that TCR signaling can induce should be amenable to study in this system. Secondly, how TCR specificity guides the activity of Treg cells in *in vivo* settings is not well defined, and in the case of the TS1xHACII model of inflammatory arthritis, we found that Treg cells that can recognize, with high affinity, the same target autoantigen that we know is recognized by $CD4^+$ T cells to initiate an autoimmune response were ineffective at preventing disease, while adoptive transfer of polyclonal Treg cells could inhibit disease

development. Moreover, disease developed in these mice despite the presence of elevated levels of endogenous Foxp3$^+$ Treg cells (as has been observed in human arthritis patients), and why these endogenous Treg cells do not prevent disease is not clear. Lastly, we have found that Treg cells that had been selected based on specificity for a self-peptide could proliferate and differentiate in response to that peptide when it was encountered as a viral antigen. However, since Treg cells appear to be generated in response to self-peptides, can they also regulate an immune response to a viral infection through recognition of self-peptides at the site of inflammation? Can viral antigens with which they are only weakly crossreactive activate Treg cells and allow them to modulate antiviral immunity? And once they have differentiated, do they become long lived and affect immune responses to subsequent infection? If manipulation of the Treg cell repertoire is to be used to treat diseases in human patients, it will be important to understand how TCR signaling can direct their activity in these different settings.

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