Adaptation of TCR Repertoires to Self-Peptides in Regulatory and Nonregulatory CD4$^+$ T Cells[1]

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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-DM$^{o/o}$ 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.**

The establishment of an effective yet self-tolerant repertoire of T cells depends on an interplay between TCRs and MHC molecules carrying an array of peptides derived from self-proteins. The diversity of MHC/peptide complexes conditions positive and negative selection of immature T cells in the thymus and molds the repertoire of TCR V regions displayed on mature T cells found in the periphery (1). Such selection and molding has been best demonstrated for CD4$^+$ T cells in transgenic and mutant mice wherein the diversity of peptides carried by MHC class II molecules was reduced to a single peptide by several means: covalently coupling a peptide to the MHC molecules themselves (2–6), or harnessing the obligate role of the H2-DM molecule in peptide editing, such that the class II molecules in H2-DM-deficient mice are complexed almost exclusively with the CLIP peptide derived from their "invariant chain" chaperone (7–9). In all of these cases, the thymic output of CD4$^+$ T cells was perturbed, although the repertoire remained surprisingly diverse, and the T cells contained clones responsive to wild-type (WT)[6] APCs bearing the normal range of self-peptides, against which T cells are tolerized in normal mice (10–12).

Some potentially autoreactive T cells do escape negative selection in the thymus, and are controlled by peripheral mechanisms of tolerance induction. Among these mechanisms is active control by a subset of CD4$^+$ T cells, commonly termed regulatory T (Treg) cells, that dampen immune and inflammatory responses (13). The hallmark of this lineage is expression of the forkhead/winged helix transcription factor *Foxp3* (14–17); Treg cells also have a characteristic gene expression signature (18–20). In mice, *Foxp3* is expressed by CD4$^+$CD25$^+$ T cells that have regulatory activity upon transfer; deficiencies in *Foxp3* are the cause of the lymphoproliferation and multiorgan autoimmunity in *scurfy* mutant mice and human immunodysregulation, polyendocrinopathy, enteropathy, X-linked patients (21). The expression of *Foxp3* has been shown in several studies to be both necessary and sufficient for Treg cell function.

The Treg lineage originates in the thymus, seemingly derived from CD4$^+$CD8$^+$ thymocytes, as are conventional CD4$^+$ T cells, but perhaps also from earlier precursors (18, 22, 23). It is unclear, however, how immature thymocytes commit to this alternative differentiation pathway. The repertoire of Treg cells is known to be enriched in autoreactive T cells (24–29). In some experimental systems, Treg cell differentiation appeared to be induced when the TCRs on immature thymocytes were engaged by agonist ligands

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[6] Abbreviations used in this paper: WT, wild type; Treg, regulatory T; Tconv, conventional lineage T; LTD, Limited; LN, lymph node; ACE, abundance-based coverage estimator; MH, Morisita-Horn; SP, single positive; DP, double positive.

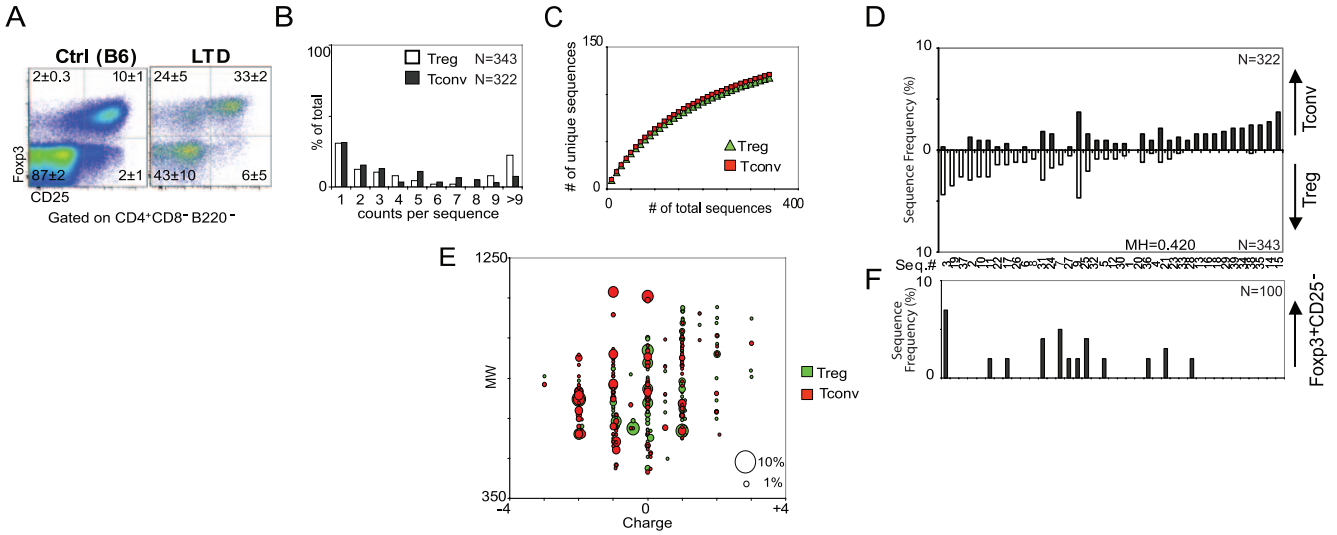


FIGURE 1. Comparison of peripheral Treg and Tconv cells. *A*, Flow cytometric analysis of LN cells from LTD mice. Numbers represent average frequencies of populations over three independent experiments. *B*, Frequency distribution of TCR sequences, based on the number of occurrences in Treg or Tconv population ($n = 343$ and 322, respectively). *C*, "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 their relative frequencies in either population, and the numbers correspond to the amino acid sequences listed in Fig. 2. *E*, Mass/charge plot of CDR3α motifs. Each circle represents an individual CDR3α motif, with an area proportional to its frequency in Treg (green) or Tconv (red), positioned in the plane according to its overall charge and m.w. (MW); white circles: area scale for 1 and 10% frequency. *F*, Frequency of individual TCR sequences in the FoxP3$^+$CD25$^-$ population, alignment as in *D*.

(25–28); in others, enrichment of Treg cells seemed rather to reflect a heightened resistance of FoxP3$^+$ cells vs conventional cells to clonal deletion (29–31). Some investigators have suggested that the selection of Treg cells might be favored in particular stromal 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-DMa* (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 repertoire, and its profound adaptation to altered negative selection in single-peptide mice provide an interesting counterpoint 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 (H57-bio/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 Vα2$^+$Vβ5$^+$CD4$^+$CD8α^-B220$^-$ and either CD25$^+$ or CD25$^-$, before resorting as individual cells into wells of 96-well PCR plates containing 10 μl of reverse transcriptase buffer (50 mM Tris-HCl, 75 mM KCl, and 3 mM $MgCl_2$), 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 (CCTCTTCTTGCG AAACTCAAATTCATCTACG), 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 Vα2 adding 45 μl of *Taq* buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 mM $MgCl_2$), 500 μM dNTP, containing 2.5 U of *Taq* polymerase, 400 ng of primers for Vα2 (36) or *Foxp3* (CT GCATCGTAGCCACCAGTA and AAGTAGGCGAACATGCGAGT, first round, 35 cycles; CCACCAGTACTCAGGGCAGT and TGAGTGT CCTCTGCCTCTCC, 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.

Vα2 sequence analysis

A total of 25 μl of the Vα2 amplification was combined with 3 μl of 10× shrimp alkaline phosphatase reaction buffer (200 mM Tris-HCl (pH 8.0) and 100 mM $MgCl_2$), 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

(Dana-Farber/Harvard Cancer Center High-Throughput Sequencing Core). Raw sequencing files were filtered for sequence quality and processed in automated fashion by custom-designed PERL scripts to translate the DNA sequence into amino acids, recognize the Jα segment used and the CDR3α boundaries, and determine CDR3α length and composition. The sequence information for each cell were then tabulated in a Microsoft Access database, together with the cell's origin, surface phenotype, and *Foxp3* RT-PCR result. Statistical and population frequency analyses were performed with the EstimateS software (http://viceroy.eeb.uconn.edu/estimates), with 500 iterations for abundance-based coverage estimator (ACE) bootstrap calculations.

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 *TCR*β gene, which prevents the use of endogenously encoded TCRβ chains, such that most T cells express the transgene-encoded Vβ5 V region; 2) a *TCR*α LTD minilocus, which contains a TCRVα2.3 region that, to be expressed, needs to be rearranged to either a Jα2 or Jα26 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 *TCR*α gene, such that assembly of an $\alpha\beta$TCR can only occur after a productive rearrangement of the *TCR*α 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. 1*A*). FoxP3$^+$ cells accounted for 55.71 $\pm$ 3.80% of CD4$^+$ T cells vs 12.03 $\pm$ 0.82% in control mice. Thus, the invariant elements of the $\alpha\beta$TCR 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 repertoires in these mice were analyzed by sorting of Vβ5$^+$Vα2$^+$ cells of different origins and phenotypes into microtiter plates, PCR amplification of the joining region and sequencing. Because intracellular staining is incompatible with single-cell RT-PCR, Treg cells were identified by sorting CD4$^+$CD25$^+$ (most of which were Treg) and CD4$^+$CD25$^-$ cells (mainly Tconv), and splitting each cell's cDNA to allow independent amplification of TCRα and FoxP3 transcripts. Treg cells were defined as CD4$^+$CD25$^+$ cells that also scored positive in the *Foxp3* RT-PCR (which was the case for 84.5% of CD4$^+$CD25$^+$ cells from LTD mice, pointing to the efficacy of the FoxP3 PCR); Tconv cells were those CD4$^+$CD25$^-$ cells that were also negative for *Foxp3*. Strong precautions were taken to guard against FoxP3 false-positives, by sorting no cell into one-third of the plate, all of which were then amplified; a plate with any of these wells positive was then automatically discarded (false-positives should thus be 3–5% or less). Routinely, young mice were analyzed to avoid interference from the endogenous retroviral superantigen that affects cells using the Vβ5 region in mice of 10 wk or more (40). The sequences were entered into a database for easier sorting and comparison and the compiled data are available as Table S1.[7]

[7] The online version of this article contains supplemental material.

Seq. #	Ja	CDR3	AA Seq	Thymus Treg	Thymus Tconv	LN Treg	LN Tconv
1	2	11	CAAKRGLSGKLTF	0	0	0.6	0.6
2	2	11	CAAMWGLSGKLTF	0	0	2.6	0
3	2	11	CAARGGLSGKLTF	2.6	0	3.8	1.6
4	2	11	CAHTGGLSGKLTF	0	0	0.3	0.9
5	2	12	CAAPRGGLSGKLTF	2.6	0	0.9	0.9
6	2	12	CAARPGGLSGKLTF	0	0	1.2	0
7	2	12	CAARTGGLSGKLTF	0	0	1.7	1.6
8	2	14	CAAKGRTGGLSGKLTF	0	0	1.2	0.3
9	2	14	CAAPFNTGGLSGKLTF	0	0	0.6	0.3
10	2	14	CAAPLNTGGLSGKLTF	0	0	2.9	1.2
11	2	14	CAAPPNTGGLSGKLTF	0	0	2.6	0.9
12	2	14	CAARGNTGGLSGKLTF	0	0	0.9	0.9
13	2	15	CAASLMNTGGLSGKLTF	0	0	0	1.6
14	2	16	CAASEGWNTGGLSGKLTF	0	0	0	2.8
15	2	16	CAASIGWNTGGLSGKLTF	0	0	0	3.7
16	26	9	CAADDNYQLIW	0	0	0	1.6
17	26	9	CAAFDNYQLIW	6.6	1.2	1.5	0.3
18	26	9	CAAGDNYQLIW	0	1.2	0	1.6
19	26	9	CAAHDNYQLIW	6.6	1.2	4.4	0.3
20	26	9	CAARDNYQLIW	1.3	2.4	0	0
21	26	9	CAASDNYQLIW	0	4.9	1.2	2.2
22	26	9	CAAYDNYQLIW	2.6	1.2	2.6	0.9
23	26	10	CAAFDDNYQLIW	0	0	0.9	0.9
24	26	10	CAALDDNYQLIW	17.1	32.9	2.9	1.9
25	26	10	CAAMDDNYQLIW	13.2	26.8	4.7	3.7
26	26	10	CAARIGNYQLIW	0	0	1.5	0.6
27	26	10	CAARKDNYQLIW	2.6	0	1.5	0
28	26	10	CAARPDNYQLIW	1.3	1.2	0	0.9
29	26	10	CAASDDNYQLIW	0	0	0	1.9
30	26	10	CAASRGNYQLIW	0	1.2	0.9	0.6
31	26	10	CATMDDNYQLIW	0	0	0.9	0
32	26	10	CAVMDDNYQLIW	2.6	0	2.0	1.6
33	26	11	CAAERDDNYQLIW	0	2.4	0.3	1.2
34	26	11	CAAIPSDNYQLIW	0	0	0	2.2
35	26	11	CAASGDDNYQLIW	0	0	0	2.5
36	26	11	CAASKGDNYQLIW	1.3	1.2	1.2	1.6
37	26	11	CAASRWDNYQLIW	0	0	3.5	0
38	26	11	CAASTNDNYQLIW	0	0	0	2.2
39	26	12	CAASRGDDNYQLIW	0	0	0.3	2.5
Total				76	82	343	322

FIGURE 2. CDR3α sequence frequencies in LTD mice. Amino acid sequences at the TCRα junctional positions, truncated to show the CDR3α stretch only (CDR3α defined as the amino acids that are bordered by the invariant C residue in *TCRV*α genes and the F/W-G-X-G Jα motif). Amino acid variations from the parental OT-1 TCR sequence are highlighted. The frequencies (in percentages) are given for thymus and LN, Treg, and Tconv cells. The total numbers of TCRα sequences sampled are given at the bottom.

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

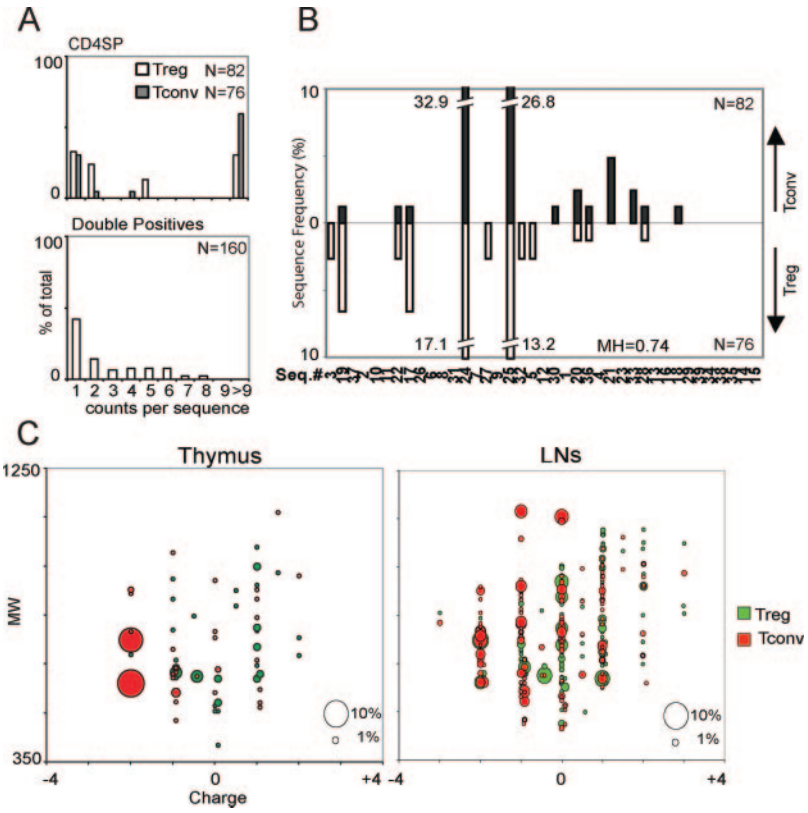


FIGURE 3. TCR sequences from the thymi of LTD mice. *A*, Frequency distribution of TCRα sequences in CD4SP (*upper*) and DP (*lower*) populations. *B*, Frequency of individual TCRα sequences in thymic CD4SP ($CD25^+FoxP3^+$ and $CD25^-FoxP3^-$, Treg and Tconv, respectively). *C*, CDR3α mass/charge plot for thymic CD4SP Treg and Tconv, coded as for Fig. 1 (LN data from Fig. 1 reproduced for comparison).

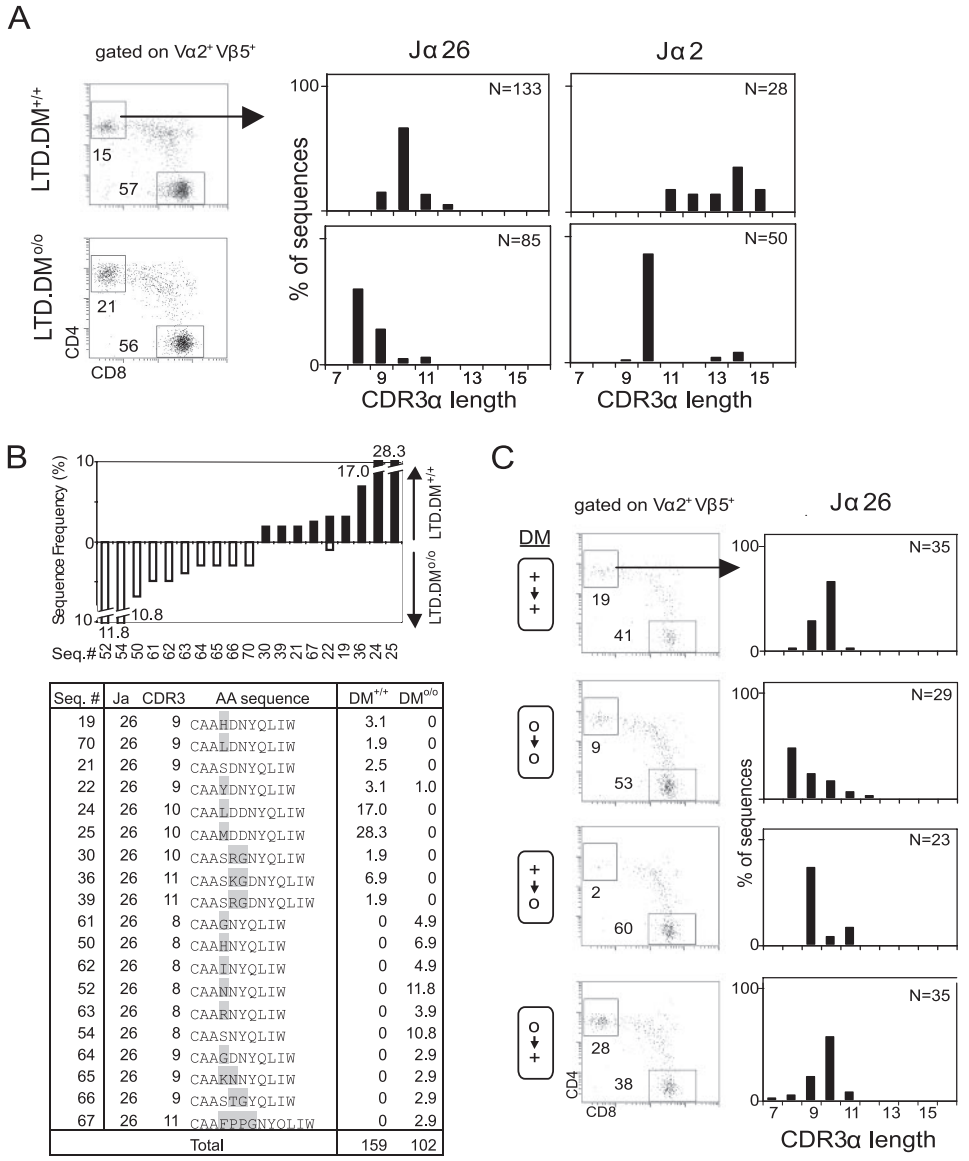
artifactual PCR contamination. Both the Treg and the Tconv populations contained diverse TCR repertoires. The same frequency distribution was found in the two cases, which largely followed a power-law distribution, as observed previously in LTD mice and other repertoires (36, 41–43); a few of the sequences were found in numerous instances and many only rarely (Fig. 1*B*). 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. 1*C*; 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 $\pm$ 32 vs 356 $\pm$ 20).

The repertoires of the Treg and Tconv populations had a distinct composition, yet also showed significant overlap (Fig. 1*D*): 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. 1*E* 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. 1*A* 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. 1*F*, 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. 1*F*). 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.



Seq. #	Ja	CDR3	AA sequence	DM+/+	DMo/o
19	26	9	CAAHDNYQLIW	3.1	0
70	26	9	CAALDNYQLIW	1.9	0
21	26	9	CAASDNYQLIW	2.5	0
22	26	9	CAAYDNYQLIW	3.1	1.0
24	26	10	CAALDDNYQLIW	17.0	0
25	26	10	CAAMDDNYQLIW	28.3	0
30	26	10	CAASRGNYQLIW	1.9	0
36	26	11	CAASKGDNYQLIW	6.9	0
39	26	11	CAASRGDNYQLIW	1.9	0
61	26	8	CAAGNYQLIW	0	4.9
50	26	8	CAAHNYQLIW	0	6.9
62	26	8	CAAINYQLIW	0	4.9
52	26	8	CAANNYQLIW	0	11.8
63	26	8	CAARNYQLIW	0	3.9
54	26	8	CAASNYQLIW	0	10.8
64	26	9	CAAGDNYQLIW	0	2.9
65	26	9	CAAKNNYQLIW	0	2.9
66	26	9	CAASTGYQLIW	0	2.9
67	26	11	CAAFPPGNYQLIW	0	2.9
Total				159	102

FIGURE 4. Comparison of TCR sequences from WT and DM-deficient LTD mice. *A*, Flow cytometric analysis (*left*) of TCR^{high} thymocytes from WT or DM-deficient LTD mice, and the distribution of CDR3α lengths in Jα26-bearing TCRαs, from sorted CD4SP cells (*right*). *B*, Frequency of individual TCRα sequences in thymic CD4SP in DM$^{+/+}$ and DM$^{o/o}$ LTD mice (CDR3α sequences and percentages 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 CDR3α lengths in Jα26-bearing TCRα in each combination.

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$^+$CD8$^-$ 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$^+$CD8$^+$ 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. 3*A*, *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 TCRα 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. 3*A*, *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. 3*B*) 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 SPs.

The plots displayed in Fig. 3*C* emphasize the difference in distribution brought about in positive selection and colonization of the peripheral LNs. In addition, the charge/m.w. distribution of Treg sequences reproduced that seen in peripheral cells, with a broader range and an enrichment in positively charged CDR3α motifs in Treg cells.

TCR sequences in animals with reduced peptide diversity

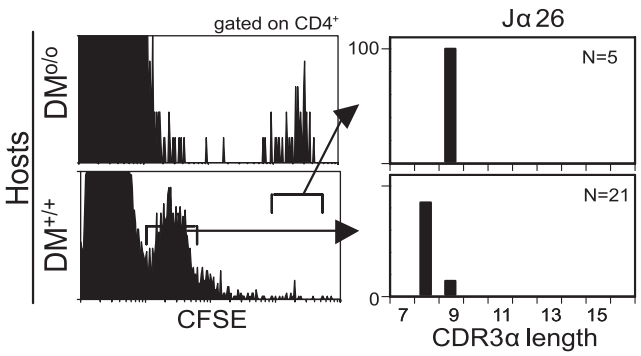
The TCR repertoire in LTD mice is selected through interaction of the TCRs with MHC class II A^b molecules carrying a broad panel of self-peptides, and it is likely that the different peptides within this panel influence the selection of different sequences. We asked

how a reduction in the peptide repertoire presented by A^b 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 A^b 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. 4*A*) 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.$DM^{o/o}$ 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. 4*A*). Most of the Jα26-containing CDR3α sequences from $DM^{o/o}$ 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. 4*A*). A similar picture emerged when sequences from LN $CD4^+$ T cells were analyzed (data not shown). In addition, the repertoires of LTD.$DM^{o/o}$ and WT animals were almost entirely distinct, with essentially no overlap between the CDR3α found in $DM^{o/o}$ or $DM^{+/+}$ mice (only sequence no. 22 was shared; Fig. 4*B*); even those sequences from LTD.$DM^{o/o}$ 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 A^b/CLIP molecule in LTD.$DM^{o/o}$ mice, or negative selection by diverse Fl^b/peptide complexes in normal LTD mice. To test these possibilities, we constructed reciprocal bone marrow chimeras using donors or hosts of the $DM^{o/o}$ or $DM^{o/+}$ 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. 4*C* (*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 $DM^{o/o}$ epithelium. The CDR3α sequences found in these cells indicated that short loops were incompatible with a diverse peptide repertoire presented by A^b in either the radioresistant or the bone marrow-derived compartments; the only combination where the short loops were detected was the $DM^{o/o}\rightarrow DM^{o/o}$ chimera (Fig. 4*C*, *right*; data not shown). These findings suggest that the peculiar TCRα sequences of LTD.$DM^{o/o}$ mice are part of TCRs that are negatively selected by DM-dependent peptide cargo or conformations of A^b, and that these can be presented by either bone marrow-derived or epithelial cells.

To test whether the $CD4^+$ T cells selected on $DM^{o/o}$ cells were indeed reactive against the peptides presented by A^b on $DM^{+/+}$ cells, we transferred CFSE-labeled LTD.$DM^{o/o}$ LN cells into



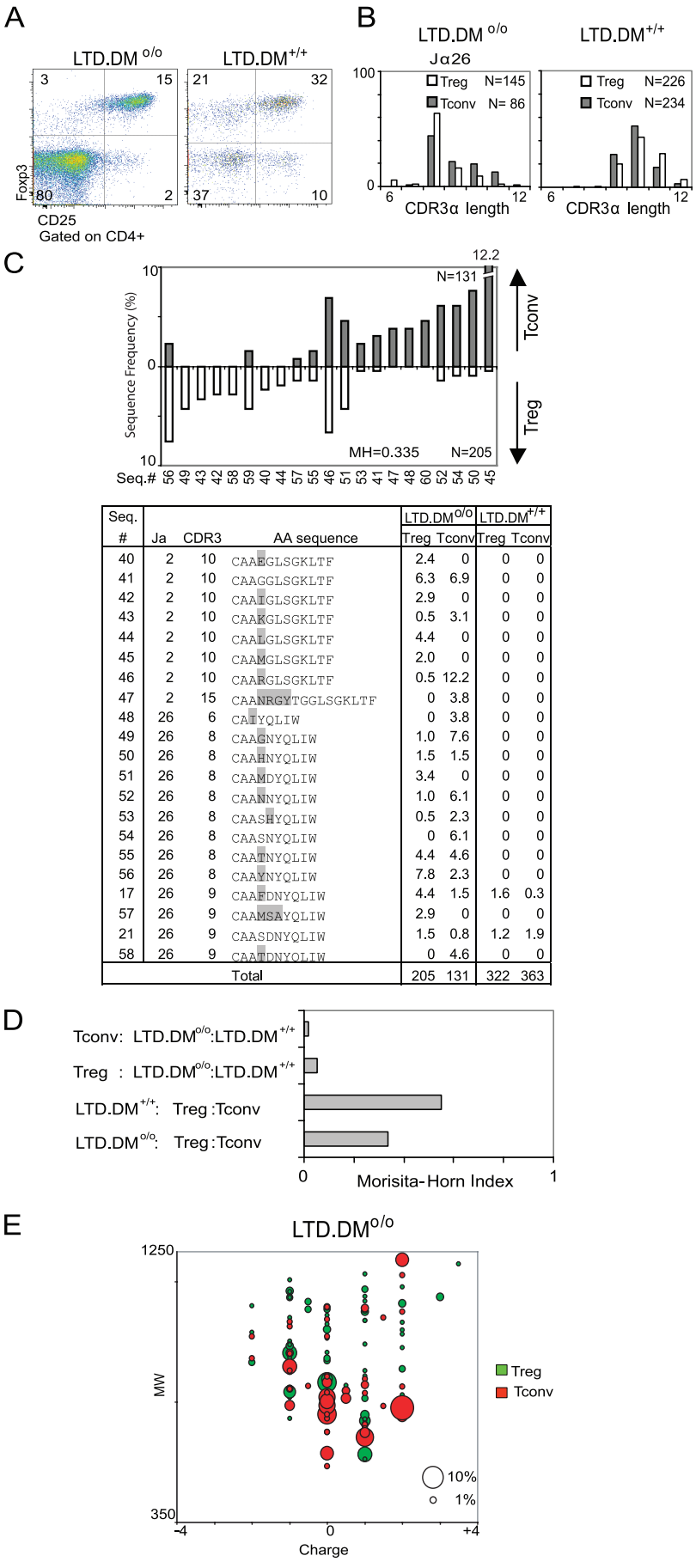
Ja	CDR3	AA Seq	Usually Found in $DM^{+/+}$	Usually Found in $DM^{o/o}$	Divided	Non-Divided
26	8	CAAANYQLIW			1	
26	8	CAAFNYQLIW			1	
26	8	CAAGNYQLIW		x	1	
26	8	CAAHNYQLIW		x	1	
26	8	CAAIHYQLIW		x	1	
26	8	CAANNYQLIW		x	2	
26	8	CAASDYQLIW		x	1	
26	8	CAASNYQLIW		x	6	
26	8	CAASSYQLIW			3	
26	8	CAPINYQLIW			1	
26	9	CAAADNYQLIW			1	1
26	9	CAADDNYQLIW	x		1	1
26	9	CAAFDNYQLIW	x	x	1	2
26	9	CAASDNYQLIW	x	x	1	1
26	9	CAASNNYQLIW			1	
		Total			23	5

FIGURE 5. TCR sequences from LTD.$DM^{o/o}$ cells transferred into WT mice. Transfer of CFSE-labeled $CD4^+$ cells from LTD.$DM^{o/o}$ donors into DM-deficient or normal hosts (*left*); divided and undivided cells were sorted from $DM^{+/+}$ hosts and their TCR sequences determined. The CDR3α lengths in Jα26-bearing TCRα are plotted on the right. Actual amino acid sequences of Jα26-bearing TCR sequences, their presence in LTD mice, $DM^{o/o}$ or $DM^{+/+}$, and count per group are tabulated below.

$DM^{o/o}$ or $DM^{+/+}$ 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.$DM^{o/o}$ 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.$DM^{o/o}$ mice (Fig. 5, *bottom*). These results were confirmed by in vitro studies, where $CD4^+$ T cells from LTD.$DM^{o/o}$ LNs also reacted strongly to WT A^b 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.$DM^{o/o}$ animals are highly reactive against A^b 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.$DM^{o/o}$ mice contained a robust population of Treg cells, albeit in slightly lower proportions than in LTD mice, and



Seq. #	Ja	CDR3	AA sequence	LTD.DM$^{o/o}$ Treg	LTD.DM$^{o/o}$ Tconv	LTD.DM$^{+/+}$ Treg	LTD.DM$^{+/+}$ Tconv
40	2	10	CAAEGLSGKLTF	2.4	0	0	0
41	2	10	CAAGGLSGKLTF	6.3	6.9	0	0
42	2	10	CAAIGLSGKLTF	2.9	0	0	0
43	2	10	CAAKGLSGKLTF	0.5	3.1	0	0
44	2	10	CAALGLSGKLTF	4.4	0	0	0
45	2	10	CAAMGLSGKLTF	2.0	0	0	0
46	2	10	CAARGLSGKLTF	0.5	12.2	0	0
47	2	15	CAANRGYTGGLSGKLTF	0	3.8	0	0
48	26	6	CAIYQLIW	0	3.8	0	0
49	26	8	CAAGNYQLIW	1.0	7.6	0	0
50	26	8	CAAHNYQLIW	1.5	1.5	0	0
51	26	8	CAAMDYQLIW	3.4	0	0	0
52	26	8	CAANNYQLIW	1.0	6.1	0	0
53	26	8	CAASHYQLIW	0.5	2.3	0	0
54	26	8	CAASNYQLIW	0	6.1	0	0
55	26	8	CAATNYQLIW	4.4	4.6	0	0
56	26	8	CAAYNYQLIW	7.8	2.3	0	0
17	26	9	CAAFDNYQLIW	4.4	1.5	1.6	0.3
57	26	9	CAAMSAYQLIW	2.9	0	0	0
21	26	9	CAASDNYQLIW	1.5	0.8	1.2	1.9
58	26	9	CAATDNYQLIW	0	4.6	0	0
Total				205	131	322	363

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$^{o/o}$ or DM$^{+/+}$. *B*, Distribution of CDR3α lengths for TCRs containing Jα26. *C*, Frequencies of individual TCR sequences in Treg and Tconv cells of DM$^{o/o}$ or DM$^{+/+}$ 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$^{o/o}$ mice.

with the more habitual balance of CD25 expression (Fig. 6*A*). TCRα sequences were determined from single FoxP3$^+$CD25$^+$ or FoxP3$^-$CD25$^-$ cells from LTD.DM$^{o/o}$ mice (205 and 131 cells, respectively, from three LTD.DM$^{o/o}$ mice). The shortening of CDR3α sequences occurred similarly in Tconv and Treg cells (shown in Fig. 6*B* for Vα26-bearing sequences), and the clear distinction between the repertoires of DM$^{+/+}$ and DM$^{o/o}$ mice applied to Treg as well as Tconv cells (Fig. 6*C*). Within LTD.DM$^{o/o}$ 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. 6*D*), 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.DM$^{o/o}$ mice (Fig. 6*E*).

Thus, the repertoire adaptation of CD4$^+$ T cells to the unique peptide conditions in LTD.DM$^{o/o}$ 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 A^b/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 A^b 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.DM$^{o/o}$ 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.DM$^{o/o}$ 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-I 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.$DM^{o/o}$ 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.$DM^{o/o}$ 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 A^b 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 $DM^{o/o}$ background comprised a completely different set of sequences, with distinctly shorter CDR3α 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 CDR3α shortening. The connection between low peptide diversity and CDR3α loop length is not necessarily a constant, however, because TCR sequences from mice expressing the TCRα minilocus with normally variable TCR-Vβ5 chains of endogenous origin did not show such an effect (our unpublished data). In this vein, a previous analysis of CDR3α sequences in TCRβ-transgenic animals had demonstrated an imprint of the reduced peptide diversity on the T cell repertoire in $DM^{o/o}$ 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^+ animals? Cells with short loops were strongly reactive against peptides or A^b conformations found on $DM^{+/+}$ cells, and are thus likely to be strongly selected against in the $DM^{+/+}$ 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-derived component. These results are reminiscent of early experiments demonstrating the tolerogenic potential of thymic epithelium for $CD4^+$ T cells (51–54).

Tregs: not just any self-reactivity will do

If the particular subset of TCRs appearing in LTD.$DM^{o/o}$ mice is reactive to normal A^b:peptide complexes of $DM^{+/+}$ mice, and if Treg cells are enriched in autoreactive TCRs, then one might have expected that the short CDR3α loops would be reflected in Treg TCRs of $DM^{+/+}$ mice. This was not the case, however, as the overlap between TCRs in LTD.$DM^{+/+}$ Tregs and LTD.$DM^{o/o}$ Tconv was negligible. This finding suggests that their avidity might be too high to survive as Tregs in the $DM^{+/+}$ environment. Treg cells are not immune to negative selection via excessive TCR stimulation (25, 30, 31, 55). However, one would expect that the diversity of TCR sequences in LTD.$DM^{o/o}$ mice entail a wide range of affinities for A^b:peptide complexes in $DM^{+/+}$ mice and it is surprising that all of the LTD.$DM^{o/o}$ TCRs would be negatively selected in the LTD.$DM^{+/+}$ mouse. This notion that only a subset of autoreactive TCRs is compatible with selection toward the Treg 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 LTD 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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