Inventories of naive and tolerant mouse CD4 T cell repertoires reveal a hierarchy of deleted and diverted T cell receptors

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Deletion or Treg cell differentiation are alternative fates of autoreactive MHCII-restricted thymocytes. How these different modes of tolerance determine the size and composition of polyclonal cohorts of autoreactive T cells with shared specificity is poorly understood. We addressed how tolerance to a naturally expressed autoantigen of the central nervous system shapes the CD4 T cell repertoire. Specific cells in the tolerant peripheral repertoire either were Foxp3+ or displayed anergy hallmarks and, surprisingly, were at least as frequent as in the nontolerant repertoire. Despite this apparent lack of deletional tolerance, repertoire inventories uncovered that some T cell receptors (TCRs) were lost from the CD4 T cell pool, whereas others mediated Treg cell differentiation. The antigen responsiveness of these TCRs supported an affinity model of central tolerance. Importantly, the contribution of different diverter TCRs to the nascent thymic Treg cell population reflected their antigen reactivity rather than their frequency among precursors. This reveals a multilayered TCR hierarchy in CD4 T cell tolerance that separates deleted and diverted TCRs and assuring that the Treg cell compartment is filled with cells of maximal permissive antigen reactivity.

T cell tolerance | clonal deletion | clonal diversion | regulatory T cell | MHC class II tetramer

TCR model antigen transgenic systems showed that Treg cell differentiation, similar to clonal deletion, can be instructed by TCR agonists (2, 3), and this was later confirmed for polyclonal T cells and natural self-antigens (4). In some models, low antigen levels favored Treg cell generation, whereas high antigen doses favored deletion, suggesting that Treg cell differentiation occurs within an avidity window between positive selection and deletion (5–8). Other observations, however, are difficult to reconcile with a reductionist, purely signal strength-based model. For instance, concomitant deletion is seen in essentially all TCR transgenic models of neoantigen-driven Treg cell diversification (2, 3, 7, 9), and it remains possible that Treg cell differentiation entails stochastic/selective elements (10).

Global TCR repertoire analyses supported the idea that Treg cell differentiation is instructed by self-antigen recognition, although some investigators came to opposing conclusions (11–13). Repertoire analyses also indicated that Aire-dependent expression of tissue-restricted antigens (TRAs) in thymic epithelial cells (TECs), a phenomenon that had previously been associated with deletional tolerance (14–17), also shapes the Treg cell repertoire (18–20).

MHCII tetramers were employed to trace minute cohorts of CD4 T cells when their cognate antigens were transgenically expressed to emulate a ubiquitous or TRA-like expression pattern (21, 22). Widespread antigen expression was associated with deletion, whereas a more restricted TRA-like expression ensued in the emergence of Foxp3+ Treg cells. While these findings suggested that the mechanism of tolerance is somehow dictated by the antigen expression pattern, it remains to be seen whether this is a generally applicable rule (23).

TCR transgenics, global repertoire sequencing, and MHC tetramers each entail distinct limitations. Monoclonal systems with unphysiologically high frequencies of antigen-specific cells may not faithfully recapitulate T cell fate decisions in polyclonal settings (24, 25). Large-scale repertoire analyses are limited in their potential to relate cell fate to antigen specificity. MHC tetramers may fail to reveal holes in the tolerant repertoire, as the loss of certain TCRs might be numerically compensated by expansion of cells carrying others TCRs. Moreover, although the emergence of Foxp3+ cells among Tetramer (Tet)+ cells indicates that Treg cell induction occurs, it remains unclear whether this selectively applies to cells bearing distinct TCRs.

Significance

Central tolerance can generate holes in the CD4 T cell repertoire or divert cells into the Foxp3+ regulatory (Treg) cell lineage. Little is known with regard to how these diametrically different cell fate decisions of autoreactive cells shape the size and composition of polyclonal cohorts of antigen-specific T cells. Here we generated inventories of very rare autoreactive T cells in the naive versus tolerant polyclonal repertoire and identified T cell receptors (TCRs) that were lost, whereas others mediated Treg cell differentiation. The antigen responsiveness of these TCRs revealed a TCR hierarchy that not only separates deleted from diverted TCRs but also generates a Treg cell compartment with high antigen reactivity.

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Here we addressed how clonal deletion and Treg induction impact not only the size but also the TCR composition of polyclonal CD4 T cells recognizing the natural CNS autoantigen myelin proteolipid protein (PLP). BL/6 mice are largely resistant to PLP-induced experimental autoimmune encephalomyelitis (EAE), suggesting a robust state of antigen-specific tolerance. In line with this, immunization of Plp1KO mice, to which PLP is a foreign antigen, elicits a CD4 T cell response against 2 epitopes spanning amino acids 11 to 19 and 240 to 248, whereas Plp1WT mice are functionally tolerant to these epitopes (26, 27). We assessed how far this unresponsiveness of the censored repertoire reflected the deletion, Treg cell diversion, or anergy of PLP-specific CD4 T cells and whether these tolerance modes selectively applied to cells carrying distinct TCRs.

Results

PLP-specific CD4 T Cells Are Not Reduced in the Tolerant Repertoire but Contain Foxp3+ Treg Cells. Using a combination of tetramer staining and magnetic enrichment (SI Appendix, Fig. S1) (28), we enumerated PLP11–19 (Tet-1) or PLP240–248 (Tet-3) specific CD4 T cells in pooled spleen and lymph node cells of Plp1KO and Plp1WT mice. The uncensored peripheral repertoire of Plp1KO mice contained 14.0 ± 3.0 Tet-1+ CD4 T cells and 4.4 ± 1.2 Tet-3+ cells (Fig. 1A and B). In Plp1WT mice, PLP11–19- or PLP240–248-specific cells were not diminished; surprisingly, there was even a tendency toward elevated numbers of Tet+ cells in the tolerant repertoire, although these differences did not reach significance (Fig. 1A and B). Whereas Foxp3+ cells were barely detectable among tetramer-positive T cells of Plp1KO mice, 30 to 40% of cells specific for either epitope were Foxp3+ in Plp1WT mice (Fig. 1C and D).

Also in the thymus, the number of Tet-1- or Tet-3-positive cells did not significantly differ between Plp1KO or Plp1WT mice (Fig. 2A and B). The Tet-1+ or Tet-3+ CD4 SP population of Plp1KO mice was essentially devoid of Foxp3+ cells. By contrast, in Plp1WT mice, around 10% of CD4 SP cells specific for epitope 1 or 3 expressed Foxp3 (Fig. 2C and D).

Treg Cells and Anergy in a Repertoire of Reduced Complexity. Our findings were consistent with the idea that tolerance of PLP11–19- and PLP240–248-specific CD4 T cells predominantly operated through Treg cell induction, which was in line with previous observations with TRA-like expressed model antigens (21, 22). However, it remained possible that clonal deletion also shaped the composition of PLP-specific cells, yet was numerically masked, for instance, by compensatory expansion of other PLP reactive cells. Distinguishing these possibilities requires a comprehensive comparison of TCRs of Tet+ cells in Plp1KO versus Plp1WT mice. However, the sheer number of TCR α- and β-chain combinations that recognize a given antigen limits the feasibility of such an approach in fully polyclonal repertoires. Several hundred naive cells specific for a foreign antigen each expressed a different TCR (29, 30). It is likely that the same applies to PLP-specific CD4 T cells. Together with the paucity of Tet+ cells, this poses a significant hurdle to a conclusive comparison of antigen-specific TCRs in the absence or presence of a given autoantigen.

To circumvent these inherent limitations of fully polyclonal TCR repertoires, we introduced a transgenic TCR β chain derived from a PLP11–19-specific TCR (27). The rationale was to reduce the repertoire complexity to the diversity of TCRα rearrangements and impose a bias toward higher numbers of PLP11–19-specific cells. The genotype of these compound transgenic mice (TCRβ-PLP1TG::Tcrα−/-::Foxp3GFP) will in the following be referred to as “Fixed-β.”

As expected, essentially all T cells in Fixed-β mice expressed the transgenic TCR Vβ6 chain (SI Appendix, Fig. S2). The peripheral repertoire of Fixed-β mice on Plp1KO background contained on average 78 ± 16 Tet-1+ CD4 T cells, which is about 5-fold more than in the corresponding fully polyclonal repertoire (Fig. 3A, compare Fig. 1A). Thus, the fixed β chain indeed imposed a certain repertoire bias, but the resulting frequency of PLP11–19-specific CD4 T cells was not unphysiologically high (31–33).

In line with our findings in the fully polyclonal repertoire, Fixed-β:Plp1WT mice had higher numbers of peripheral Tet-1 CD4 T cells than their PLP-deficient counterparts (Fig. 3A), and more than 50% of Tet-1+ cells were Foxp3+ (Fig. 3B). Among cells from Plp1WT mice that did not express Foxp3, the majority were FR4+CD73+, a characteristic of anergic CD4 T cells (Fig. 3C) (34, 35), whereas essentially all Tet-1+Foxp3− cells in Plp1KO mice displayed a naïve FR4+CD73− phenotype (Fig. 3B and C). Moreover, most Tet-1+Foxp3+ cells in Plp1WT mice were CD44hi and had lower levels of CD5 as compared to Tet-1−Foxp3− cells from Plp1KO mice, corroborating the notion that they were antigen-experienced and intrinsically tolerant rather than naive and ignorant (SI Appendix, Fig. S3).

![Fig. 1. PLP-specific peripheral CD4 T cells in Plp1KO or Plp1WT mice.](image-url)
Evidence for Clonal Deletion and Anergy Induction in the Thymus.

The CD4 SP compartment of Foxp3KO mice contained around 750 Tet-1+ cells, essentially all of which were Foxp3+. In Plp1WT mice, Tet-1+ CD4 SP cells were significantly reduced, and a substantial fraction of cells were Foxp3+ (Fig. 4 A and B). Around 30% of the thymic Treg cell compartment consists of recirculating cells from the periphery (36). These lack CCR7 expression (37). More than 80% of Foxp3+ Tet-1+ cells in Plp1WT mice were CCR7+, indicating that they were recently induced Treg cells (Fig. 4C).

CD4 SP cells can be subdivided into sequential maturation stages according to expression of CD69 and MHCI (CD69+MHCI−→CD69−MHCI+→CD69+MHCI+) (38). Tet-1+ cells were significantly reduced within the most mature CD69+MHCI+ subset of Plp1WT mice (2.7 ± 1.0% vs. 11.3 ± 2.1%; P = 0.0044) (Fig. 4D). Together with the lower total number of Tet+ thymi, this suggested that concomitant to the diversion of some cells into the Treg cell lineage, other cells were deleted at a relatively late stage of CD4 SP maturation. Moreover, the remaining mature Tet+ cells in Plp1WT thymi contained a substantial proportion of cells that were FR4+CD73+, consistent with anergy being a third antigen-instructed cell fate option of PLP-specific thymocytes.

Clonal Composition of Tet+ Cells in the Presence or Absence of PLP Expression. If deletion and diversion into the Treg cell lineage selectively applied to cells carrying different TCRs, some TCRs from the uncensored repertoire were expected to preferentially contribute to the Treg cell population in Plp1WT mice, whereas other TCRs would disappear from the repertoire. To investigate this, we generated an uncensored reference library of PLP11−19−specific TCRs from Foxp3− cells in Plp1KO mice by single-cell TCRa sequencing (39). These cells are neither exposed to tolerogenic forces nor subject to peripheral homoeostatic influences of noncognate nature. We obtained TCRa sequences from 529 Tet-1+ CD4 SP cells, and of these, a total of 488 TCRa nucleotide sequences encoded for TCRa rearrangements that were each present at a frequency of at least 1% (Fig. 5A). Four public TCR entities, in the following referred to as TCR A, B, C, and D (SI Appendix, Fig. S4), were present at frequencies between 4 and 26% in the thymus and together accounted for roughly 50% of the Tet-1+ population in both the thymus and the periphery (Fig. 5A).

We next addressed the frequency of the TCRs A, B, C, and D in mice with a conditional deletion of Plp1 in thymic epithelial cells (Plp1ATAEC). We previously showed that expression of PLP in TECs is necessary for central tolerance (26, 27). Indeed, the Tet-1+ CD4 SP cell population in Plp1ATAEC mice was significantly larger as compared to Plp1WT mice and essentially devoid of Foxp3+ cells, confirming that both manifestations of central tolerance in Plp1WT thymi required PLP expression in TECs (SI Appendix, Fig. S5 A and B). The relative abundance of the TCRs A to D was similar to that in Plp1KO mice (Fig. 5B). Thus, the 4 public PLP-specific TCRs were present at stereotypic frequencies in 2 independently generated uncensored inventories.

In the thymus of Foxp3−:Plp1WT mice, the relative abundance of the TCRs A to D among Tet-1− Foxp3− CD4 SP cells resembled their distribution in the uncensored reference inventories (Fig. 5C). By contrast, the TCR composition of the Tet-1− Treg cell population was strikingly different. TCR A was strongly overrepresented, whereas TCRs B and C were absent or substantially underrepresented, respectively. Only TCR D was similarly abundant among both Foxp3+ and Foxp3− CD4 SP cells from Plp1WT mice (and as in the uncensored repertoire). This revealed a differential propensity of TCRs A, B, C, and D to divert CD4 SP cells into the Treg cell lineage in the presence of cognate antigen.

The TCR composition of peripheral Tet-1− Treg cells in Fixed-β:Plp1WT mice was very similar to the nascent Tet-1− Treg cell repertoire in the thymus (Fig. 5D, compare Fig. 5C). Again, TCR A was far more abundant than TCR D, and TCRs B and C were extremely rare or absent. Remarkably, the distribution of the TCRs A, B, C, and D among peripheral Foxp3− CD4 T cells (most of which displayed anergy hallmarks) was essentially a mirror image of that on Treg cells; that is, TCRs A and D were dominant, while TCRs B and C were largely lacking (Fig. 5D).
rather than deletion (21, 22). However, an inspection of the TCR TRA-specific CD4 T cells. Moreover, our findings sug-
poorly reflected whether and to what extent deletion shaped this
was still detectable among CD4 SP thymocytes from
activity was absent from the tolerant repertoire. Although TCR C
mice, we deem it likely that its absence from the peripheral T cell
Discussion
Our findings corroborate the idea that CD4 T cell tolerance to
TRAs may preferentially operate through Treg cell induction rather than deletion (21, 22). However, an inspection of the TCR composition of PLP-specific T cells indicated that the mere number of Tet+ cells in tolerant and nontolerant repertoires only poorly reflected whether and to what extent deletion shaped this autoantec T cell population (41). Moreover, our findings sug-
esther that anergy is a third antigen-instructed cell fate option of
In line with a central prediction of the affinity model of clonal
delusion versus Treg cell induction (1, 10), the tip of PLP re-
activity was absent from the tolerant repertoire. Although TCR C
was still detectable among CD4 SP thymocytes from Plp1 WT
mice, we decte it likely that its absence from the peripheral T cell
pool at least in part resulted from thymic deletion. We showed
previously that central tolerance to PLP involves direct antigen
presentation by mTECs (27). Typically, TRAs are expressed by a
small fraction of mTECs. This imposes spatial and temporal
imitations on the likelihood with which a given antigen is en-
countered by developing T cells. Hence, the TCR inventory of
Foxp3− thymocytes may to a considerable degree be derived from
cells that are upstream of antigen encounter and the ensuing
cell fate decision. In line with loss of PLP-specific deleter TCRs
such as TCR C occurring at a very late CD4 SP cell stage, there
was a strong reduction of mature Tet+ CD4 SP cells in Plp1 WT
mice, whereas more immature CD4 SP stages were unaffected by
the presence of cognate antigen. The paucity of mature thymic
Tet+ cells in Plp1 WT mice precluded testing the prediction that
TCR C should be largely absent from the mature CD4 SP cell
compartment.

The contribution of distinct diverter TCRs to the Treg cell
population reflected their relative antigen responsiveness rather
than their frequency in the uncensored thymic precursor pool.
One possible explanation is that mature Treg cells of the highest

Fig. 3. Number and phenotype of PLP11−19-specific peripheral CD4 T cells in
Plp1 KO or Plp1 WT mice expressing a fixed TCRβ chain. (A) Flow cytometry of
pooled spleen and lymph node cells after enrichment of PLP11−19-specific
CD4 T cells, gated on CD4+CD8+ cells. The calculated mean number ±
SEM of tetramer-positive cells/mouse is indicated (n ≥ 11 each). The graph on
the right shows a summary. Each data point represents an individual mouse.
(B) Foxp3GFP and CD25 expression in tetramer-positive cells from pooled
spleen and lymph node cells. Numbers indicate the mean frequency ± SEM
of Foxp3+CD25+ cells (n ≥ 11 each). The graph on the right shows the
FR4 and CD73 expression on gated Foxp3+ PLP11−19-specific CD4 T cells from
pooled spleen and lymph node cells. The dot plot on the right shows FR4
and CD73 expression in total Foxp3− peripheral CD4 T cells for comparison
(n ≥ 6 each).

Discussion

In line with a central prediction of the affinity model of clonal
delusion versus Treg cell induction (1, 10), the tip of PLP re-
activity was absent from the tolerant repertoire. Although TCR C
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The contribution of distinct diverter TCRs to the Treg cell
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One possible explanation is that mature Treg cells of the highest

Fig. 4. Number and phenotype of PLP11−19-specific CD4 SP thymocytes in
Plp1 KO or Plp1 WT mice expressing a fixed TCRβ chain. (A) Flow cytometry of
thymocytes after enrichment of PLP11−19-specific cells, gated on
Dump "CD8" CD4+ cells. The calculated mean number ± SEM of tetramer-
positive cells/thymus is indicated (n ≥ 12 each). The graph on the right shows a
summary. Each data point represents an individual mouse. (B) Foxp3GFP
and CD25 in Tet-1+ Foxp3+ cells. The calculated mean number ± SEM of
Foxp3−CD25− cells (n ≥ 12 each). The graph on the right shows FR4
and CD73 expression in total Foxp3− peripheral CD4 T cells for comparison
(n ≥ 6 each).

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relative reactivity may possess a competitive advantage in the periphery (42). However, we deem it equally if not more likely that the correlation between a TCR’s relative reactivity and its abundance among Treg cells reflects a differential efficacy of Treg lineage specification itself, as it is already evident in the nascent thymic Treg cell repertoire. Consistent with this idea, the extent of Treg cell differentiation upon injection of precursors with different OVA-specific TCRs into OVA expressing thymi directly correlated with their in vitro reactivity to antigen (43). In TCR transgenic models, cells with identical TCRs compete for a limiting niche during thymic Treg cell differentiation (24, 25). It will be interesting to address whether Treg cell precursors with higher-affinity TCRs may outcompete lower-affinity cells for a shared developmental niche or whether each clonal specificity occupies a private niche whose size increases with TCR reactivity. Continuous TCR stimulation fuels Treg cell fitness and function (44, 45). Hence, filling of the Treg cell repertoire with cells of the highest permissive TCR affinity may be crucial for optimal immune regulation.

Global TCR sequencing revealed some overlap between the TCR repertoires of Foxp3-positive and Foxp3-negative cells (12, 46, 47). It was concluded that a substantial number of autoreactive cells evade from tolerance induction in the thymus, yet are accompanied by Treg cells of identical specificity that prevent their unwanted activation (10). Reminiscent of this buddy hypothesis, the distribution of TCRs among Foxp3-negative Treg cells reflects a differential efficacy of Treg lineage induction, and the strikingly similar TCR composition of Foxp3+ and anergic Treg cells raises the possibility that their developmental trajectories are interconnected (48). Downstream of the initial TCR stimulus, Treg cell precursors compete for nonantigenic factors such as IL-2 to compete their differentiation (1). It is tempting to speculate that at least some of the primed Treg Precursors that lose this competition exit the thymus in an anergic state.

In sum, our findings reveal a multilayered TCR hierarchy that shapes the size, composition, and functional state of an autoreactive
T cell repertoire. Optimizing the efficacy of the generation of TCR inventories of rare antigen-specific cells will pave the way toward deciphering interindividual variations in the composition of autoreactive T cell cohorts, more precisely understanding where and when deleterious TCRs are lost from the repertoire, and unraveling whether and which TRA-specific Treg cells might be strategically positioned in the vicinity of their cognate autoantigen.

Materials and Methods

Animals. Foxxp3gfp reporter mice (DEREG) (49), Tcra−/− mice (50), and Plp1−/− (51) mice have been described previously. Plp1−/− mice (27) were obtained by crossing Foss1-Cre mice (52) to carry a conditional Plp1 allele (Plp1f53). The transgenic fixed TCRβ allele was from the αβ TCR transgenic mouse strain TCR-PLP1 (27). For more details, see SI Appendix. Animal studies were approved by local authorities (Regierung von Oberbayern, Az 7-08 and 142-13).

Flow Cytometry. Single-cell suspensions of spleen, lymph nodes, or thymus were surface stained according to standard procedures. For details on antibodies, flow cytometry and software, see SI Appendix.

Generation of I-Aβ Tetramers. MHCIi tetramers were produced as described previously (33). For details, see SI Appendix.

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Enrichment of Tet+ Cells. Tet-labeled cells were enriched using anti-PE and anti-APC microbeads and magnetized columns (Miltenyi Biotech) as described previously (33). For details, see SI Appendix.

Single-Cell TCR Sequencing. TCRα-chain sequencing was performed with a protocol modified from ref. 39. For more details, see SI Appendix.

Reexpression and Functional Testing of TCRs. TCRs were reconstituted by viral transduction of NFAT-GFP reporter hybridoma cells (40) stably expressing the fixed TCRβ chain. Hybridoma cells were stimulated with titrated amounts of peptide or anti-CD3 antibody, and GFP expression was measured by flow cytometry. For more details, see SI Appendix.

Statistical Analyses. Statistical significances were calculated with Prism7 using the 2-tailed unpaired Student’s t test.

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