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Adaptation of TCR Repertoires to Self-Peptides in Regulatory and Nonregulatory CD4+ T Cells

Jamie Wong, Reinhard Obst, Margarida Correia-Neves, Grigoriy Losyev, Diane Mathis, and Christophe Benoist

Currently, it is not understood how the specificity of the TCR guides CD4+ T cells into the conventional lineage (Tconv) vs directing them to become regulatory (Treg) cells defined by the Foxp3 transcription factor. To address this question, we made use of the “Limited” (LTD) mouse, which has a restricted TCR repertoire with a fixed TCRβ chain and a TCRα chain minilocus. The TCR repertoires of Tconv and Treg cells were equally broad, were distinct, yet overlapped significantly, representing a less strict partition than previously seen between CD4 and CD8 T cells. As a group, the CDR3α motifs showed a significant trend to higher positive charge in Treg than in Tconv cells. The Tconv and Treg repertoires were both reshaped between thymus and periphery. Reducing the array of peptides presented by MHC class II molecules by introducing the H2-DMαα mutation into the LTD mouse led to parallel shifts in the repertoires of Tconv and Treg cells. In both cases, the CDR3α elements were entirely different and strikingly shortened, relative to normal LTD mice. These peculiar sequences conferred reactivity to wild-type MHC class II complexes and were excluded from the normal repertoire, even among Treg cells, indicating that some forms of self-reactivity are incompatible with selection into the Treg lineage. In conclusion, the Treg repertoire is broad, with distinct composition and characteristics, yet significantly overlapping and sharing structural constraints with the repertoire of conventional CD4+ T cells. The Journal of Immunology, 2007, 178: 7032–7041.
of their relative frequencies in either population, and the numbers correspond to the amino acid sequences listed in Fig. 2. E, “Species accumulation curve” plotting the number of unique TCR sequences observed as a function of the total number of sequences sampled (averaged from 500 random draws from the data). D, Frequency of individual TCR sequences. The bar histogram depicts the frequency in Tconv and Treg populations (filled bars above the midline and open bars below, respectively). Sequences are ordered as a function of the total number of sequences sampled (averaged from 500 random draws from the data).

(a) null mutation of presented by MHC class II molecules by crossing into the mouse toires that result from drastically limiting the range of peptides comparisons (36). We also examined the changes in both reper-
gation by an agonist peptide (32, 35). The extent to which these (LTD) mouse, which carries a minilocus transgene that imposes 


troversial (18).

ter to clonal deletion (29–31). Some investigators have sug-
gated that the selection of Treg cells might be favored in particular stromial niches (23). In addition, mature CD4+ T cells from peripheral lymphoid organs could be converted to FoxP3 positivity by exposure to TGF-β (32–34) or to chronic stimulation by an agonist peptide (32, 35). The extent to which these peripheral conversion mechanisms influence the repertoire of Treg cells in normal immune responses remains somewhat controversial (18).

In this study, we have analyzed the breadth and evolution of the repertoire of Treg cells, and its relationship to the repertoire of conventional lineage T (Tconv) cells, by using the “Limited” (LTD) mouse, which carries a minilocus transgene that imposes great limits on TCR variability and thereby facilitates sequence comparisons (36). We also examined the changes in both repertoires that result from drastically limiting the range of peptides presented by MHC class II molecules by crossing into the mouse a null mutation of H2-Dmα (DM), the key peptide editor for class II molecules (7–9). Two groups have very recently reported related studies of the Treg repertoire in different experimental systems (24, 37, 38). The high degree of TCR diversity among Treg cells of LTD mice, the particular structural characteristics of this repertoires, and its profound adaptation to altered negative selection in single-peptide mice provide an interesting counterpart to these other data sets.

Materials and Methods

Mice

LTD mice (36) and DM-deficient mice (7) have been described. For radiation chimeras, recipients were irradiated with 1000 rad and reconstituted 10 h later with 5 × 10^6 T cell-depleted (HS7-biotin-SA–magnetic beads; Miltenyi Biotec) bone marrow cells. Recipients were analyzed 5 or 6 wk later. All animals were housed and bred under specific pathogen-free conditions at the Harvard Medical School Center for Animal Research and Comparative Medicine, under protocols reviewed and approved by the Institutional Animal Care and Use Committee (protocol 02954).

Flow cytometry

Cell surface staining was performed with standard techniques. Intracellular staining for FoxP3 was performed with the PE-conjugated mAb (FJK-16s) according to the manufacturer’s protocol (eBioscience). Analysis was performed on Coulter XL (Beckman Coulter), MoFlo (DakoCytomation), or Aria (BD Biosciences) instruments. Flow cytometry data files were analyzed using Expo32 (Beckman Coulter) and FlowJo software (Tree Star). CFSE labeling and transfer were performed as described (39).

Single-cell sorting and RT-PCR

Live thymocytes and lymphocytes were first sorted in bulk as Va2+ Vβ5+ CD4+ CD8– B220 and either CD25+ or CD25–, before re-sorting as individual cells into wells of 96-well PCR plates containing 10 μl of reverse transcription buffer (50 mM Tris-HCl, 75 mM KCl, 2 mM MgCl2), 2% Triton X-100, 500 μM dNTP with 1 μg BSA, 50 ng of oligo(dT) (12–18), and 47 ng of Foxp3-specific primer (CCACTTCTTGGCAAACTCAATTTACTACGG), 8 U of RNaseOUT, and 30 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). The plates were incubated for 90 min at 37°C, then heat inactivated for 10 min at 70°C. Plates were replicated by transferring 5 μl of the cDNA into an empty plate. Nested PCR amplification was performed in the replicates for Foxp3 or Va2 adding 45 μl of Taq buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 mM MgCl2), 500 μM dNTP, containing 2.5 U of Taq polymerase, 400 ng of primers for Va2 (36) or Foxp3 (CT GCATCGTACCCACCGATAGTAAGTGCGGAGTG, first round, 35 cycles; CCAACCAGTGACCCCGGACGATA and AAGTAGGGGAACTGGCAGT, second round, 25 cycles). Contamination was monitored for all steps (sorting, reverse transcriptase, and PCR), by leaving 32 control wells empty per 96-well PCR plate sorted. The contamination rate was calculated to be <0.7% in total. Experiments showing evidence of PCR contamination were discarded.

Va2 sequence analysis

A total of 25 peptides of the Va2 amplification was combined with 3 μl of 10X shrimp alkaline phosphatase reaction buffer (200 mM Tris-HCl (pH 8.0) 100 mM MgCl2, 1 U of shrimp alkaline phosphatase (Amersham Biosciences), and 1 U of exonuclease I (New England Biolabs), and water to total 30 μl. The reaction was then heated to 37°C 30 min, 80°C 10 min, and cooled to 4°C, and the product was subjected to automated sequencing.
Results

To compare the TCR repertoires of the Treg and Tconv CD4+ lineages, we exploited the LTD mouse line, a transgenic combination whose restricted range of TCR variability facilitates the tracking of T cell populations through differentiation and maturation steps (36). These mice combine: 1) a rearranged TCRβ gene, which prevents the use of endogenously encoded TCRβ chains, such that most T cells express the transgene-encoded Vβ5 V region; 2) a TCRα LTD minilocus, which contains a TCRαV2.3 region that, to be expressed, needs to be rearranged to either a Ja2 or Ja26 element; this rearrangement uses the normal VJ imprecise joining mechanisms, and thus introduces the usual range of diversity through base removal and N-nucleotide addition; 3) homozygous knockout alleles of the endogenous TCRα gene, such that assembly of an αβTCR can only occur after a productive rearrangement of the TCRα minilocus transgene. The limited diversity that results from this combination is nevertheless compatible with the selection of populations of CD4+ and CD8+ T cells (36). These mice are lymphopenic with roughly one-tenth the number of CD4+ cells. Staining with mAb reagents directed against FoxP3 and CD25, markers of Treg cells, showed that the LTD mice contained a sizeable proportion of Treg cells (Fig. 1A). FoxP3+ cells accounted for 55.71 ± 3.80% of CD4+ T cells vs 12.03 ± 0.82% in control mice. Thus, the invariant elements of the αβTCR in LTD mice are compatible with the selection of both Tconv and Treg CD4+ T cells, and the only variable element, the CDR3 element of TCRα, must guide the differentiation toward one or the other lineage, indeed with a slightly higher preference for the Treg lineage.

As in our previous studies, TCR repertoire analyses in these mice were performed by analyzing the TCRβ gene for rearrangement. The high variability of the TCRβ repertoire makes it amenable to analysis by single-cell sequencing. Using this single-cell sequencing approach, we first compared the TCR repertoires of Treg and Tconv cell repertoires in the periphery.

Using this single-cell sequencing approach, we first compared the TCRα repertoires of Tconv and Treg cells from the lymph nodes (LN) of LTD mice. From independent runs involving six different animals, we obtained 343 and 322 sequences from LN Treg and Tconv cells, respectively. Although individual mice yielded samples that differed somewhat in TCR sequence composition, there was reproducibility, in that certain high-frequency sequences were found in all animals. These high frequency TCRs were derived from several different nucleotide sequences, even within a single mouse, indicating that they corresponded to preferential selection at the protein level rather than to preferential DNA recombination or to the amplification of a single dominant clonotype, or to

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7 The online version of this article contains supplemental material.
artificial PCR contamination. Both the Treg and the Tconv populations contained diverse TCR repertoires. The same frequency distribution was found in the two cases, which largely followed a power-law distribution, as observed previously in LTD mice and other repertoires (36, 41–43); a few of the sequences were found in numerous instances and many only rarely (Fig. 1B). In both the Treg and Tconv sets, respectively, sequences with greater than seven occurrences accounted for 30.0 and 15.2% of the TCRα repertoire, and unique sequences accounted for 30.9 and 31.4%. The overall diversity of sequences was quite comparable in the two cases, as illustrated by the “species accumulation curve” (Fig. 1C; a graphic estimate of total species diversity represented by plotting the number of unique species observed as a function of the total number of samplings), and by calculating the ACE which approximates the total diversity in a population (both techniques were originally developed to calculate the species diversity in an ecosystem from limited data sampling from a population; Refs. 24 and 44). This estimation revealed Treg and Tconv cells to have a very similar degree of diversity in the periphery (ACE count of 374 ± 32 vs 356 ± 20).

The repertoires of the Treg and Tconv populations had a distinct composition, yet also showed significant overlap (Fig. 1D); of the frequent sequences, 12 showed a >3-fold bias of frequency in one lineage vs the other, but 10 were present in both (<2-fold bias). A Morisita-Horn (MH) index, which accounts for the overall diversity and the abundance of individual TCR sequences when assessing the similarity between two populations (24, 45, 46), was calculated to be 0.420 between LN Treg and Tconv repertoires. This overlap is far greater than that observed when sequences of conventional CD4⁺ and CD8⁺ cells of LTD mice were compared (Ref. 36; MH index of 0.005). As mentioned above, overlap between the Treg and Tconv repertoires has been observed to varying extents in previous analyses (24, 38), however, the overlap detected here would be the greatest reported.

We then attempted to compare the overall characteristics of sequences preferentially found in Treg vs Tconv cells. The distribution of CDR3α lengths was nearly identical in the two populations (mean CDR3α length of 11.3 and 11.5, Treg and Tconv populations, respectively; data not shown). The plot of Fig. 1E positions each sequence according to m.w. and overall charge of the CDR3α region. A slight bias emerged, many of the CDR3α sequences preferentially found in Treg cells being distributed around a higher positive charge than the sequences of Tconv cells (Student’s t test, p = 0.001).

**TCR specificities of CD4⁺CD25⁻FoxP3⁻ Treg cells**

The cytometric analysis of Fig. 1A showed that a large proportion of Treg cells was contained in the CD25⁻ population in LTD mice. Among FoxP3⁺ Treg cells, the CD25⁻ population is usually a minority, but it has been shown to exert the same suppressive functions in vivo as its CD25⁺ counterparts, and its gene-expression profile is typical of the “Treg signature” (18). However, recent data indicate that the CD25⁻ phenotype may correspond to a different mode of selection (M. Feuerer, C. Benoist, and D. Mathis, unpublished observations). We thus asked whether the TCRα repertoire of CD4⁺CD25⁻ cells carries the imprint of a different mode of selection or activation. These sequences were derived from the individual CD4⁺CD25⁻ cells that proved positive for FoxP3 expression in the split-well RT-PCR. Overall, 100 TCR sequences representing the CD4⁺CD25⁻ FoxP3⁺ population were obtained from six mice (Fig. 1F, Table S1). CD25⁻ Treg cells appeared to be more closely related to the CD25⁺ Treg population than to CD25⁻ Tconv cells, because the TCRα sequences found most frequently in the FoxP3⁺CD25⁺ cells were those overrepresented in FoxP3⁺CD25⁻ Tregs (Fig. 1F). Thus, the FoxP3⁺CD25⁻ Treg population does not appear to harbor a distinct subset of TCR sequences, in keeping with the notion that it is a subphenotype of Treg cells.
Selection into the Tconv and Treg lineages in the thymus

We then asked whether the differences/overlap between peripheral Treg and Tconv cells reflected selection and adaptation in the periphery, including a possible conversion due to antigenic exposure, or rather the imprint of positive selection in the thymus. Thymic CD4<sup>+</sup>/CD8<sup>-</sup> single-positive (SP) cells are the earliest differentiation state where Treg and Tconv lineages can be readily distinguished. A total of 76 and 82 TCR sequences were obtained from Treg and Tconv SPs, respectively (from the thymi of two LTD mice) (Fig. 2). These data were also compared with previously obtained sequences from the CD4<sup>+</sup>/CD8<sup>-</sup> double-positive (DP) stage (36), the presumed precursor of both lineages (18). The frequency distribution in the Treg and Tconv SP populations was similar, but was somewhat different from that observed in the periphery, with a higher prevalence of two very frequent sequences that dominated both the Treg and Tconv repertoires (Fig. 3A, top panel). Here again, there was consistency in the presence of the same high frequency TCR sequences in different animals, indicating that we were examining the same general T cell selection process, and not TCR<alpha> repertoires particular to each mouse. The frequency distribution of SPs was also quite distinct from that of preselection DPs, which was composed largely of nonrepetitive sequences (36) (Fig. 3A, bottom panel), suggesting that the uneven distribution of TCRs reflects the adaptation to self-MHC structures during positive and negative selection. As in the periphery, Treg and Tconv TCR repertoires showed fairly similar overall diversity (ACE of 239 and 454, respectively). The sequences that dominated the thymic pool were also found in the peripheral populations (Fig. 3B) but, as expected from the frequency distribution, with substantial amplification in the range of repeated sequences and a reduction in the abundance of the two sequences (nos. 24 and 25) that dominated thymic CD4<sup>+</sup> SPs.

TCR sequences in animals with reduced peptide diversity

The TCR repertoire in LTD mice is selected through interaction of the TCRs with MHC class II A<beta> molecules carrying a broad panel of self-peptides, and it is likely that the different peptides within this panel influence the selection of different sequences. We asked

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**FIGURE 4.** Comparison of TCR sequences from WT and DM-deficient LTD mice. A, Flow cytometric analysis (left) of TCR<sup>high</sup> thymocytes from WT or DM-deficient LTD mice, and the distribution of CDR3<alpha> lengths in Ja26-bearing TCR<alpha>s, from sorted CD4<sup>-</sup>SP cells (right). B, Frequency of individual TCR<alpha> sequences in thymic CD4<sup>-</sup>SP in DM<sup>+/+</sup> and DM<sup>o/o</sup> LTD mice (CDR3<alpha> sequences and percent- ages tabulated below the histogram). C, Flow cytometric analysis of thymi from bone marrow chimeras (all LTD donors and nontransgenic hosts, DM genotypes of donor and host indicated on left) and the distribution of CDR3<alpha> lengths in Ja26-bearing TCR<alpha> in each combination.
how a reduction in the peptide repertoire presented by Aβ molecules would affect the CD4+ T cell repertoire of LTD mice, in particular the Tconv/Treg distinction. For this purpose, we generated animals that carried the components of the LTD mouse on a background deficient for DM. DM catalyzes peptide exchange on MHC class II molecules in endocytic-loading compartments, so that unedited Aβ molecules in DM-deficient animals present quasiexclusively the “CLIP” peptide derived from the class II-associated invariant chain (reviewed in Ref. 47). DM-deficient mice have large compartments of CD4+ cells, only slightly reduced from those of WT animals (7–9, 48); DM-deficient LTD mice also showed sizeable numbers of CD4+ cells in the thymus (Fig. 4A) and LNs (data not shown).

In a first set of sequencing experiments, single CD4+CD8− thymocytes were sorted and their TCRα transcripts sequenced as above; 135 TCRα sequences were obtained from three LTD.DMo/o animals, and were analyzed and compared with those of the corresponding population from LTD.DM+/- animals. The DM deficiency had a striking impact on the CD4+ T cell repertoire: CDR3α loops were distinctly shorter than in the DM-positive animals (Fig. 4A). Most of the Jα26-containing CDR3α sequences from DMo/o animals were 8 and 9 aa long, compared with lengths of 9–11 in WT mice; a parallel shortening was observed for sequences using Jα2 (Fig. 4A). A similar picture emerged when sequences from LN CD4+ T cells were analyzed (data not shown).

In addition, the repertoires of LTD.DM+/- and WT animals were almost entirely distinct, with essentially no overlap between the CDR3α found in DM+/- or DMo/o mice (only sequence no. 22 was shared; Fig. 4B); even those sequences from LTD.DM0/o mice that had usual lengths carried sequences never seen in any repertoire of DM+/- mice, in this or previous studies, in Treg or in Tconv cells. Interestingly, the sequences observed by Pacholczyk et al. (38) in single-peptide mice also showed a shortening of the CDR3α length. Overall, despite the reduction in the diversity of peptide available for selection, the TCR sequences remained diverse within both Treg and Tconv cells, with ACE scores of 200 to 450, only marginally reduced compared with the scores of 500 observed in LTD mice with full peptide diversity.

In theory, this very peculiar repertoire could reflect either positive selection on the Aβ/CLIP molecule in LTD.DM0/o mice, or negative selection by diverse Flβ/peptide complexes in normal LTD mice. To test these possibilities, we constructed reciprocal bone marrow chimeras using donors or hosts of the DM+/- or DMo/o genotypes (all donors were LTD, all hosts were TCRα deficient, ensuring that all recovered T cells were of donor origin). As illustrated by the flow cytometry profiles of Fig. 4C (left), the LTD system recapitulated the findings on bone marrow chimeras using DM-deficient animals (10, 11): the multiplicity of peptides presented in a WT host resulted in more efficient positive selection, and DM+/- bone marrow-derived APCs efficiently purged many cells from the repertoire, particularly when combined with positive selection on DM0/o epithelium. The CDR3α sequences found in these cells indicated that short loops were incompatible with a diverse peptide repertoire presented by Aβ in either the radiosensitive or the bone marrow-derived compartments; the only combination where the short loops were detected was the DM0/o → DM0/2 chimera (Fig. 4C, right; data not shown). These findings suggest that the peculiar TCRα sequences of LTD.DM0/o mice are part of TCRs that are negatively selected by DM-dependent peptide cargo or conformations of Aβ, and that these can be presented by either bone marrow-derived or epithelial cells.

To test whether the CD4+ T cells selected on DM0/o cells were indeed reactive against the peptides presented by Aβ on DM+/- cells, we transferred CFSE-labeled LTD.DM0/o LN cells into DM+/- or DM0/o hosts. Sixty hours later, most of the donor cells had divided in the WT but not in the DM-deficient hosts (Fig. 5, top). These cells were sorted, and their TCRα transcripts were sequenced. Most of the T cells that had divided carried the short CDR3α loops typical of LTD.DM0/o mice, while the few nondivided cells carried TCRs with longer sequences. The actual sequences of the CDR3α elements confirmed this conclusion, as cells that had divided contained the amino acid sequences unique to LTD.DM0/o mice (Fig. 5, bottom). These results were confirmed in vitro studies, where CD4+ T cells from LTD.DM0/o LN cells also reacted strongly to WT Aβ APCs (data not shown; incidentally, we observed little cross-reactivity to APCs displaying allo-MHC targets, indicating that the increased MLR cross-reactivity observed by Huseby et al. (49) is not a general feature of single-peptide mice, but might have resulted from the strong perturbations in MHC class II levels in the particular mice analyzed). These findings suggest that the short CDR3α loops that were dominant in LTD.DM0/o animals are highly reactive against Aβ molecules presenting the natural peptide set and are thus negatively selected in DM-positive mice.

**TCR sequences in Treg and Tconv cells of DM-deficient animals**

We then asked whether the reduction in the diversity of self-peptides similarly impacted Tconv and Treg repertoires. Flow cytometry after intracellular staining for FoxP3 showed that CD4+ LN cells of LTD.DM0/o mice contained a robust population of Treg cells, albeit in slightly lower proportions than in LTD mice, and...
FIGURE 6. Treg and Tconv cells from LNs of WT and DM-deficient LTD mice. A, Flow cytometric analysis of LN cells from LTD mice, DM<sup>0/0</sup> or DM<sup>+/+</sup>. B, Distribution of CDR3α lengths for TCRs containing Jα26. C, Frequencies of individual TCR sequences in Treg and Tconv cells of DM<sup>0/0</sup> or DM<sup>+/+</sup> mice (CDR3α sequences and percentages tabulated below the histogram). D, MH comparisons of populations. E, CDR3α mass/charge plot of TCRα sequences in Treg and Tconv cells of DM<sup>0/0</sup> mice.
with the more habitual balance of CD25 expression (Fig. 6A). TCRα sequences were determined from single FoxP3+/CD25+ or FoxP3+/CD25− cells from LTD.DMα/α mice (205 and 131 cells, respectively, from three LTD.DMα/α mice). The shortening of CDR3α sequences occurred similarly in Tconv and Treg cells (shown in Fig. 6B for Vα26-bearing sequences), and the clear distinction between the repertoires of DM+/+ and DMα/α mice applied to Treg as well as Tconv cells (Fig. 6C). Within LTD. DMα/α mice, there was again a significant overlap between the repertoires of Treg and Tconv cells, with a large number of shared sequences. Although the degree of overlap was somewhat reduced relative to LTD mice (Fig. 6D), with an MH index of 0.335 and a smaller number of “undecided” sequences equally represented within the Treg and Tconv TCR repertoires. The overrepresentation of CDR3α motifs with an overall positive charge was not observed in Treg cells from LTD.DMα/α mice (Fig. 6E).

Thus, the repertoire adaptation of CD4+T cells to the unique peptide conditions in LTD.DMα/α mice occurs in similar fashion for Treg and Tconv cells. The TCR repertoires of Treg cells from the single-peptide mice were far more related to those of Tconv cells from the same type of animals than they were to Treg cells from DM+/+ mice.

Discussion

To analyze the selection and dynamics of CD4+ Treg in relation to Tconv cells, we have analyzed mice with restricted repertoires of TCR elements or MHC-bound peptides. The Treg TCR repertoire was comparable to that of Tconv cells in terms of its breadth and diversity, as concerns both the thymic and peripheral cells. The Treg and Tconv repertoires were distinct but did show a significant degree of overlap, some sequences being equally frequent in both cell types. When the diversity of selecting MHC:peptide complexes was reduced to essentially Aβ/CLIP, these repertoires remained diverse, but showed a complete structural adaptation, in a parallel manner in both lineages, and largely in response to changes in negative selection imposed by self-recognition.

Distinct yet overlapping

The TCR repertoires of Treg and Tconv CD4+ T cells in the LTD mice were clearly distinct, yet also shared a number of sequences. It is generally thought that TCR specificities with marked affinity for self develop preferentially into Treg cells, whether through induced differentiation (25, 26, 28) or through resistance to clonal deletion (30, 31). This overlap, then, is puzzling. It was found both in the context of normal MHC:peptide diversity and when CLIP was essentially the sole peptide presented by Aβ molecules. Not all TCRs found in Treg cells showed the same degree of overlap, as some TCRs were unique to Tregs, while others were equally represented in Treg and Tconv cells. The overlap was also present, if slightly reduced, in single-peptide LTD.DMα/α mice. Some overlap between regulatory and nonregulatory cell repertoires was also observed recently in other experimental contexts of limited TCR diversity (24, 37, 38). Similarly, repertoire overlap was observed when the selection of Treg cells was enhanced by crossing RAG-deficient TCR-transgenic mice against lines expressing their cognate ligand; the conversion to FoxP3+ Tregs was only partially effective in such mice, and the same TCRs coexisted in Treg and Tconv cells (25, 26, 30). It is not clear why different contexts of restricted TCR diversity lead to a different degree of Treg/Tconv overlap, which was greater in this study (MH = 0.42) than in the data of Pacholczyk (38) or Hsieh et al. (37) (MH = 0.16, 0.20, respectively); one might speculate that the high degree of overlap observed in LTD mice is linked to their inefficient rate of positive selection, as these mice do have lymphopenic characteristics (36).

One might also imagine that it is affected by the superantigen which triggers T cells bearing Vβ5 TCRs, albeit in mice older than used here (40). In contrast, both Treg and Tconv repertoires should be affected in parallel and the overlap was already clear in thymic sequences. The existence of such an overlap has clear implications for how one envisages the development of the Treg lineage. It is imparted from the early stages of positive selection, because it is clearly apparent when one compares thymic repertoires, which would suggest that there might be a probabilistic element to Treg lineage commitment in the thymus. The shared TCR sequences may reside on the cusp of some theoretical threshold value between Treg and Tconv differentiation, and TCR/MHC:peptide affinities/avidities that might condition Treg and Tconv cell commitment must also overlap significantly.

We do not formally know whether the TCRs with sequences shared between Treg and Tconv cells of LTD mice are actually self-reactive. This was the case for the overlapping sequences in the study of Hsieh et al. (24), where some of the overlap TCRs did show self-reactivity, at least according to these authors’ criterion of preferential expansion in lymphopenic hosts. If the same holds true for the shared sequences of LTD mice, then many of their T cells must be self-reactive. Yet, these mice do not present with autoimmune manifestations, so this potential autoreactivity must somehow be kept in check.

The distinct distribution of TCR CDR3α motifs in Treg cells of LTD mice resulted in a structural distinction that had not been previously observed: CDR3α loops with overall positive charges were distinctly more frequent in the TCR repertoire of Treg cells than of Tconv cells. This observation was true for both thymic and peripheral CD4+ T cells. It is interesting that the Treg bias for a positively charged CDR3α is actually reversed in LTD.DMα/α mice, where they are more negatively charged overall. These observations suggest that this relative preference requires the normal complement of self-peptides, and that interaction with negatively charged peptides normally favors Treg selection. Such a preference would not be the case with the CLIP peptide, which has positively charged residues.

Repertoire reshaping

The Treg and Tconv repertoires undergo evident changes between the thymus and the peripheral LNs: all frequent TCRs found in the thymus were also represented in the periphery, supporting the generally accepted notion that peripheral Treg cells are of thymic origin, but their dominance is reduced, and the repertoire flattens somewhat. For example, two sequences that together represented 30.3 and 59.7% of thymic Treg and Tconv cells, respectively, were found only in 7.6 and 5.6% of peripheral cells. Comfortingly, the same trends were observed in our previous analyses of bulk CD4+ cells in LTD mice (36). Comparable flattening was observed in the Treg and Tconv populations, suggesting that both cell types are under the same pressures to diversify their repertoires. Such reshaping was also observed by Hsieh et al. (37) and Pacholczyk et al. (38) in other limited-repertoire systems (albeit to a lesser extent for the latter). In contrast, this evolution of the Treg and Tconv CD4+ repertoires contrasts with the behavior of CD8+T cell populations in the same LTD mice: CD8+ peripheral T cells had a repertoire distinctly narrower than that of their thymic precursors, ending with a gross overrepresentation of two TCRs (including, probably not coincidentally, the OT-1 TCR from which the minilocus derived). We have proposed that preferential expansion or retraction in the peripheral repertoire may be linked to influences from environmental Ags (36). These opposite evolutions of repertoires in CD4+ and CD8+ cells may be related to the propensity of CD8+T cells to mount more expansive responses than those...
ever achieved by CD4+ cells (50). Whatever their origin, it is quite interesting that Treg cell repertoires track closely with repertoires of Tconv cells, with the same proportional reduction of the dominant shared sequences in the two cases.

**Adaptation of the repertoires in single-peptide mice**

The TCR sequences from LTD mice on DM-positive and -deficient backgrounds allowed an interesting correlation to be made between the selecting peptide complexity and TCR diversity. One might have imagined that a reduction in the diversity of selecting peptides in the thymus would restrict positive selection to a smaller variety of TCR specificities. This, however, was not the case, and the diversity of both the Treg and Tconv repertoires was nearly as high in LTD.DM<sup>+/+</sup> mice as in their LTD counterparts. This comparable TCR diversity irrespective of the MHC:peptide repertoire was also observed by Pacholczyk et al. (38) in their parallel version of LTD mice. One interpretation is that the reduction in MHC:peptide diversity similarly affects both positive and negative selection, and that the window of allowable TCRs remains of similar width; that Treg and Tconv repertoires both remain broad in LTD_DM<sup>+/+</sup> mice is consistent with this view. The implication, though, is that the diversity of self MHC:peptide is clearly not rate limiting for repertoire selection.

An alternative explanation might be that the TCR repertoire is molded by interactions with the invariant components of the Aβ molecule, and that the peptide makes a relatively minor contribution to the energetics of the overall interface. This is unlikely to be the case, however, because the alteration in peptide range had a profound impact on the nature of the TCRs selected. LTD TCRs selected in the DM<sup>+/+</sup> background comprised a completely different set of sequences, with distinctly shorter CDR3<sub>α</sub> lengths. The cause for this shortening is unclear, but it is interesting that the sequences of Pacholczyk et al. (38) in cells of single-peptide mice showed a similar CDR3<sub>α</sub> shortening. The connection between low peptide diversity and CDR3<sub>α</sub> loop length is not necessarily a constant, however, because TCR sequences from mice expressing the TCRs minilocus with normally variable TCR-V<sub>α</sub>/V<sub>β</sub> chains of endogenous origin did not show such an effect (our unpublished data). In this vein, a previous analysis of CDR3<sub>α</sub> sequences in TCRγ-transgenic animals had demonstrated an imprint of the reduced peptide diversity on the T cell repertoire in DM<sup>+/+</sup> mice, but of a different nature, with a restricted loop length rather than an actual shortening (48). Adaptation to the structural constraints imposed by the single peptide is almost certainly at the root of the repertoire adaptation, and these structural constraints must impact similarly on the selection events that lead to Treg and Tconv differentiation.

Why, however, are TCRs with such short loops absent from the repertoire of LTD.DM<sup>+/+</sup> animals? Cells with short loops were strongly reactive against peptides or Aβ conformations found on DM<sup>+/+</sup> cells, and are thus likely to be strongly selected against in the DM<sup>+/+</sup> mice. The bone marrow chimera experiments showed that this negative selection occurred whether DM was expressed on the radio-resistant thymic epithelium or the hemopoietic-depletion lineage may explain why only some of the TCR/Ag double-transgenic combinations lead to robust Treg compartments (26, 28–30, 56). Similarly, Hsieh et al. (37) analyzed the autoreactive TCRs found in the activated T cells of Foxp3-deficient scurfy mice. Several of these did match those of Treg cells in normal mice, but this concordance was only partial, and some autoreactive TCRs were not found in Treg cells of Foxp3-proficient mice.

In conclusion, the glimpse offered by the restricted repertoire of the LMT mouse shows the Treg repertoire to be broad, showing distinct composition and characteristics, yet significantly overlapping and sharing structural constraints with the repertoire of conventional CD4+ T cells.

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