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The Role of TCR Specificity in Naturally Arising CD25$^+$ CD4$^+$ Regulatory T Cell Biology

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Abstract CD25$^+$ CD4$^+$ T cells (T_R) are a naturally arising subset of regulatory T cells important for the preservation of self-tolerance and the prevention of autoimmunity. Although there is substantial data that TCR specificity is important for T_R development and function, relatively little is known about the antigen specificity of naturally arising T_R. Here, we will review the available evidence regarding naturally arising T_R TCR specificity in the context of T_R development, function, and homeostasis.

1 Introduction

A fundamental finding regarding the significance of T cell receptor specificity for the development of CD25$^+$ CD4$^+$ regulatory T cells (T_R) is that T_R are

not observed in TCR transgenic mice lacking RAG genes (Hori et al. 2002; Itoh et al. 1999, Olivares-Villagomes et al. 1998). The presence of functional RAG genes does permit the development of $CD25^+$ T_R in TCR transgenic mice, presumably via expression of endogenously rearranged TCR chains. The likely explanation for the lack of $CD25^+$ T cell development in these monoclonal TCR transgenic mice is that the transgenic $CD4^+$ TCRs reported so far most likely originated from $CD25^-$ T cells. This is inferred from the well-known inability of T_R to proliferate or produce IL-2 in response to TCR engagement in vitro (Takahashi et al. 1998; Thornton and Shevach 1998), which would favor the use of TCRs in these transgenic mice from $CD25^-$, and not $CD25^+$, T cells expanded after in vivo immunization and in vitro re-stimulation. Thus, these data demonstrate that a particular TCR specificity is required to facilitate T_R development.

In addition to affecting T_R development, TCR specificity likely controls T_R function. In vitro studies using both polyclonal and TCR transgenic T_R clearly show that activation through the TCR is required for suppression of $CD25^-$ $CD4^+$ T cell proliferation via a contact-dependent mechanism (Takahashi et al. 1998; Thornton and Shevach 1998). Similar in vivo studies have been performed using T_R isolated from TCR transgenic mice (Apostolou et al. 2002; Walker et al. 2003a). In these experimental models, TCR transgenic T_R encounter with its cognate peptide ligand can be conveniently controlled, with the caveat that the transgenic TCR interaction with its cognate peptide may be of higher affinity than those interactions involving naturally arising T_R TCRs. Taken together, these data suggest that T_R may have antigen specificity different from conventional $CD25^-$ T cells, and that this TCR specificity is required for their development and function. In this review, we will discuss the currently available evidence for the antigen specificity of T_R and hypothesize how this specificity may direct T_R development and dictate the activation of T_R to suppress the immune response.

2
The Antigen Specificity of Naturally Arising T_R

The prevailing hypothesis regarding the TCR specificity of naturally arising regulatory T cells is that they recognize self-antigen, and that this interaction is important for T_R development and function to suppress autoimmunity. This model was originally prompted by two studies in the 1990s that indirectly suggested that naturally arising T_R recognize tissue-specific self-antigens. Initial studies by Taguchi and colleagues suggested that the functional maintenance of $CD4^+$ T cells capable of protection against prostatitis or oophoritis required

the presence of the corresponding organ, as adoptive transfer of T cells from male mice were more effective at preventing neonatal thymectomy-induced autoimmune prostatitis than oophoritis, and vice versa for T cells from female mice (Taguchi et al. 1994). Studies from Mason's group extended this observation by demonstrating that ablation of the thyroid gland resulted in the selective functional loss of T cells within the $CD4^+$ population capable of preventing radiation-induced autoimmune thyroiditis, but not diabetes (Seddon and Mason 1999). Curiously, thyroid ablation did not result in the loss of protective thymic $CD4^+$ T cells. Although the $CD4^+$ T cell population was not fractionated in these studies to ensure that the suppressing cells were indeed $CD25^+$ T_R, these data support the hypothesis that tissue-specific antigen recognition by T_R is necessary for their survival, development, and/or expansion in the periphery, as the tissue-protective $CD4^+$ T cell population is functionally lost in the absence of the target organs studied.

Studies of TCR transgenic models offer additional support for the hypothesis that T_R may recognize self-antigen. These models rely on the expression of the cognate ligand for the transgenic TCR as a neo-self-antigen driven by another transgene (Jooss et al. 2001; Jordan et al. 2001; Walker et al. 2003a). In one well-characterized model, a high level of peptide expression resulted in the deletion of TCR transgenic T cells, whereas a moderate level resulted in partial deletion, with the development of $CD25^+$ cells resembling T_R in approximately 50% of the remaining cells (Jordan et al. 2001). Thus, these data serve as a direct demonstration that regulatory T cells could develop due to interactions with self-peptide:MHC complexes.

Where does this TCR interaction with antigen occur? Early reports suggested that $CD25^+$ T cells originate in the thymus, as animals thymectomized at day 3 of life develop spontaneous autoimmunity which could be rescued by the adoptive transfer of normal $CD25^+$ regulatory T cells (Sakaguchi et al. 1995). Thus, the development of autoreactive cells relative to T_R is favored under the conditions of early thymectomy, and these observations suggested that the cause of autoimmunity in day 3-thymectomized mice was the insufficient export of T_R from the thymus during the first few days ex utero. Further work directly demonstrated that regulatory T cells are indeed generated in the thymus, and that these thymic $CD25^+$ $CD4^+$ mature cells are capable of suppressor function as revealed by adoptive transfer experiments (Itoh et al. 1999; Seddon and Mason 2000). Consistent with these earlier reports, it was found in one of the TCR transgenic models described above that the expression of cognate peptide by radiation-resistant thymic stromal cells alone was sufficient for the generation of $CD25^+$ TCR transgenic T cells in bone marrow chimera studies (Jordan et al. 2001). Development of $CD25^+$ T cells with suppressor capabilities has also been observed in mice that express class II

only on thymic epithelium and not bone marrow-derived cells (Bensinger et al. 2001). Thus, these data form the current paradigm for T_R development, which maintains that T_R develop due to an interaction with self-antigen in the thymus at an avidity range between positive and negative selection (reviewed in Maloy and Powrie 2001).

However, there are some data that suggest that a simple avidity threshold model does not satisfactorily explain T_R development. For example, it has been argued that regulatory T cell development depends on a high affinity interaction between TCR and peptide:MHC class II complexes. This was suggested based on the use of two transgenic TCRs with a 100-fold difference in the sensitivity of the response to the cognate hemagglutinin (HA) peptide as assessed by an in vitro proliferation assay (Jordan et al. 2001). As described above, enhanced $CD25^+$ T_R development was observed in transgenic mice expressing a higher affinity anti-HA TCR. In contrast, increased development of $CD25^+$ T cells was not observed in mice co-expressing a lower-affinity HA-specific transgenic TCR with several transgenic constructs driving varying levels of HA peptide expression, even though mild to marked deletion of T cells expressing this lower-affinity TCR was observed in the double transgenic mice. Although it remains possible that the transgenic mice utilized in this study were unable to express the HA-peptide at levels optimal for development of T_R expressing this lower-affinity TCR, these data do suggest that TCR engagement by a higher-affinity ligand may result in a qualitatively different signal required for regulatory T cell development. Thus, these results question a simple avidity model for T_R development.

Very recently, an alternative view of the role of TCR-ligand interactions in T_R development has been offered by the Mathis and Benoist group (van Santen et al. 2004). Using mice co-expressing a transgenic TCR (tgTCR) and its cognate peptide ligand encoded by a tet-inducible transgene, these investigators observed increasing percentages of $CD25^+$ $tgTCR^+$ T cells in the thymus corresponding to the level of the TCR ligand induced upon doxycycline treatment. However, there was a relatively small increase in the absolute numbers of thymic $CD25^+$ T cells, despite their significantly elevated frequency. This model implies that thymic T_R precursors are relatively insensitive to deletion (Fig. 1), which may be due to previously reported up-regulation of pro-survival factors, e.g., OX40, GITR, TNF-RII (Gavin et al. 2002; McHugh et al. 2002), and that the development of regulatory T cells is not instructed by TCR signals, but is determined either stochastically or influenced by non-TCR signals. Nevertheless, the existing data supporting this alternative stochastic-selective model of T_R development, in our opinion, do not dispute an essential role for TCR signaling in T_R development and cannot definitively rule out the original instructive model.

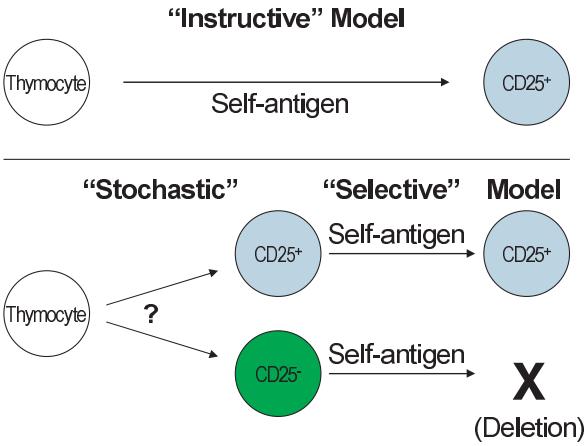

Fig. 1 Models for the role of TCR signals in regulatory T cell development in the thymus. The "instructive" model (*top*) suggests that regulatory T cell development results from specific TCR signals due to encounter with self ligands, whereas the "stochastic selective" model argues that regulatory T cell precursors develop due to stochastic expression of non-TCR signals, or factors such as Foxp3, affecting regulatory T cell commitment. Engagement of TCR by high-affinity ligands would result in a selective increase in the frequency of regulatory versus non-regulatory T cells of the same specificity due to preferential deletion of non-regulatory T cells upon encounter with self-ligands. Regulatory T cells would be relatively resistant to deletion in this model. However, a hybrid model based on "instructive" TCR signals for recruitment into the T_R phenotype coupled with preferential "selection" or survival of $CD25^+$ T cells may represent the most likely mechanism of thymic T_R development

3 Antigenic Specificity of Induced Regulatory T Cells

Our discussion so far has focused on studies addressing the role of TCR specificity in regulatory T cells that arise naturally in the absence of immune challenge. Other studies have examined the antigen specificity of regulatory T cells elicited under inflammatory conditions. Such T cells have been described as "adaptive" regulatory T cells (reviewed in Bluestone and Abbas 2003). These studies have added further support for an important functional role of the recognition of self-antigens by T_R. For example, in a transgenic model of diabetes elicited upon the induction of the pro-inflammatory cytokine TNFα and co-stimulatory molecule CD80 in pancreatic islet β cells, it was shown that as few as 2000 $CD25^+$ T cells isolated from the draining pancreatic lymph nodes were capable of delaying onset of diabetes upon adoptive

transfer into a prediabetic host (Green et al. 2002). Thus, these suppressive $CD25^+$ T cells appear to be elicited by the pro-inflammatory environment in the pancreatic islets, although it is unknown whether these cells represent expanded naturally arising T_R or $CD25^-$ T cells converted into T_R. The putative specificity of these T cells for islet cell antigen(s) is underscored by the fact that adoptive transfer of tenfold higher numbers of $CD25^+$ T cells isolated from non-pancreatic lymph nodes was unable to protect recipient mice from diabetes. It must be noted, however, that in addition to TCR specificity, potential differences between the activated "adaptive" regulatory T cells in the draining lymph nodes at the site of inflammation and the naturally arising $CD25^+$ T_R found elsewhere, such as increased suppressor activity or different cytokine and chemokine receptor profiles, could also account for these observations.

Other evidence for the existence of adaptive regulatory T cells specific for self-antigens and their significant biological role comes from studies of tumor immunity. Initial observations suggested that the presence of $CD25^+$ regulatory T cells can diminish anti-tumor responses, but it was not clear whether this effect was antigen-specific (Shimizu et al. 1999). Several recent studies have suggested that adaptive $CD25^+$ T cells may suppress tumor immunity by recognizing self-antigens. For example, $CD25^+$ T cells with suppressor ability can be elicited by gene gun immunization with autoantigens identified in the SEREX screen (Nishikawa et al. 2003). In another example, human T cell clones with a phenotype resembling regulatory T cells were isolated from tumor-infiltrating lymphocytes of melanoma patients (Wang et al. 2004). Some of these clones were identified to be reactive to the self-protein LAGE1. However, it also remains unclear in these experiments whether these cells arose from naturally arising T_R or were elicited from the $CD25^-$ T cell population. Although the lineage relationship between naturally arising and adaptive regulatory T cells has not been definitively addressed, these reports on the self-reactivity of adaptive regulatory T cells are consistent with the self-reactivity of naturally arising T_R described above.

4 The Paradox of Foreign Antigen Recognition by Regulatory T Cells

The above description of self-reactivity within the naturally arising regulatory T cell population fits with the original identification of regulatory T cells as a critical mechanism for the prevention of autoimmunity. However, it has become increasingly evident that T_R play an important role in the regulation of virtually all immune responses. While initial studies focused on defining the progression of a variety of autoimmune responses in the absence or presence

of regulatory T cells, more recent studies have examined the role of T_R in the regulation of immune responses to foreign antigens.

For example, it has been reported that infection of mice with *Helicobacter hepaticus* results in the generation of both $CD25^+$ and $CD25^-$ cells capable of producing IL-10 in response to bacterial antigens and suppressing *Helicobacter*-induced inflammatory colitis (Kullberg et al. 2002). As in aforementioned studies, it is not clear whether these IL-10-producing T cells originate from naturally arising $CD25^+$ T_R or differentiate from the $CD25^-$ T cell population. As *Helicobacter* is considered to be a commensal microorganism in immunocompetent hosts, it is intriguing to hypothesize that gut flora may significantly influence the TCR repertoire of "naturally arising" $CD25^+$ T cell populations (as well as $CD25^-$ populations) by facilitating selective expansion of T_R and $CD25^-$ T cell clones bearing TCR reactive to bacterial antigens. Along the same line, studies of oral tolerance to foreign antigens have demonstrated the ability to generate $CD25^+$ T cells with regulatory properties from $CD25^-$ T cells (Thorstenson and Khoruts 2001).

Thus, a portion of the normal naturally arising regulatory $CD25^+$ T cell population in the periphery may actually contain adaptive $CD25^+$ T cells produced upon interactions with foreign antigens. In support of this hypothesis, several groups have reported in vitro generation of $CD25^+$ T cells with regulatory properties from peripheral $CD25^-$ T cells in both human and murine models (Chen et al. 2003; Nagler-Anderson et al. 2004; Walker et al. 2003b). In the latter, TGF-β was shown to play an essential role in the "peripheral conversion" of murine $CD25^-$ T cells into $CD25^+$ T cells with regulatory properties (Chen et al. 2003). Furthermore, von Boehmer's group has also reported on the generation of $CD25^+$ T_R from peripheral $CD25^-$ TCR transgenic x RAG-deficient T cells upon chronic provision of constant levels of the cognate peptide antigen using an osmotic peptide pump as a delivery device (Apostolou and von Boehmer 2004). Thus, these studies serve as a proof of principle that under certain circumstances, such as high levels of TGF-β, $CD25^+$ regulatory T cells can arise from peripheral $CD25^-$ T cells upon encounter with their cognate antigen.

To complicate matters further, recognition of foreign antigen by the naturally arising $CD25^+$ T cell population has also been demonstrated using immunization with hapten 2,4-dinitrofluorobenzene, or infection with *Candida albicans* or *Leishmania major* (Belkaid et al. 2002; Dubois et al. 2003; Montagnoli et al. 2002). For example, adoptive transfer studies revealed that the *Leishmania*-reactive $CD25^+$ T cells accumulating at the sites of infection were derived primarily from the naturally arising $CD25^+$, and not $CD25^-$, donor T cells. In fact, it was observed that persistent immunologic memory to *Leishmania* as well as *Candida* requires the presence of these adaptive T_R

originating from the naturally arising T_R population, arguing for an important biological role for regulatory T cells in the down-modulation of immune responses to pathogens.

These reports describing reactivity to foreign antigens within the naturally arising T_R population appear to be at odds with the prevalent paradigm of T_R development commencing upon recognition of high-affinity self-antigens in the thymus. One scenario that might account for both self- and foreign-antigen reactivity of T_R would be that the TCR specificity requirements for T_R

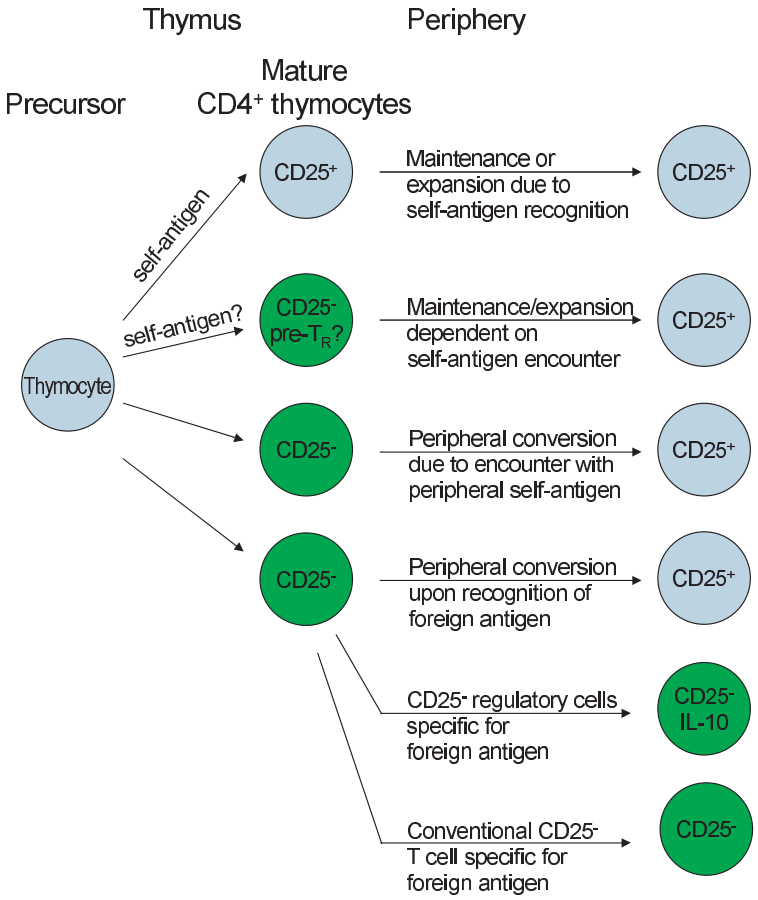

Fig. 2 Development of $CD25^+$ regulatory T cells. Potential TCR-ligand interactions that may result in generation of $CD25^+$ T cells with regulatory properties in the thymus (*left*) and in the periphery (*right*)

development is analogous to conventional $CD25^-$ T cell development, except that T_R are simply positively selected based on higher-avidity interactions with self-peptide:MHC class II complexes. A logical extension of this hypothesis is that the T_R TCR repertoire may be functionally as diverse as the $CD25^-$ TCR repertoire, allowing for recognition of a wide variety of foreign antigens. The available evidence regarding the diversity of the T_R TCR repertoire does not exclude such a possibility, as it has been shown that Vα and Vβ usage is similar between $CD25^+$ and $CD25^-$ $CD4^+$ T cells (Takahashi et al. 1998). The actual diversity of the T_R TCR repertoire, however, has not been extensively studied until now (see below).

The possibility of a foreign antigen inducing peripheral conversion of $CD25^-$ T cells into $CD25^+$ regulatory T cells as well as stimulating the expansion of naturally arising $CD25^+$ T cells may significantly complicate our view of the development and function of naturally arising T_R (Fig. 2). Thus, it is plausible that there may be several subsets within the peripheral T_R population in regards to specificity of their TCR and their origin. Some peripheral T_R may develop in the thymus as a result of increased avidity recognition of self-antigen, whereas others may have been elicited from $CD25^-$ T cells under special conditions, e.g., upon chronic exposure to a foreign or self-antigen in the presence of TGF-β. Finally, thymically derived T_R expanded upon encounter with a high-affinity foreign or self-antigen might also be found within the naturally arising T_R population. The relative size of each of these putative subsets and their functional potential are, however, unknown.

5
T_R Appear to Have a Diverse TCR Repertoire That Is Different from the $CD25^-$ TCR Repertoire

To reconcile the findings suggesting that naturally arising regulatory T cells display TCRs having an increased affinity for self-ligands with the observations suggesting that T_R TCRs may also recognize foreign antigens, our group has recently attempted to compare the TCR repertoires displayed by T_R and $CD25^-$ $CD4^+$ T cells and to test whether the naturally arising T_R population recognizes self-peptide:MHC class II complexes with greater avidity than that of the $CD25^-$ T cell population (Hsieh et al. 2004). To directly address these issues, we have analyzed the naturally arising $CD25^+$ and $CD25^-$ TCR repertoires represented by variable TRAV14 (Vα2) TCRα chains paired with a fixed TCRβ chain in TCRβ transgenic mice. Importantly, T cells were selected in these mice by a highly diverse wild-type array of peptide:MHC class II complexes. Based on the observations regarding $CD25^+$ T_R development

in TCR transgenic mice with or without RAG expression discussed above, we expected that individual randomly generated TCRα chains will facilitate thymocyte differentiation into either the $CD25^+$ or $CD25^-$ subset.

Direct sequence analyses of the TCR repertoire represented by Vα2 TCRα chains paired with a fixed TCRβ chain suggested that the T_R TCR repertoire is diverse, similar to to the $CD25^-$ T cell subset (Hsieh et al. 2004). In agreement with these results, a remarkable diversity in the T_R TCR repertoire has also been observed using CDR3 spectra-typing analysis of human $CD25^+$ T cells from peripheral blood (Kasow et al. 2004).

This diversity may explain the apparent ability of the naturally arising regulatory T cell population to participate in regulation of immune responses to pathogens such as *Leishmania*. Although T_R were shown to inhibit a sterilizing immune response in the *Leishmania* infection model, thereby allowing for the maintenance of functional "memory" T cells, these and other analogous results provide insufficient support for the idea that the naturally arising T_R population evolved to control infectious immunity. From a general perspective, the potential benefits of preserving a chronic low level infection to maintain functional memory T cells over a sterilizing immune response to pathogens are not immediately obvious. Furthermore, it is possible that T_R involvement in responses to pathogens may be happenstance due to the diversity of the regulatory T cell receptor repertoire and the shared features of inflammation associated with both chronic infection and autoimmunity.

6
A Large Proportion of Peripheral $CD25^+$ TCRs Have Greater Self-Reactivity than $CD25^-$ TCRs

The aforementioned paradigm of regulatory T cell development implies that the $CD25^+$ and $CD25^-$ TCR repertoires are different, as they are selected based on a different avidity for self-antigen. Our sequencing analyses of the TCR repertoire represented by a variable TRAV14 associated with a transgenic TCRβ chain is consistent with this prediction, as we find that there is an overlap estimated at less than 25% between the TCRs isolated between both subsets (Hsieh et al. 2004).

Although increased self-reactivity within the naturally arising regulatory T cell population has been proposed at least a decade ago based on indirect experimental approaches (Taguchi et al. 1994), definitive proof of this hypothesis has been elusive. In vitro studies showed that regulatory T cell recognition of endogenous self-peptide:MHC class II complexes is incapable of inducing suppressor function, and that additional TCR signal is required,

e.g., by anti-TCR antibody or mitogen (Takahashi et al. 1998; Thornton and Shevach, 1998). However, there is substantial concern regarding the extent that established methods to assess T_R-mediated suppressor function in vitro represent the physiologic situation in vivo. Thus, this in vitro finding does not exclude the possibility that naturally arising T_R function based on recognition of such self-antigen:MHC class II ligands in vivo. Other in vitro evidence in support of $CD25^+$ T cell self-reactivity obtained by limiting dilution cloning in the presence of syngeneic antigen-presenting cells is hard to interpret because of the difficulty of assessing the cloning efficiency of T_R and the possible contamination of T_R population with activated T cells with up-regulated CD25 expression (Romagnoli et al. 2002). Thus, these in vitro data neither strongly support nor exclude the possibility that T_R recognize self-antigens with greater avidity than $CD25^-$ T cells.

Direct characterization of naturally arising T_R interactions with self-antigens in vivo has also proven difficult, primarily because for some time the only available readout for T_R function in vivo was the prevention of induced or spontaneous pathology. However, it was found independently by several groups that TCR transgenic T_R that develop in the presence of the TCR's cognate peptide ligand encoded by another transgene can proliferate in response to the same neo-self-antigen in vivo (Cozzo et al. 2003; Klein et al. 2003; Walker et al. 2003a; Yamazaki et al. 2003). The proliferative responses of adoptively transferred TCR transgenic T_R in these experimental systems as assessed by dilution of CFSE fluorescence was strictly antigen-specific.

Proliferation within the naturally arising polyclonal T_R populations has also been described in vivo in CSFE dilution or BrDU-labeling experiments (Fisson et al. 2003; Tang et al. 2003). Extrapolating the data described above from TCR transgenic models to these data might suggest then, that naturally arising regulatory T cells are proliferating because of their TCR self-reactivity. However, it is not clear from these data what the precursor frequency of the proliferating cells is. Moreover, the differing proliferative capacity of the $CD25^+$ and $CD25^-$ T cell subsets may be explained by distinct properties unrelated to TCR specificity, such as expression of chemokine receptors or cytokine receptors such as IL-2R. This consideration makes interpretation of these experiments complicated. Although the demonstration of an increased basal level of proliferative turnover within the naturally arising $CD25^+$ T cell population in normal animals is an interesting and important observation, it can be considered only as circumstantial evidence for the self-reactivity of the naturally arising regulatory T cell population.

We have recently addressed these caveats by directly testing whether T_R-derived TCRs exhibit greater self-reactivity than TCRs derived from $CD25^-$

$CD4^+$ T cells (Hsieh et al. 2004). These TCRs were identified in our sequencing studies of TRAV14 TCR-α chain expressing $CD25^+$ and $CD25^-$ CD4 T cells isolated from TCRβ chain transgenic mice. In these experiments, we retrovirally transduced the corresponding TCRα chains into monoclonal $CD25^-$ T cells that express the original transgenic TCRβ chain and are specific for a known foreign peptide antigen. Thus, the transfer of TCRα chains resulted in the recreation of the TCRs from T_R or $CD25^-$ $CD4^+$ T cells and allowed for the meaningful comparison of TCR specificities between the subsets as the intrinsic proliferative capacity and signaling properties of the recipient cells are held constant. The extent and rate of expansion of T_R and $CD25^-$ TCR-transduced T cells adoptively transferred into lymphopenic hosts were used as the most sensitive in vivo readout for the reactivity of TCRs for self-peptide:MHC class II complexes. Using this approach, we found that 40% of the individually expressed T_R TCRs conferred the ability to rapidly expand in vivo while none of the ten $CD25^-$ $CD4^+$ TCRs tested did so. These data therefore suggest that a large proportion of naturally arising T_R TCRs recognize constitutively presented peripheral self-antigens with greater avidity than $CD25^-$ TCRs.

7 What Is the Tissue Distribution of T_R Target Self-Antigens?

We already discussed earlier studies suggesting that regulatory T cells need to specifically recognize tissue-derived self-antigens for their survival and/or functional activity in the periphery (Seddon and Mason 1999; Taguchi et al. 1994) and subsequent work supporting tissue specificity of T_R-mediated protection from autoimmunity (Green et al. 2002; Walker et al. 2003a). However, the recognition of tissue-specific antigens by some T_R does not exclude the recognition of ubiquitously presented self-antigens by others. Such recognition is predicted by TCR transgenic models in which regulatory T cell development is directed by a transgene driving expression of the cognate antigen in a variety of tissues (Cozzo et al. 2003). Furthermore, the development of regulatory T cells in H-2M-deficient mice, which express primarily a single peptide-MHC class II complex, CLIP:I-A^b, or in mice expressing Eα peptide covalently bound to I-A^b molecules, strongly argues for the existence of T_R recognizing ubiquitously presented self-peptides expressed in high copy numbers (Bensinger et al. 2001; Pacholczyk et al. 2002). Our laboratory has obtained analogous results (M. Gavin, J. Fontenot, and A.R., unpublished observations) in studies of previously described single-peptide mice (Barton et al. 2002).

Our aforementioned studies of the regulatory T cell receptor repertoire formed by variable TRAV14 (Vα2) TCRα chains and a fixed TCRβ chain are consistent with the existence of T_R specific for both tissue-specific and ubiquitously expressed MHC class II-bound self-peptides. The latter notion is supported by our observation that complex pools of $CD25^+$- but not $CD25^-$-derived TCRs, conferred the ability of T cells to proliferate in vitro to autologous splenic APCs (Hsieh et al. 2004). When tested individually, however, the proliferative response was observed upon expression of some but not all $CD25^+$ TCRs. TCR recognition of these ligands also appeared to be peptide-specific rather than peptide-promiscuous, as APCs with drastically skewed repertoire of peptides bound to MHC class II molecules failed to induce proliferative in vitro responses (Fig. 3). Thus, these data imply that T_R TCR recognition is peptide-specific and a subset of naturally arising T_R recognizes ubiquitously presented self-peptides with a sufficiently high affinity to be detected in vitro.

On the other hand, T cells transduced with some $CD25^+$, but not $CD25^-$ TCRs, were capable of inducing tissue-specific pathology, e.g., bronchiolitis and lung perivasculitis identified histologically upon adoptive transfer of these T cells into lymphopenic hosts (C.H. and A.R., unpublished observations). In preliminary experiments, we have also observed alveolitis induced by adoptively transferred activated T cells transduced with a single $CD25^+$ TCR. Although it is formally possible that organ-specific pathology, i.e., autoimmunity, in these experiments may result from reactivity of TCR with ubiquitously expressed self-peptides, a more straightforward interpretation of these observations is that some T_R TCR recognize tissue-specific antigens.

How might antigen specificity affect regulatory T cell development? Development of tissue-specific regulatory T cells is likely to require an encounter with the tissue-specific antigen in the thymus, as suggested by an experimental model where TCR transgenic T_R precursors recognize transgene-encoded cognate antigen expressed in the thymus under the rat insulin promoter (Walker et al. 2003a). Presumably, expression of these tissue-specific antigens would be under the control of the AIRE gene. Although the numbers of naturally arising $CD25^+$ $CD4^+$ regulatory T cells are normal in AIRE-deficient mice (Anderson et al. 2002), detailed analysis of the effect of AIRE deficiency on the specificity of regulatory T cells generated in the thymus and their ability to suppress tissue-specific autoimmunity has not been reported. It is expected that the putative tissue-specific T_R would expand and suppress local autoimmune responses upon antigen encounter in the draining lymph nodes and/or peripheral tissues. It can also be hypothesized that the extent of T_R expansion and suppression would correlate with the level of the corresponding self-antigen presented, allowing for more suppression during periods of increased

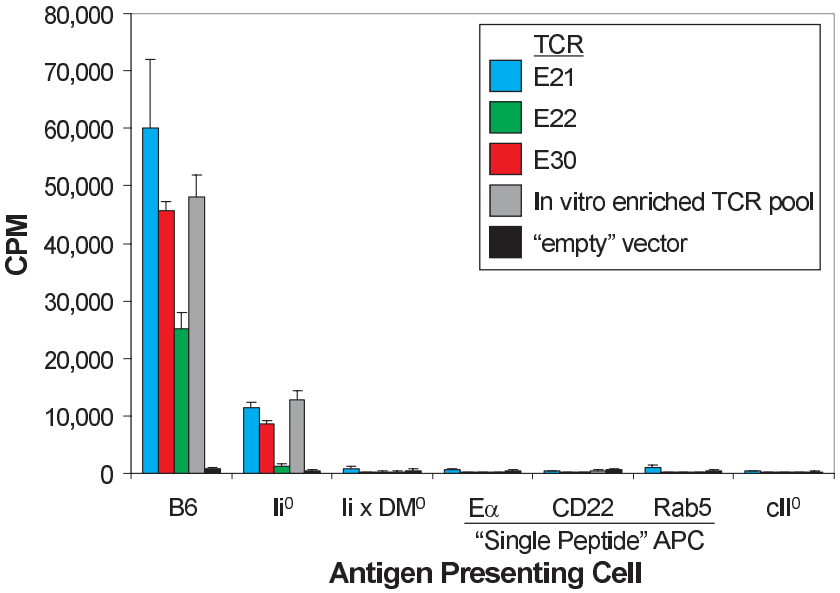

Fig. 3 Recognition of ubiquitous self-peptides displayed by autologous APCs in a peptide-specific manner. Three different individual TRAV14 TCRα chains originally isolated from CD25$^+$ T_R from TCli-β TCRβ transgenic mice were retrovirally transduced into TCli-$\alpha\beta$ TCR transgenic RAG-deficient T cells to reconstitute the original TCRs as described (Hsieh et al. 2004). TCR clones E21, E22, and E30 were isolated from an in vitro-enriched TCR pool obtained by serial passage of T cells transduced with TCRs from a CD25$^+$-derived TCR library in the presence of irradiated autologous splenocytes and IL-2. Retrovirally transduced T cells were rested over 14 days, and then restimulated in the presence of irradiated APCs described below. Incorporation of ^{3}H-thymidine was assessed between 48 and 72 h. T cells transduced with the three individual TCRs or with a pool of TCR (positive control) proliferated, albeit to a differing degree, in response to syngeneic B6 splenic APCs. In contrast, TCli-$\alpha\beta$ TCR transgenic RAG-deficient T cells transduced with the empty vector (negative control) failed to mount a significant proliferative response. The notion that some T_R may recognize ubiquitously expressed self peptides is supported by the observation that two of three individual TCR responded to Ii-deficient APCs. In the absence of Ii, surface expression of MHC class II molecules is decreased five- to tenfold, and they harbor a significantly restricted repertoire of peptides derived primarily from proteins endogenously synthesized by the APCs (Kovats et al. 1998). In contrast, none of the tested TCR was able to recognize Ii x DM double-deficient APCs, which in comparison to Ii-deficient APCs, exhibit a drastic reduction in diversity and expression level of class II-bound self-peptides, but maintain the same overall surface MHC class II expression. Similarly, no response was observed to APCs from previously characterized Eα-dbl^o, Rab-dbl^o, and CD22-dbl^o mice. These single-peptide APCs display wild-type levels of surface MHC class II molecules bound almost exclusively with a single peptide derived from I-Eα, Rab5, or CD22 proteins (Barton et al. 2002)

self-antigen expression or availability, and less suppression during periods of decreased self-antigen expression, permitting tunable suppression of inflammation associated with infection and autoimmunity. Thus, self-recognition would serve as a sensor for a cell-extrinsic negative feedback loop in which regulatory T cells would protect small areas of the body against inadvertent immune responses to excessively presented self-antigens. In a situation of increased efficiency of self-antigen presentation due to infection-associated tissue damage, concurrent innate immune activation via TLR signals induced by microbial ligands during acute infection has been proposed to permit initiation of the adaptive immune response by abrogating or rendering resistance to T_R-mediated suppression (Pasare and Medzhitov 2003). However, we would predict that down-modulation of TLR signals during chronic infection would allow T_R to effectively limit inflammation, as discussed above.

In contrast to tissue-specific regulatory T cells, the potential biological role of T_R recognizing ubiquitously presented antigen appears less obvious. In this regard, we would like to put forward a hypothesis that these T cells make up a significant portion, if not the majority, of the T_R population and provide basal protection against relatively weak low-affinity autoimmune responses of a broad specificity, but cannot efficiently protect from autoimmunity mediated by high-affinity tissue-specific effector T cells. A related intriguing notion is that perhaps these T_R act to preserve tolerance to ubiquitous antigens, such as nuclear antigens potentially involved in systemic lupus erythematosus.

In conclusion, it has become evident that the antigen specificity of naturally arising regulatory T cells is very complex, as their TCR repertoire is arguably as diverse as that of $CD25^-$ T cells. The naturally arising regulatory T cell population found in the periphery is likely largely comprised of the classic, thymus-derived T_R with an increased avidity for self peptide:MHC class II complexes. However, a number of peripheral T_R may also be generated upon repeated or chronic encounters with foreign antigens, such as orally derived or inhaled antigens, as well as commensal non-pathogenic microbes, or self-antigens. These subsets of naturally arising T_R with different antigen specificities for self- or non-self-antigens may then serve to prevent unnecessary tissue damage associated with autoimmunity or chronic infection. Nevertheless, it is clear that there is still much to learn regarding regulatory T cell antigen specificity and its impact on the development, peripheral survival and expansion, and suppressive function mediated by this T cell subset, which is critically important for the maintenance of immune homeostasis. Development of transgenic mice expressing T_R-derived TCR and identification of their foreign and self-peptide ligands will be necessary to further our understanding of the role of TCR-ligand interactions in the development and function of naturally arising regulatory T cells.

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