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Nonself-Antigens Are the Cognate Specificities of Foxp3$^+$ Regulatory T Cells

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SUMMARY

The majority of regulatory Foxp3$^+$CD4$^+$ T cells naturally arises in the thymus. It has been proposed that T cell receptors (TCRs) on these cells recognize self-MHC class II-peptide complexes with high or higher affinity and that their specificities mirror specificities of autoreactive T cells. Here, we analyzed hundreds of TCRs derived from regulatory or nonregulatory T cells and found little evidence that the former population preferably recognizes self-antigens as agonists. Instead, these cells recognized foreign MHC-peptide complexes as often as nonregulatory T cells. Our results show that high-affinity, autoreactive TCRs are rare on all CD4$^+$ T cells and suggest that selecting self-peptide is different from the peptide that activates the same regulatory T cells in the periphery.

INTRODUCTION

Central tolerance eliminates thymocytes expressing T cell receptors (TCRs) with high affinity to self-antigens, but routinely, a small fraction of potentially pathogenic T cells escapes this process. In the periphery, autoreactive T cells are controlled by active suppression mechanism carried out by regulatory CD25$^+$CD4$^+$ T (Treg) cells that express transcription factor Foxp3 (Hori et al., 2003; Fontenot et al., 2003; Khattri et al., 2003; Shevach et al., 2006). Foxp3 was initially considered as a lineage specification factor that determines the fate of thymic precursors of Treg cells (Fontenot et al., 2005a; Fontenot et al., 2005b). However, as recently shown in different mouse models with a fluorescent reporter knockin gene, Foxp3 does not act as a master switch for Treg cell lineage commitment (Gavin et al., 2007; Wan and Flavell, 2007). Instead, it stabilizes the phenotype and suppressive function of anergic thymocytes by altering the wide array of Treg cell-specific genes (Gavin et al., 2007; Zheng et al., 2007). Thus, although Foxp3 and the suppressive functions of Treg cells can now be correlated with its late expression in mature CD4$^+$CD8$^-$ thymocytes (Fontenot et al., 2005a), the signals inducing the lineage commitment of these cells and Foxp3 expression remain elusive. It has been proposed that some of the thymocytes that recognize self-MHC-peptide complexes with high affinity, instead of dying during negative selection or becoming autoreactive T cells, would acquire Foxp3 and differentiate to Treg cells (Coutinho et al., 2005; Fontenot and Rudensky, 2005; Liu, 2006; Cabarrocas et al., 2006).

The direct evidence implying that precursors of Treg cells require high-affinity, self-reactive TCRs to become Treg cells came from the TCR transgenic mouse model that coexpressed cognate peptide in the thymus (Jordan et al., 2001). In this model, interactions between TCR and class II MHC that is bound with strong but not weak agonist peptide lead to the selection of Foxp3$^+$ Treg cells with regulatory properties (Caton et al., 2004; D'Cruz and Klein, 2005; Kim and Rudensky, 2006). It was also shown that expression of agonist peptides induces a nondeletional differentiation to Foxp3$^+$ Treg cells in the thymic medulla and that in the periphery suboptimal dose of cognate antigen can convert naive, TCR transgenic cells to Foxp3$^+$ Treg cells (Apostolou et al., 2002; Watanabe et al., 2005; Liu, 2006; Aschenbrenner et al., 2007). However, another study that used different, intrathymically expressed agonist peptide could not reproduce selection of Treg cells, suggesting that factors other than agonist peptides may induce natural differentiation of Treg cells (van Santen et al., 2004).

If higher-affinity TCR-MHC-peptide interactions control natural Treg differentiation, one should also expect that these cells abundantly express self-reactive TCRs. Indeed, experiments performed in TCRβ transgenic mice, in which conventional CD4$^+$ T cells were transduced with TCRs derived from Treg cells, showed that in lymphopenic hosts and in cocultures with autologous APCs, these transductants proliferate more quickly than transductants expressing TCRs cloned from naive T cells (Hsieh et al., 2004). Moreover, when these TCRβ transgenic mice were crossed with Foxp3-deficient mice that are prone to autoimmunity, the Treg and autoreactive Foxp3$^-$CD25$^+$ cells but not the Foxp3$^-$CD25$^-$ cells expressed TCRs with overlapping antigenic specificities (Hsieh et al., 2006). These results imply that Treg cells express TCRs with higher affinity to self and that these TCRs overlap with TCRs expressed by pathogenic, self-reactive T cells. However, this proposition remains uncertain because the contribution of Foxp3-deficient Treg cells in the pathogenesis of *scurfy* male mice is still unclear and transfer of these cells does not induce autoimmune diseases in lymphopenic

hosts (Lin et al., 2007). In addition, the incidence of TCRs specific to foreign antigens on these cells has also been confusing. There are multiple experimental reports that Treg cells recognize exogenous antigens derived from microbes, parasites, neoantigens, or allogeneic tissues, but it is unclear whether these responses are antigen specific or rely on inherent crossreactivity, dual TCRs, or a bystander effect (Belkaid et al., 2002; Suvas et al., 2003; Ochando et al., 2005; Tuovinen et al., 2006; Lerman et al., 2004; Larkin et al., 2007). Interestingly, analyses of TCRs in mice expressing a TCRβ transgene or miniature repertoire of TCRs found that dominant TCRs on naive T and regulatory T cells overlap to some extent (Hsieh et al., 2004; Pacholczyk et al., 2006; Wong et al., 2007) and there is an increased diversity of TCRs on Treg cells, suggesting that this repertoire encodes multiple antigen specificities (Pacholczyk et al., 2006). Therefore, here we wanted to comprehensively analyze antigenic specificities of Foxp3$^+$ Treg cells and to determine whether their TCRs are inherently autoreactive.

For this purpose, we characterized hundreds of TCRs originally expressed by CD4$^+$Foxp3$^+$ Treg or CD4$^+$Foxp3$^-$ naive T cells and concluded that the frequency of TCRs with high affinity for self- versus nonself-antigens is similar within the two CD4$^+$ populations. Our results also demonstrate that Treg cells maintain a highly diverse TCR repertoire that included most of the TCRs dominantly expressed by naive T cells. In contrast, many TCRs expressed by Treg cells were not found on naive CD4$^+$ T cells. Finally, we showed that an outbreak of immune wasting disease in lymphopenic mice relied on the host's class II MHC ability to present foreign and not self-antigens. Therefore, when wasting disease is induced by T cells bearing Treg-derived TCRs (Hsieh et al., 2004), these TCRs recognize nonself-antigens at least as efficiently as TCRs expressed on naive T cells.

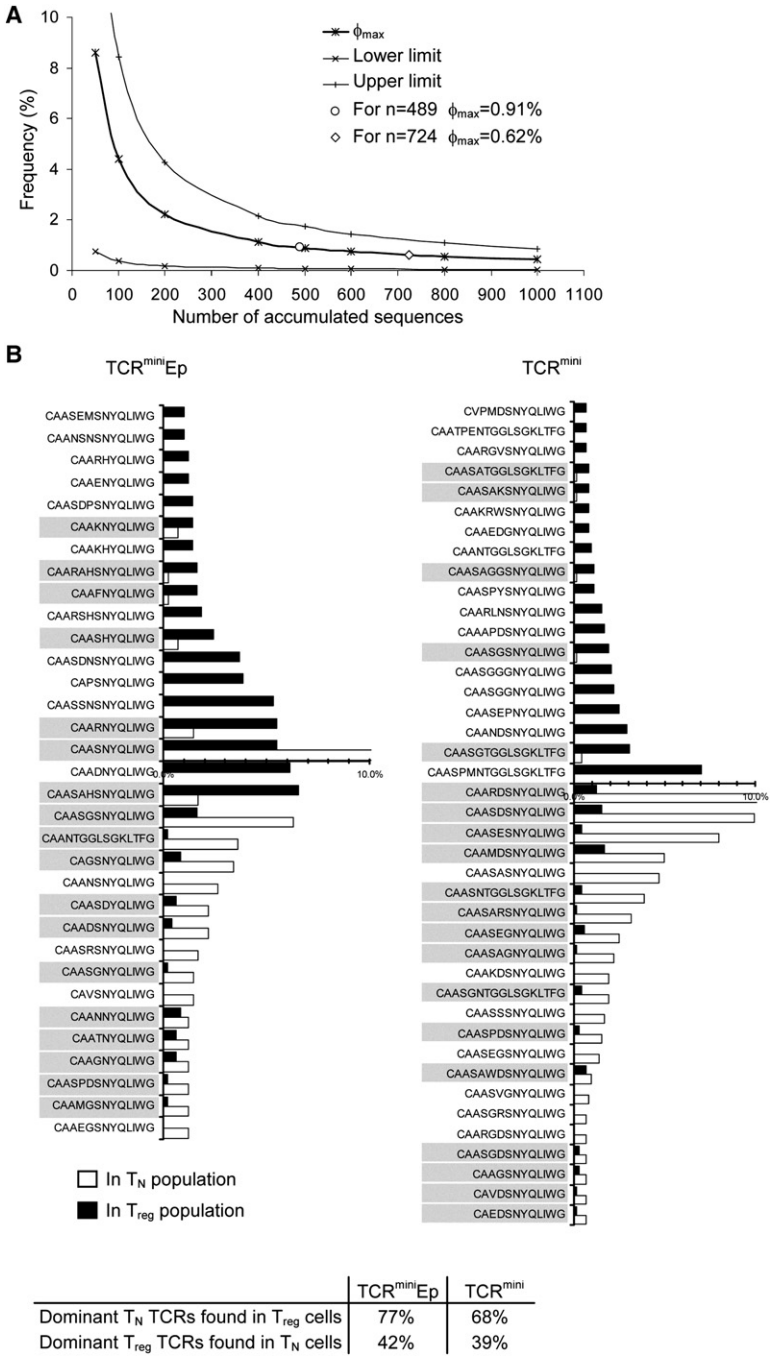
RESULTS

Naive T and Treg Cells Express Many Identical TCRs that Are Asymmetrically Distributed

It has been reported that naive T and Treg cells express predominantly different repertoires of TCRs, suggesting that these populations are separated on the basis of the affinity of individual TCRs to self-MHC-peptide ligands. However, naive T and Treg cells share a portion of TCRs that, depending on the experimental model and evaluation method, varies from 10%–42% (Hsieh et al., 2006; Pacholczyk et al., 2006; Wong et al., 2007). These analyses included many infrequent TCRs, possibly underestimating the overlap between compared regulatory and nonregulatory populations. To determine the nature of this overlap, we took advantage of the recently described TCR^{mini} mouse model, in which sequences of CDR3 regions of TCRα chains are utilized for tracking individual T cell clones. In these animals, T cells express one transgenic TCRβ chain and many TCRα chains derived from a minilocus that can rearrange one Vα2 segment and two Jα26 or Jα2 segments during T cell development in the thymus. In the TCR^{mini} mice used here, Foxp3$^+$ Treg and Foxp3$^-$ naive T cells are naturally selected in the thymus on A^b molecules bound with many endogenous peptides (TCR^{mini} mice) or a single covalently linked Ea(52-68) peptide (TCR^{mini}Ep mice). First, we looked at how many of the "most frequent" TCRα transcripts from naive T cell clones were present at least once in the Foxp3$^+$ Treg cell population. As a cutoff value for determination of frequent TCR clones, we calculated the maximum frequency φ_{max} (with a 95% confidence) of a TCR clone whose sequence could not be found after accumulation of "n" TCR sequences in a sample (Figure 1A) (Baron et al., 2003). On the basis of this evaluation, we could assume that we found all TCR clones in the tested repertoire with a frequency greater than 0.62% (0.91% for TCR^{mini}Ep). As shown in Figure 1B, we found 41 different TCRs that had a frequency above φ_{max} value from TCR^{mini} mice (32 from TCR^{mini}Ep) and then compared their frequencies within the naive T and Treg repertoires. A total of 68% of TCRs that were dominantly used by naive T cells were also found on Treg cells, although with very different frequencies. In contrast, 39% of the dominant TCRs for Treg cells were identified on naive T cells. Furthermore, when a similar comparison was performed for TCRs retrieved from TCR^{mini}A^{b}Ep mice, 77% of the dominant TCRs from naive T cells were also found on Treg cells, whereas 42% of TCRs from Treg cells were present on naive T cells (Figure 1B). Thus, when we concentrated TCR analysis on T cell clones with high frequency, the overlap between Treg and naive T populations became substantially greater than initially reported (Pacholczyk et al., 2006). In addition, more TCRs from naive T cells were found on Treg cells, suggesting that TCR repertoire expressed by Treg cells may span over a large portion of the naive T cell repertoire. This result corroborates with our previous finding that Treg cells have higher diversity of TCRs, suggesting that observed variation of dominant TCRs expressed on naive T and Treg cells can arise from asymmetric distribution of the same TCRs, not separate selection of different TCRs. The overlap between these two TCR repertoires appeared to be even more significant in TCR^{mini}Ep mice, in which the diversity of different self-class-II-MHC-peptide complexes is minimal and positive selection of CD4$^+$ T cells proceeds on one peptide covalently bound to A^b molecules. This expression of identical TCRs on Treg and naive T cells argued against the hypothesis that naive T and Treg cell lineages separate on the basis of the different affinities of TCRs for the selecting MHC-peptide ligands.

Homeostatic Expansion of T Cells In Vivo

Naive T cells that express TCRs with higher affinity to self-ligands have improved homeostatic expansion (Ge et. al., 2001). It was also shown that after adoptive transfer to lymphopenic hosts, naive T cells transduced with TCRs cloned from Treg cells expand more quickly than ones transduced with TCRs derived from naive T cells (Hsieh et al., 2004). This observation has been interpreted in favor of the hypothesis that Treg cells frequently recognize self-MHC-peptide complexes with higher affinity and that this



	TCRminiEp	TCRmini
Dominant T_N TCRs found in T_{reg} cells	77%	68%
Dominant T_{reg} TCRs found in T_N cells	42%	39%

Figure 1. Overlap of TCRs between Naive T and Treg Cell Populations

(A) The maximum frequency (ϕ_{max}) of a CDR3α transcript whose sequence did not appear after accumulation of n sequences, with 95% confidence, was computed on the basis of Equation 1. Upper and lower limits of the 95% confidence interval for a clone with ϕ_{max} frequency were calculated on the basis of Equation 2. Both equations are shown in Experimental Procedures.

(B) Observed frequency of most dominant naive and Treg clones found in TCR^{mini} or TCR^{mini}Ep mice. We calculated percentage of overlap by dividing shared clones by a total number of dominant clones of a given population. Shown amino acid sequences represent fragments of CDR3 regions including amino acids beginning with the third amino acid after the invariant C residue in all TCRAV genes (Y-L/F-C-A-X-1) and spanning the amino acid immediately preceding the TCRAJ motif (2-F/W-G-X-F-G-T). Clones shared between populations are highlighted by gray boxes. The table below the graphs shows at what frequency dominant clones of one population were found at least once in the second population (for both type of mice).

feature of Treg-derived TCRs enhances their homeostatic fitness. Because natural Treg cells require self-peptide-MHC complexes for homeostatic expansion and proliferate in vivo in the absence of exogenous antigen, we examined whether the reported higher affinity of Treg cells to self-peptides will enhance the peripheral expansion of these cells in a nonlymphopenic host.

To investigate this issue, we chose the most frequent TCRs for the naive T and Treg clones and followed their expansion ratios between the thymus and the periphery. If increased self-reactivity would help Treg cells expand better, one should expect a greater proportion of Treg than naive T clones expanding after leaving the thymus. The same fraction of both naive T and Treg cell populations, roughly one-third, expanded, contracted, or did not significantly change their frequency after migration from the thymus to the periphery (Figure 2). It suggested that the overall fitness required for the survival of naive T and Treg cells in nonlymphopenic mileu is very similar.

It is also possible that Treg cells, because of their anergic nature, cannot proliferate beyond a particular threshold level. To investigate this possibility, we followed the

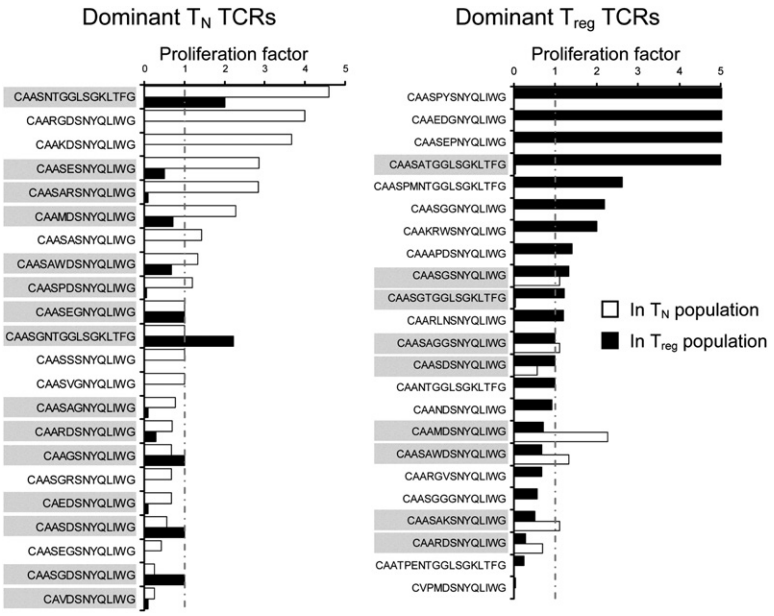


Figure 2. Relative Extrathymic Proliferation of Dominant T Cell Clones from TCR^{mini} Mice

The proportions of peripheral proliferation of most frequent T cell clones (shown in Figure 1) was calculated by division of the peripheral frequency of a given clone by its thymic frequency. CDR3α sequences are aligned from the most expanded to the least proliferated clones. Clones shared between populations are highlighted by gray boxes.

fate of naive T cell clones expressing TCRs found also on Treg cells (Figure 2). If Treg cells were selected on the basis of elevated affinity to self-MHC-peptide complexes, the naive clones expressing these TCRs should expand better than naive T cell clones bearing TCRs not expressed by Treg cells. We found that a similar number of naive T cell clones increased or decreased regardless of whether these cells had "shared" or "nonshared" TCRs, demonstrating that naive T cell clones bearing "regulatory" TCRs do not proliferate more quickly (Figure 2). Altogether, these results implied that expression of Treg-like TCRs on naive T or Treg cells does not grant a T cell a homeostatic advantage in a nonlymphopenic environment.

Generation of CD4$^+$ T Cells with TCRs Derived from Treg Cells

The inherent anergy of Treg cells in vitro impedes the study of their antigenic specificities for self- and nonself-antigens. So far, only a few TCRs derived from Treg cells have been cloned and individually transduced into nonregulatory T cells for analysis (Hsieh et al., 2004). This elegant approach disconnects the TCR from an anergic parental Treg cells, thereby allowing the TCR specificity to be tested in vivo and in vitro, but it is not suitable for examining many different TCRs on a per-cell basis. Furthermore, most of the TCRs analyzed so far were cloned from CD25$^+$ T cells with unknown status of Foxp3, thereby permitting unintentional contamination with TCRs derived from Foxp3$^-$CD25$^+$ autoreactive T cells. To overcome these difficulties, we generated T cell hybridomas from Treg cells and confirmed their origin by comparing their TCRs with TCRs found on single-cell-sorted Foxp3$^+$ Treg cells. CD4$^+$ T cell hybridomas are extensively used for identifying low-affinity agonists, revealing self-antigens responsible for survival and thymic selection, or for detecting low-abundant, endogenous peptides, demonstrating that it is an established method of studying the TCRs' specificity (Grubin et al., 1997; Grakoui et al., 1999; Felix et al., 2007).

To produce hybridomas from Treg cells, we sorted CD4$^+$CD25$^+$ T cells and cultured them by using a protocol optimized for propagating Foxp3$^+$ T cells in vitro. In brief, sorted CD4$^+$CD25$^+$ T cells were cultured in vitro for 7–10 days without losing expression of Foxp3 and CD25 molecules (Figure 3A). Importantly, after the expansion phase, the distribution of CDR3 regions of TCRα chains between sorted and expanded Treg cells remained very similar (82% overlap), demonstrating that the expansion did not alter the original distribution of TCRs expressed on Treg cells (Figure 3B). Expanded Treg cells could be efficiently immortalized by fusion with thymoma BW5147$\alpha^-\beta^-$. After fusion, Treg-derived hybridomas lost Foxp3 expression and other surface markers expressed by dividing Treg blasts but acquired the ability to secrete IL-2 upon TCR stimulation—a common feature of conventional CD4$^+$ T cell hybridomas. To further ensure that Treg-derived hybridomas express TCRs from CD25$^+$Foxp3$^+$ Treg cells, we compared their TCR sequences with sequences obtained from a database of single-cell-sorted Treg cells. As shown in Figure 3C, after sequencing only 40 TCRs, we found the most dominant TCRs originally expressed by Treg cells, and this finding demonstrated that the hybridomas expressed a true representation of TCRs found on Treg cells. Finally, to examine whether "regulatory origin" limits IL-2 secretion upon TCR stimulation, we directly compared the amounts of IL-2 produced by naive T- and Treg-derived hybridomas. Regardless of origin, all T cell hybridomas produced comparable amounts of IL-2, demonstrating that this assay is appropriate for screening antigenic specificities of TCRs derived from Treg cells (Figure 3D). To ensure maximum sensitivity, we assayed only CD4^{hi} TCR^{hi} hybridomas and used either bone-marrow-derived CD11c^{hi} A$^{b\ hi}$ DCs or freshly prepared, nonirradiated

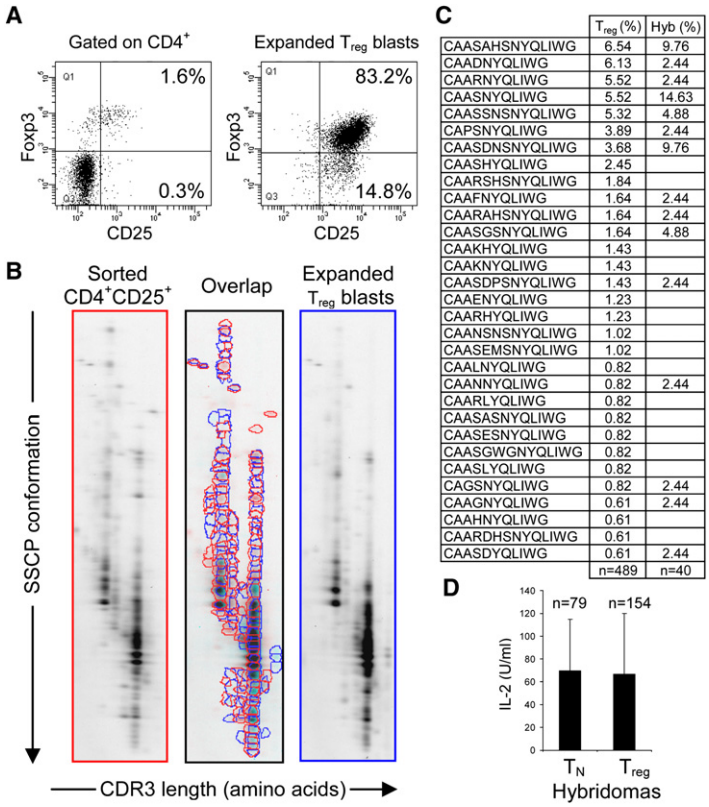


Figure 3. TCRs on T Cell Hybridomas Derived from Expanded Treg Blasts Accurately Represent TCRs Found on Treg Cells

(A) Expression of Foxp3 by Treg cells isolated from lymph nodes of TCR^{mini}Ep mice and 7–10 days after in vitro culture of these cells. Cells were surface stained with antibodies against CD4, CD8, and CD25 molecules; this was followed by intracellular staining with Foxp3 antibody.

(B) 2D-F-SSCP revealed extensive (82%) overlap of CDR3 regions of TCRα chains from freshly sorted and expanded Treg cells. cDNA was synthesized from isolated RNA of both types of cells. Runoff PCR was done with primer specific for TCRVα2, coupled with Cy3 (fluorescent dye). First, fluorescent PCR products were run in the first dimension with capillary electrophoresis in denaturing PAGE. The second dimension was run in a nondenaturing SSCP slab gel. Resulting 2D gels show diversity, distribution, and frequency of dominant CDR3α regions from indicated populations. The intensity of individual dots corresponds to the real frequency of a particular rearrangement in the original sample. Spots migrating with different speed on the second dimension SSCP gel represent different VαJα rearrangements.

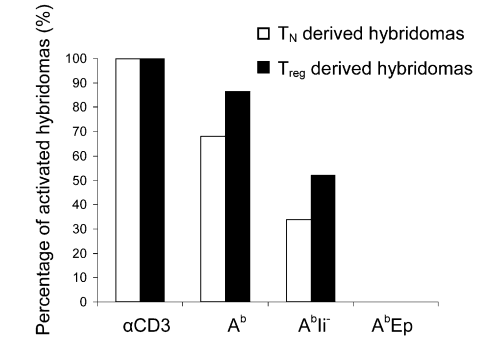
(C) TCR CDR3α sequences retrieved from Treg-derived hybridomas (Hyb) were compared with the most abundant CDR3α sequences found in Treg cells in TCR^{mini}Ep mice (Treg).

(D) Hybridomas derived from naive T or Treg cells of TCR^{mini}Ep mice were cocultured with splenocytes from C57BL/6 TCRα$^{-/-}$ mice. Responding hybridomas were compared for the ability to produce IL-2. Production of IL-2 secreted by activated hybridomas was measured by HT-2 proliferation with MTT assay. Results are shown as arithmetical mean ± SD.

splenocytes as the APCs. So far, we established more than 1000 T cell hybridomas, of which approximately half was derived from Treg cells and the other half was derived from naive T cells. These hybridomas were obtained from the following three different types of mice: the TCR^{mini}Ep, TCR^{mini}, and conventional C57BL/6. Use of the database of TCR^{mini} sequences obtained from single-cell sorted Treg cells allowed us to identify the origin of the TCRs sequenced from the T cell hybridomas (Pacholczyk et al., 2006).

Hybridomas Derived from Treg Cells of TCR^{mini}Ep Mice Recognize Nonself-Antigens

It has been suggested that TCRs expressed by Treg cells may recognize abundant peripheral self-antigens with an affinity that is above that required for homeostatic expansion (Hsieh et al., 2004; Hsieh et al., 2006). To determine the fraction of Treg cells that recognizes ubiquitously expressed self-antigens, we first examined the antigen specificities of T cell clones derived from naive T and Treg cells from TCR^{mini}Ep mice. The advantage of this model is that all self-MHC class II molecules in the thymus and periphery remain bound with a single peptide and that the resulting repertoire of TCRs is tolerant to A^{b}Ep complex but lacks tolerance to natural, endogenously processed peptides. Also, more than two-thirds of T cell hybridomas obtained from CD4^{+} T cells produce IL-2 when cocultured with APCs derived from C57BL/6 mice (Ignatowicz et al., 1996). This feature of TCRs selected by A^{b}Ep complex allowed us to precisely test the specificity for self-(A^{b}Ep) and nonself-MHC-peptide (A^{b}WT) complexes. As shown in Figure 4A, 68% and 87% of hybridomas derived from naive T or Treg cells, respectively, become activated after overnight coculture with APCs expressing nonself A^{b}WT complexes, but none of these hybridomas produced IL-2 after incubation with self-APCs. Furthermore, the same TCR^{mini}Ep-derived hybridomas were incubated with APCs derived from invariant chain-deficient (A^{b}Ii^{-}) mice that express a lower number of molecules loaded with a reduced number of Ii-independent, endogenous peptides. Here, hybridomas derived from naive T and Treg cells also responded to nonself-APCs with similar, though expectedly lower, frequency. Importantly, recognition of nonself-antigens was observed regardless of whether TCRs on T cell hybridomas represented a pool of "shared" TCRs or TCRs that are only found on Treg cells (Table 1). In summary, these experiments showed that irrespective of the origin of the antigen(s), the frequency of TCRs directed to nonself-antigens is similar between naive T and Treg cells and that autoreactive TCRs specific to abundant self-MHC-peptide complexes are rare on all CD4^{+} T cells.



		A^b	A^bIi^-	A^bEp(self)
$TCR^{mini}Ep$	Hyb_{TN}	n=126	n=89	n=126
	Hyb_{Treg}	n=178	n=119	n=178

Figure 4. Treg-Derived Hybridomas from $TCR^{mini}Ep$ Mice Frequently Recognize Foreign but Not Self-MHC-peptide Complexes

Hybridomas were cocultured overnight with splenocytes or bone-marrow-derived dendritic cells (BMDCs) from C57BL/6 (A^b), Ii-, or A^bEpIi^- (AEp) mice. Production of IL-2 secreted by activated hybridomas was measured by HT-2/MTT assay. In each experiment, all hybridomas were separately tested for response to anti-CD3 stimulation in the presence of autologous APCs so that the percentages of hybridomas able to respond to activation could be determined. The box beneath the bar graph shows the number of hybridomas (n) used in each test.

Treg Cells Selected on A^bWT Complexes Rarely Recognize Self-Antigens

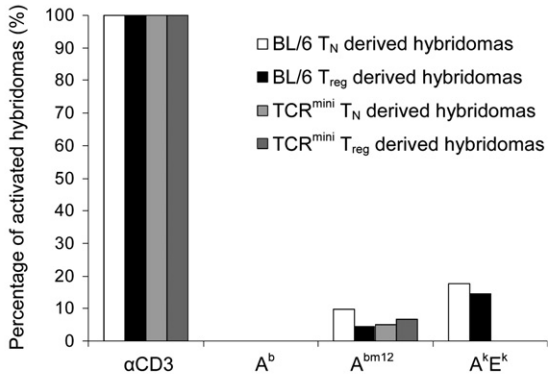
One could argue that ubiquitous expression of A^bEp complexes in $TCR^{mini}Ep$ mice enhances deletion of autoreactive TCRs, but in normal animals expressing A^bWT complexes, negative selection will be less efficient and incidence of autoreactive TCRs on Treg cells could be higher. Therefore, we made another set of hybridomas from TCR^{mini} and C57BL/6 mice that express A^bWT molecules and tested their ability to respond to APCs expressing self- and nonself-MHC-peptide complexes. When hybridomas from TCR^{mini} mice were cocultured with autologous APCs, none of them produced IL-2, indicating that the frequency of Treg cells with potentially autoreactive TCRs is very low (Figure 5A). To detect the responses of these hybridomas to nonself-antigens, we tested their ability to become activated by APCs expressing A^{bm12} molecules. The A^{bm12} molecule is similar to autologous A^b molecules, but because of the mutation in antigen-binding groove, the A^{bm12} molecules remain bound with a different set of endogenously processed peptides (Bill et al., 1989). As shown in Figure 5A, hybridomas derived from Treg and naive T cells recognized the A^{bm12} APCs but not other allogeneic APCs expressing A^k and E^k complexes. These results showed that TCRs expressed on Treg cells recognize different nonself-peptides bound to MHC, but they are not overtly reactive to the backbone of class II MHC complexes (Felix et al., 2007). Moreover, a similar frequency of A^{bm12}-specific TCRs was observed on hybridomas derived from naive T and Treg cells, confirming that these TCR repertoires have comparable ability to sense nonself-APCs.

Because we have not found autoreactive TCRs in naive T or Treg cell populations, we decided to take advantage of the TCR^{mini} repertoire that allows for backtracking receptors to their occurrence in vivo. We sequenced TCRs on hybridomas derived from $TCR^{mini}Ep$ that responded in vitro to A^b molecules bound with endogenous peptides, and then we searched for identical TCRs in our database of almost 1500 TCRs sequenced from $CD4^+$ T cells derived from TCR^{mini} mice. Although there are only a few TCRs shared between these mice, we identified six TCRs

Table 1. Reactivities of Most Dominant Treg TCRs from $TCR^{mini}Ep$ Mice

		Hybridomas Reactive to	
Treg-Derived CDR3α Sequences from $TCR^{mini}Ep$ Mice	Used by Cells	A^bWT(Nonself)	A^bEp(Self)
CAASAHSNYQLIWG	T_N/Treg	+	–
CAADNYQLIWG	Treg	+	–
CAARNYQLIWG	T_N/Treg	+	–
CAASNYQLIWG	T_N/Treg	+	–
CAASSNSNYQLIWG	Treg	+	–
CAPSNYQLIWG	Treg	+	–
CAASDNSNYQLIWG	Treg	+	–
CAASHYQLIWG	T_N/Treg	N/A	N/A
CAARSHSNYQLIWG	Treg	N/A	N/A
CAAFNYQLIWG	T_N/Treg	+	–
CAARAHSNYQLIWG	T_N/Treg	+	–
CAASGSNYQLIWG	T_N/Treg	–	–

Twelve dominant Treg TCRs identified by single-cell RT-PCR were compared with TCRs found on T cell hybridomas. On the basis of reactivities of these hybridomas, antigenic specificity was assigned to the particular CDR3α sequence.



		A^b (self)	A^{bm12}	A^kE^k
BL/6	Hyb_{TN}	n=106	n=82	n=62
	Hyb_{Treg}	n=117	n=108	n=96
TCR^{mini}	Hyb_{TN}	n=124	n=75	n=52
	Hyb_{Treg}	n=134	n=90	n=66

Figure 5. Hybridomas Derived from Naive T and Treg Cells Respond with the Same Frequency to Stimulation with Allogeneic APCs but Not Autologous APCs

Hybridomas derived from naive and Treg cells from TCR^{mini} or C57BL/6 mice were stimulated by splenocytes or BMDCs derived from autologous C57BL/6 (A^b), allogenic CBA Ca/J (A^kE^k), or bm12 (A^{bm12}) mice. All hybridomas were tested for response to anti-CD3 stimulation in the presence of autologous APCs. Production of IL-2 was measured by HT-2 proliferation with MTT assay. For determining percentages of hybridomas responding to APCs, only hybridomas that responded to the anti-CD3 stimulation were considered. The box beneath the bar graph shows the number of hybridomas (n) used in each test.

that were highly reactive to A^bWT from TCR^{mini}Ep mice, and these TCRs were found in TCR^{mini} mice as well (Table 2). Interestingly, when we examined on what type of T cells these TCRs have been found in TCR^{mini} mice, two TCRs were expressed on naive T cells and five were expressed on Treg cells. Each of these autoreactive TCRs was rare in TCR^{mini} mice, suggesting that although some of TCRs expressed by Treg cells are potentially autoreactive, their frequency on naive T and Treg cells was low.

To extrapolate our finding from TCR^{mini} mice to wild-type Treg cells, we also tested antigenic specificities of T cell hybridomas obtained from C57BL/6 mice. As shown in Figure 5A, approximately 10% of the hybridomas responded to allogeneic APCs irrespectively of whether these TCRs originated from naive T or Treg cells. However, none of the hybridomas tested here produced IL-2 after incubation with autologous APCs. This result confirmed our previous findings from TCR^{mini} mice that in general, $CD4^+$ T cells rarely express autoreactive TCRs that recognize self-class II MHC-peptide complexes as agonists.

Wasting Disease Induced by Adoptive Transfer of Effector T Cells Is Caused by Nonself-Antigens

Inflammatory bowel disease (IBD) is an immune-mediated disease that manifests with inflammation on the colonic and rectal mucosa (ulcerative colitis) and with Crohn's disease that affects both the small and large intestines. The genetic and environmental factors responsible for precipitation of the disease are linked with an aberrant inflammatory response toward commensal bacteria. There is an established experimental protocol for inducing IBD in mice; in this protocol, transfer of $CD4^+CD45RB^{hi}$ T cells into immune-deficient mice, such as *Rag2*$^{-/-}$ or SCID mice, leads to colitis, which causes a wasting disease (reviewed in Coombes et al. [2005]). It has also been shown that expansion of nonregulatory T cells engineered for expressing TCRs cloned from $CD25^+$ T cells causes wasting disease, whereas expansion of T cells expressing TCRs cloned from $CD25^-$ T cells does not (Hsieh et al., 2004). These results were interpreted as evidence that repertoire of TCRs expressed by Treg cells is enriched in autoreactive specificities. However, so far there is no direct evidence that self-antigens are responsible for initiation of wasting disease in the transfer model.

To determine whether recognition of self-antigens can cause wasting disease, we sorted naive $CD4^+CD45RB^{hi}$ T cells from TCR^{mini} and TCR^{mini}Ep mice and transferred them into lymphopenic A^bWT *TCRα*$^{-/-}$ or A^bEp *TCRα*$^{-/-}$

Table 2. The CDR3α Regions of Autoreactive TCRs Identified in TCR^{mini} Repertoire

	Distribution on $CD4^+$ T Cells in TCR^{mini} Mice	
Sequences of TCRs Reactive to A^bWT Found in TCR^{mini}Ep and TCR^{mini} Mice	Treg	T_N
CAASNYQLIWG	+	
CAASAHSNYQLIWG	+	
CAGSNYQLIWG		+
CAARSHSNYQLIWG	+	+
CAASDYQLIWG	+	
CAASGWDSNYQLIWG	+	

TCRs on TCR^{mini}Ep Treg-derived hybridomas reactive to antibodies were compared against database containing CDR3α sequences retrieved by single-cell RT-PCR from T cells in TCR^{mini} mice. Of the six CDR3 sequences identified so far, five were expressed on $Foxp3^+$ Treg cells and two were found on $Foxp3^-$ T cells.

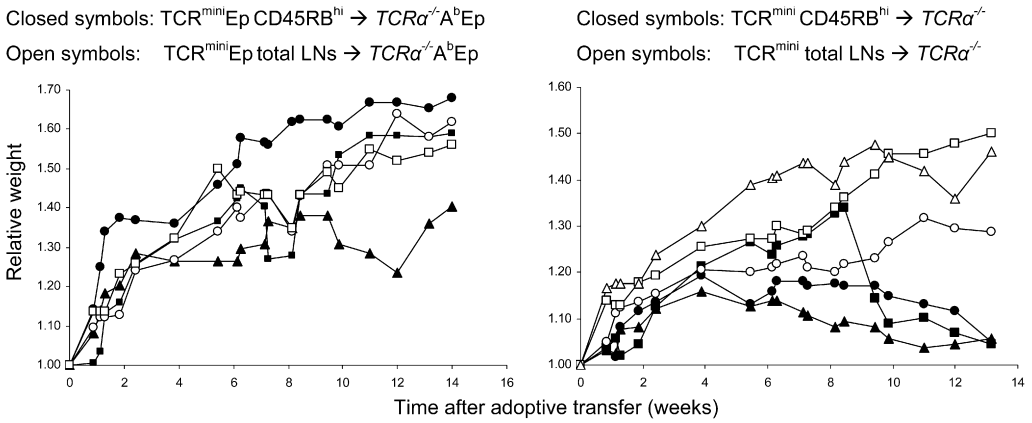


Figure 6. The CD4+CD45RB^{hi} T Cells Isolated from TCR^{mini} but Not TCR^{mini}Ep Mice Cause Wasting Disease upon Adoptive Transfer into Autologous Lymphopenic Hosts

T cells isolated from lymph nodes were FACS sorted with CD4 and CD45RB antibodies. A total of 4 × 10^5 of CD4$^+$CD45RB^{hi} T cells (purity greater than 99%) from TCR^{mini} or TCR^{mini}Ep mice were injected intraperitoneally to A^{b}WT *TCRα*$^{-/-}$ or A^{b}Ep *TCRα*$^{-/-}$ lymphopenic hosts, respectively. The ratio of the starting weight of recipient mice is shown. Data are representative of two independent experiments with two or three mice per group. Weight loss was associated with colon inflammation, prolapse, bowel-wall thickening, and typical hunched-back appearance (data not shown).

mice, respectively. The host mice that received CD4$^+$CD45RB^{hi} T cells from TCR^{mini} mice started losing weight 4–5 weeks after adoptive transfer (Figure 6); this trend continued until mice had to be sacrificed. To ensure that the observed weight loss was a result of colitis, we confirmed lesions in the intestine macroscopically (data not shown). Interestingly, transfer of CD4$^+$CD45RB^{hi} T cells from TCR^{mini}Ep mice into A^{b}Ep *TCRα*$^{-/-}$ mice did not result in colitis. Because mice expressing covalent A^{b}Ep complexes do not present other antigens, our results support previous findings that recognition of bacterial antigens of commensal flora is required for initiation of wasting disease upon transfer of effector CD4$^+$ T cells. Importantly, this experiment also implied that the previously reported precipitation of wasting disease by a transfer of T cells expressing Treg-derived TCRs is caused not by a higher frequency of autoreactive TCRs but rather by sufficient frequency of nonself-specificities on Treg cells.

DISCUSSION

We report that contrary to the general belief, nonself-antigens constitute a primary source of peptides recognized by TCRs that are expressed on Treg cells and that have an affinity high enough for inducing an antigenic response. Moreover, these TCRs do not show a major enrichment in high-affinity, autoreactive specificities. This conclusion was obtained on the basis of the following two approaches: an analysis of TCR usage and homeostatic fitness of naive T and Treg clones in vivo and an analysis of antigenic specificities of TCRs derived from Foxp3$^+$ Treg cells.

Our comparative analysis showed that 70% of the most frequent TCRs on naive T cells were also found on Treg cells, but with very different frequencies. This disparity in the allocation of shared TCRs was already set in the thymus, suggesting that thymic, not peripheral, events enforce this asymmetric distribution. A similar observation of shared TCRs between regulatory and nonregulatory populations was also reported elsewhere, in which seven out of ten most frequent TCRs on CD25$^-$ and CD25$^+$ CD4$^+$ T cells was shared between both analyzed populations (Hsieh et al., 2006). This substantial fraction of shared TCRs suggests that naive T and Treg cells have, in fact, many shared affinities. In addition, 40% of dominant TCRs on Treg cells were also found on naive T cells. Possibly, this overlap could be larger but remained partially hidden because of vastly different frequencies of the same TCRs and probably a higher diversity of TCRs on Treg cells (Pacholczyk et al., 2006). This could also indicate that TCRs used by naive T cells constitute a subset of TCRs expressed on Treg cells.

The main role of Treg cells is to suppress autoreactive T cells, and this suggests that TCRs on Treg cells may recognize self-antigens as agonists. However, we found that T cells expressing TCRs derived from a Treg cell population did not become activated and produced IL-2 upon exposure to self-MHC-peptide ligands. Moreover, the majority of these T cell clones responded to nonself-antigens that represent endogenously processed peptides and allogenic MHC-peptide complexes. Regardless of whether TCRs were derived from Treg or naive T cells subpopulations, the frequency of TCRs recognizing nonself-peptides was similar. Our data corroborate previous findings that partial reversal of the anergic state of Treg cells by coculture in the presence of IL-2 or IL-15 allows them to respond to allogeneic, but not syngeneic, APCs in both mice and humans (Dieckmann et al., 2001; Pacholczyk et al., 2002). Here, the only way we were able to identify potentially autoreactive TCRs specific for A^{b}WT complexes in TCR^{mini} mice was by crossreferencing A^{b}WT-reactive TCRs originally found on hybridomas from TCR^{mini}Ep to

the database of almost 1500 TCRs previously cloned from TCR^{mini} mice. This result demonstrated that the frequency of high-affinity, self-reactive TCRs in the Treg cell population is very low, although it may be marginally higher than on naive T cells. One could argue whether T cell hybridomas are an appropriate tool for detecting autoreactive TCRs. However, T cell hybridomas made from autoreactive T cells continued to manifest the original self-reactivity in vitro and for years T cell hybridomas were used for detecting weak and strong agonists, sometimes at very low levels (Hampl et al., 1997; Grubin et al., 1997). In general, the self-reactive hybridomas are produced either after antigen priming or from autoimmune prone strains but not directly from naive TCR repertoire, which is expected to have a very low frequency of autoreactive T cells (Yanoma et al., 1988; Buzas et al., 2003).

It has been hypothesized that TCRs on Treg cells "draw from the same pool of TCRs as autoreactive T cells" and that a majority of TCRs on Treg cells have pathogenic potential (Hsieh et al., 2006). This conclusion was based on the observation that CD25$^+$ Treg cells from wild-type mice express TCRs similar to the receptors expressed on pathogenic autoreactive CD4$^+$CD25$^+$ T cells from Foxp3$^-$ mice. Indeed, in the recently produced Foxp3-GFP knockin mice the "disabled" Treg cells expressing GFP left the thymus and partially accumulated in nonlymphatic tissues, suggesting direct involvement of these cells in pathogenesis (Gavin et al., 2007; Lin et al., 2007). However, the role of "disabled" Treg cells in the autoimmune disease observed in Foxp3-deficient mice is unclear because these cells remain anergic, cannot provoke an autoimmune disease upon adoptive transfer to a lymphopenic host, and deteriorate in the absence of other T cells (Lin et al., 2007). Furthermore, when the same questions were addressed in *scurfy* mice expressing transgenic GFP under the control of Foxp3 regulatory sequences, the proportion of GFP$^+$ in CD4$^+$ T cells in sick *scurfy* and healthy B6 males was similar, and the number of GFP$^+$ T cells activated was no greater than that of GFP$^-$ T cells (P.K. and M. Kuczma, unpublished data). Concurrently, the depletion of Foxp3-expressing T cells in adult animals leads to autoimmune disease that has a severity similar to the disease observed in the *scurfy* mice and that is entirely driven by autoreactive T cells from the pool of Foxp3$^-$ T cells (Kim et al., 2007). Other evidence for pathogenic autoreactive specificities of Treg cells is based on an adoptive transfer of naive T cells that are retrovirally transduced with Treg-derived TCRs and cause autoimmunity manifested by wasting disease (Hsieh et al., 2004). However, onset of colitis depends on an overly aggressive T cell response to a subset of commensal enteric bacteria, and the transfer of naive T cells into germ-free animals does not cause the disease (Gad, 2005; Sartor, 2006). The wasting disease described here was induced by adoptively transferred naive T cells only in animals in which class II MHC was able to present foreign antigens, but not in animals in which class II MHC was preoccupied with covalently bound self-peptide, confirming that in such an experimental setup, nonself-antigens are a necessity. Hence, the fact that naive T cells with "regulatory" TCRs cause wasting disease, as described in other reports (Hsieh et al., 2004), appears to suggest that specificities directed toward nonself (bacterial)-antigens are well represented in the Treg repertoire.

In general, conventional T cells require weak interactions with self-MHC-peptide complexes to pass thymic selection and to survive in the periphery and require higher-affinity interactions with nonself-MHC-peptide ligands to induce activation and effector functions. In contrast, it has been proposed that a commitment of Treg cells is driven by higher-affinity interactions between TCR and self-MHC-peptide complexes (Jordan et al., 2001; Apostolou et al., 2002; Hsieh et al., 2004) and that Treg cells recognize ubiquitous self-ligands with an avidity above that required for selection and survival (Hsieh et al., 2006). These interactions induce Treg phenotype and Foxp3 expression and skew the specificity of TCR repertoire toward self-antigens. Consequently, naive T and Treg cells express different sets of TCRs that recognize nonself- or self-antigens, respectively. Therefore under these circumstances, the fact that Treg cells may use the same self-ligand(s) for positive selection in the thymus and effector function in the periphery is fundamentally different from the conventional model of T cell selection and activation. Furthermore, if lineage commitment of Treg cells depends on higher TCR affinity for self-ligand(s), the repertoire of TCRs on Treg cells would be less diverse than the repertoire on naive T cells. This is because for a given antigen, there will be fewer TCRs with a higher affinity than TCRs with lower affinity. However, our data show that Treg cells may not only have higher diversity of TCRs than conventional T cells but also share a substantial portion of TCRs with that population (Figure 1) (Pacholczyk et al., 2006).

Our findings question the direct role of TCR signaling in the lineage commitment of Treg cells but support the view that recognition of self-MHC-peptide complexes is required for these cells to survive in the thymus and periphery. Subsequently, we would like to offer an alternative model in which commitment of Treg precursors occurs prior to thymic selection by the TCR (Pacholczyk et al., 2002; van Santen et al., 2004; Pennington et al., 2006) and even weak interactions with self-ligands can induce survival of thymic Treg cells. In this model, precursors of Treg (pre-Treg) cells possess a unique physiological status and exist as a fraction of late CD4$^-$CD8$^-$ (DN) or cortical CD4$^+$CD8$^+$ (DP) thymocytes. This condition may be triggered by a "near-death experience," different proliferation propensity prior to the DP stage, or a currently unknown signal(s) delivered upon the contacts of thymocyte with other thymocytes or thymic stromal cells. Different physiological status of pre-Treg cells, not signaling through the TCR, will determine their lineage commitment and TCR distribution. Positive selection of both lineages proceeds on the same pool of self-ligands, generating unbiased TCRs distribution. Both naive T and Treg repertoires are primarily directed to recognize nonself-antigens with small fractions of potentially autoreactive TCRs. After positive selection, the TCR repertoires on Treg cells is wider because physiological status of pre-Treg thymocytes can

make positive selection of Treg cells more promiscuous. Some Treg cells can express autoreactive TCRs, but in contrast to the current dogma, the self-peptide(s) involved in positive selection of Treg cells is not responsible for activation of their suppressor functions in the periphery. In support of a "precomittment" hypothesis, it has been recently reported that Foxp3 expression can occur in preselected $CD4^-CD8^-$ thymocytes, when these cells develop in the absence of transconditioning by $CD4^+CD8^+$ thymocytes (Pennington et al., 2006).

EXPERIMENTAL PROCEDURES

Mice

$TCR^{mini}A^bWT$ mice were obtained as previously described (Pacholczyk et al., 2006). We generated $TCR^{mini}A^bEp$ mice by crossing $V\alpha J\alpha/\beta^{Tg}$ A^bWT mice with mice deficient for the invariant chain and endogenous $A\beta^b$ chain but expressing a single covalent A^bEp complex (Ignatowicz et al., 1996). To eliminate expression of endogenous TCRα chains, we crossed all TCR^{mini} mice used in this study with mice deficient in endogenous TCRα genes. All TCR^{mini} mice were 6 to 8 weeks old and were heterozygous for the TCRα mini locus so that expression of a single TCR could be ensured.

All mice were housed under specific pathogen-free conditions in the animal care facility at the Medical College of Georgia. All work involving animals was conducted under protocols approved by the Animal Care and Use Committee at Medical College of Georgia.

Cell Preparation and Flow-Cytometry Analysis

Single-cell suspensions were prepared from lymph nodes by mechanical disruption through nylon mesh. The following antibodies were used for flow-cytometry analyses: anti-CD4-APC, anti-CD8-PerCP, anti-CD25-FITC, anti-CD45RB-PE, anti-Vα2-PE, anti-Vβ14-FITC, anti-TCRβ-APC, and anti-Foxp3-APC (all from BD PharMingen or eBioscience). Intracellular staining for Foxp3 expression was performed with intracellular staining kit, in accordance with the manufacturer's protocol (eBioscience). FACS analysis was performed with BD FACSCanto and Diva software (BD PharMingen). Dead cells were excluded by gating of forward and side scatter.

Generation of T Cell Hybridomas

For generating conventional T cell hybridomas, $CD4^+$ T cells sorted from lymph nodes were cultured for 3 days in culture medium in the presence of plate-bound aCD3 mAb and autologus-irradiated splenocytes. On day 3, dead cells were removed by gradient centrifugation on Lymphocyte Separation Media (Cellgro). The recovered live cells were expanded in the presence of IL-2 for 4 days, and T cell blasts were converted into T cell hybridomas as previously described (Ignatowicz et al., 1996). To generate T cell hybridomas derived from regulatory T cells, we sorted $CD4^+CD25^+$ lymphocytes and induced their proliferation by aCD3 and aCD28 antibodies fixed on the solid surface in the presence of exogenous IL-2 for 10 days (N.S. and M.I., unpublished data). Expanded $CD25^+$ T cell blasts were also converted into T cell hybridomas.

HT-2 Assay

T cell hybridomas were tested for their responsiveness toward self- and nonself-APCs by HT-2 assay (Ignatowicz et al., 1996). In brief, 10^5 hybridoma cells were incubated with 5×10^5 splenocytes or 10^5 bone marrow dendritic cells, from different mice, as indicated. After 24 hr, the amount of secreted IL-2 was measured with the detector HT-2 cell line. The proliferation of HT-2 cells in response to IL-2 was measured with 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay.

MoFlo Sorting of $CD4^+CD45^{hi}$ T Cells and Adoptive Transfer to Lymphopenic Hosts

Lymphocytes were stained with appropriate antibodies and were subjected to double sorting on MoFlo sorter (Cytomation) (Pacholczyk et al., 2006). Purity of the $CD4^+CD25^+$ or $CD4^+CD45RB^{hi}$ populations was >99%. $TCR\alpha^{-/-}$ mice were injected intraperitoneally with sorted $CD4^+$ T cell subpopulations in PBS. Mice received 4×10^5 $CD45RB^{hi}$ $CD4^+$ cells. After adoptive transfer, mice weight was monitored on a weekly basis.

RT-PCR and Two-Dimensional, Fluorescent, Single-Stranded Conformational Polymorphism

RNA was isolated from sorted populations with Trizol according to the manufacturer's protocol (Invitrogen). cDNA synthesis was done with M-MLV reverse transcriptase and Random Hexamers (all from Promega). cDNA was amplified in a standard PCR reaction with Vα2-specific primers (Va2_287 s, CA_29r). A total of 2 μl of PCR product was used as a template for a runoff reaction with a fluorescent primer Va2_287 s labeled on the 5′ end with Cy3 (synthesized by IDT). The denatured fluorescent products were then subjected to 2D electrophoresis as previously described (Pacholczyk et al., 2006) We acquired fluorescent images by scanning the slab gel in a Typhoon 9410 imager (Amersham-Pharmacia). Images were analyzed with Image Master 5.0 Platinum software (Amersham-Pharmacia).

Estimation of Most Frequent TCRs

Maximum frequency ϕ_{max} of possibly yet unseen TCR clone was calculated on the basis of the following equation published elsewhere (Baron et al., 2003):

$$n\left(\phi_{max} - 1.65\sqrt{\frac{\phi_{max}(1-\phi_{max})}{n-1}}\right) = 1, \quad (1)$$

in which n is a total number of accumulated sequences. To estimate boundaries of a real frequency (ϕ_{clon}) of a TCR clone with observed frequency $f_{clon} = \phi_{max}$, we used the following equation:

$$\phi_{clon} \in \left[f_{clon} - 1.96\sqrt{\frac{f_{clon}(1-f_{clon})}{n-1}}, f_{clon} + 1.96\sqrt{\frac{f_{clon}(1-f_{clon})}{n-1}}\right]. \quad (2)$$

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