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 T_{reg} 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 T_{reg} 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 T_{reg} 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 assures that the T_{reg} 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

T cell receptor (TCR) stimulation of thymocytes can result in their deletion but can also specify the diversion of MHCII-restricted T cells into the Foxp3$^+$ regulatory T (T_{reg}) cell lineage (1). The determinants that specify these alternative cell fates are incompletely understood, and little is known about how clonal deletion and clonal diversion shape the composition of polyclonal cohorts of autoreactive cells of shared antigen specificity.

TCR/model antigen transgenic systems showed that T_{reg} 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 T_{reg} cell generation, whereas high antigen doses favored deletion, suggesting that T_{reg} 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 T_{reg} cell diversion (2, 3, 7, 9), and it remains possible that T_{reg} cell differentiation entails stochastic/selective elements (10).

Global TCR repertoire analyses supported the idea that T_{reg} 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 T_{reg} 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$^+$ T_{reg} 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 T_{reg} 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$^+$ T regulatory (T_{reg}) 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 T_{reg} cell differentiation. The antigen responsiveness of these TCRs revealed a TCR hierarchy that not only separates deleted from diverted TCRs but also generates a T_{reg} cell compartment with high antigen reactivity.

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Here we addressed how clonal deletion and T_{reg} 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 *Plp1*^{KO} 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 *Plp1*^{WT} mice are functionally tolerant to these epitopes (26, 27). We assessed how far this unresponsiveness of the censored repertoire reflected the deletion, T_{reg} 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$^+$ T_{reg} Cells. Using a combination of tetramer staining and magnetic enrichment (*SI Appendix*, Fig. S1) (28), we enumerated $PLP_{11–19}$ (Tet-1) or $PLP_{240–248}$ (Tet-3) specific CD4 T cells in pooled spleen and lymph node cells of *Plp1*^{KO} and *Plp1*^{WT} mice. The uncensored peripheral repertoire of *Plp1*^{KO} mice contained 14.0 ± 3.0 Tet-1$^+$ CD4 T cells and 4.4 ± 1.2 Tet-3$^+$ cells (Fig. 1 *A* and *B*). In *Plp1*^{WT} mice, $PLP_{11–19}$– or $PLP_{240–248}$–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. 1 *A* and *B*). Whereas Foxp3$^+$ cells were barely detectable among tetramer-positive T cells of *Plp1*^{KO} mice, 30 to 40% of cells specific for either epitope were Foxp3$^+$ in *Plp1*^{WT} mice (Fig. 1 *C* and *D*).

Also in the thymus, the number of Tet-1– or Tet-3–positive cells did not significantly differ between *Plp1*^{KO} or *Plp1*^{WT} mice (Fig. 2 *A* and *B*). The Tet-1$^+$ or Tet-3$^+$ CD4 SP population of *Plp1*^{KO} mice was essentially devoid of Foxp3$^+$ cells. By contrast, in *Plp1*^{WT} mice, around 10% of CD4 SP cells specific for epitope 1 or 3 expressed Foxp3 (Fig. 2 *C* and *D*).

T_{reg} Cells and Anergy in a Repertoire of Reduced Complexity. Our findings were consistent with the idea that tolerance of $PLP_{11–19}$– and $PLP_{240–248}$–specific CD4 T cells predominantly operated through T_{reg} 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 *Plp1*^{KO} versus *Plp1*^{WT} 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 $PLP_{11–19}$–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 $PLP_{11–19}$–specific cells. The genotype of these compound transgenic mice (TCRβ-PLP1^{TG}::*Tcra*$^{+/–}$::*Foxp3*^{GFP}) 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 *Plp1*^{KO} 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. 3*A*, compare Fig. 1*A*). Thus, the fixed β chain indeed imposed a certain repertoire bias, but the resulting frequency of $PLP_{11–19}$–specific CD4 T cells was not unphysiologically high (31–33).

In line with our findings in the fully polyclonal repertoire, Fixed-β::*Plp1*^{WT} mice had higher numbers of peripheral Tet-1$^+$ CD4 T cells than their PLP-deficient counterparts (Fig. 3*A*), and more than 50% of Tet-1$^+$ cells were Foxp3$^+$. Among cells from *Plp1*^{WT} mice that did not express Foxp3, the majority were FR4$^+$CD73$^+$, a characteristic of anergic CD4 T cells (Fig. 3*C*) (34, 35), whereas essentially all Tet-1$^+$Foxp3$^–$ cells in *Plp1*^{KO} mice displayed a naive FR4$^–$CD73$^–$ phenotype (Fig. 3 *B* and *C*). Moreover, most Tet-1$^+$Foxp3$^–$ cells in *Plp1*^{WT} mice were CD44^{hi} and had lower levels of CD5 as compared to Tet-1$^+$Foxp3$^–$ cells from *Plp1*^{KO} mice, corroborating the notion that they were antigen-experienced and intrinsically tolerant rather than naive and ignorant (*SI Appendix*, Fig. S3).

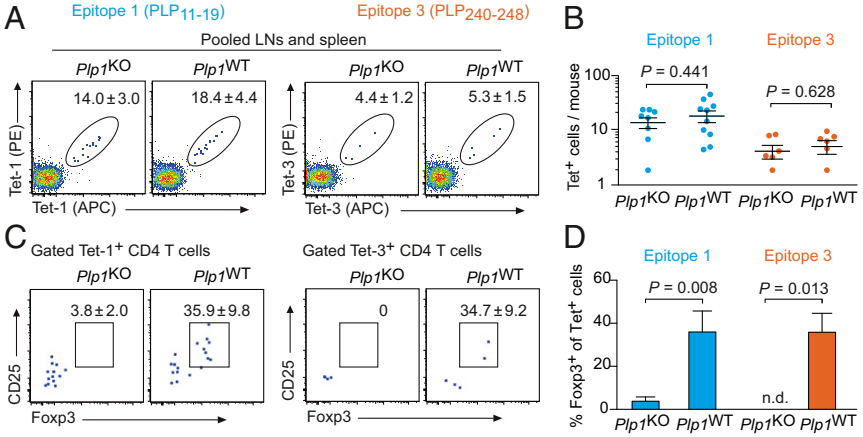

Fig. 1. PLP-specific peripheral CD4 T cells in *Plp1*^{KO} or *Plp1*^{WT} mice. (*A*) Analysis of pooled spleen and lymph node cells after enrichment of $PLP_{11–19}$– or $PLP_{240–248}$–specific CD4 T cells. Dot plots are gated on CD4$^+$CD8$^–$Dump$^–$ cells (*SI Appendix*, Fig. S1). The calculated mean number ± SEM of tetramer-positive cells/mouse is indicated ($n \geq 6$ each). (*B*) Number of $PLP_{11–19}$– or $PLP_{240–248}$–specific CD4 T cells/mouse. Each data point represents an individual mouse. (*C*) Foxp3^{GFP} and CD25 expression in peripheral tetramer-positive cells. Numbers indicate the mean frequency ± SEM of Foxp3$^+$CD25$^+$ cells ($n \geq 6$ each). (*D*) Summary of data in *C*.

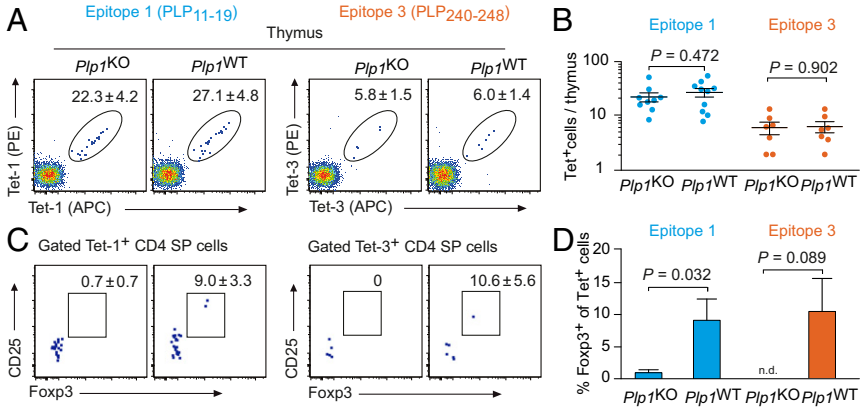

Fig. 2. Number and phenotype of PLP-specific CD4 SP thymocytes in *Plp1*^{KO} or *Plp1*^{WT} mice. (*A*) Flow cytometry of thymocytes after enrichment of $PLP_{11–19}$– or $PLP_{240–248}$–specific cells, gated on $CD4^+CD8^-Dump^-$ cells. The calculated mean number ± SEM of tetramer-positive cells/thymus is indicated ($n \geq 7$ each). (*B*) Summary of data in *A*. Each data point represents an individual thymus. (*C*) Foxp3^{GFP} and CD25 expression in tetramer-positive CD4 SP cells. Numbers indicate the mean frequency ± SEM of $Foxp3^+CD25^+$ cells ($n \geq 7$ each). (*D*) Summary of data in *C*.

Evidence for Clonal Deletion and Anergy Induction in the Thymus. The CD4 SP compartment of Fixed-β::*Plp1*^{KO} mice contained around 750 Tet-1$^+$ cells, essentially all of which were Foxp3$^-$ (Fig. 4 *A* and *B*). In *Plp1*^{WT} 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 T_{reg} cell compartment consists of recirculating cells from the periphery (36). These lack CCR7 expression (37). More than 80% of Foxp3$^+$ Tet-1$^+$ cells in *Plp1*^{WT} mice were CCR7$^+$, indicating that they were recently induced T_{reg} cells (Fig. 4*C*).

CD4 SP cells can be subdivided into sequential maturation stages according to expression of CD69 and MHCI ($CD69^+MHCI^- \rightarrow CD69^+MHCI^+ \rightarrow CD69^-MHCI^+$) (38). Tet-1$^+$ cells were significantly reduced within the most mature $CD69^-MHCI^+$ subset of *Plp1*^{WT} mice (2.7 ± 1.0% vs. 11.3 ± 2.1%; $P = 0.004$) (Fig. 4*D*). Together with the lower total number of Tet$^+$ cells in *Plp1*^{WT} thymi, this suggested that concomitant to the diversion of some cells into the T_{reg} cell lineage, other cells were deleted at a relatively late stage of CD4 SP maturation. Moreover, the remaining mature Tet$^+$ cells in *Plp1*^{WT} 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 T_{reg} cell lineage selectively applied to cells carrying different TCRs, some TCRs from the uncensored repertoire were expected to preferentially contribute to the T_{reg} cell population in *Plp1*^{WT} mice, whereas other TCRs would disappear from the repertoire. To investigate this, we generate an uncensored reference library of $PLP_{11–19}$–specific TCRs from Tet$^+$ thymocytes from *Plp1*^{KO} mice by single-cell TCRα sequencing (39). These cells are neither exposed to tolerogenic forces nor subject to peripheral homeostatic influences of noncognate nature. We obtained TCRα sequences from 529 Tet-1$^+$ CD4 SP cells, and of these, a total of 488 TCRα nucleotide sequences encoded for TCRα rearrangements that were each present at a frequency of at least 1% (Fig. 5*A*). 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. 5*A*).

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 (*Plp1*^{ΔTEC}). 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 *Plp1*^{ΔTEC} mice was significantly larger as compared to *Plp1*^{WT} mice and essentially devoid of Foxp3$^+$ cells, confirming that both manifestations of central tolerance in *Plp1*^{WT} 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 *Plp1*^{KO} mice (Fig. 5*B*). Thus, the 4 public PLP-specific TCRs were present at stereotypic frequencies in 2 independently generated uncensored inventories.

In the thymus of Fixed-β::*Plp1*^{WT} 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. 5*C*). By contrast, the TCR composition of the Tet-1$^+$ T_{reg} 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 *Plp1*^{WT} 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 T_{reg} cell lineage in the presence of cognate antigen.

The TCR composition of peripheral Tet-1$^+$ T_{reg} cells in Fixed-β::*Plp1*^{WT} was very similar to the nascent Tet-1$^+$ T_{reg} cell repertoire in the thymus (Fig. 5*D*, compare Fig. 5*C*). 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 T_{reg} cells; that is, TCRs A and D were dominant, while TCRs B and C were largely lacking (Fig. 5*D*).

A Reactivity Hierarchy Among Deleter and Diverter TCRs. TCR A dominated the T_{reg} cell compartment of tolerant mice, indicating that it acted as an efficient diverter TCR. By contrast, TCR C, albeit being at least as abundant in the uncensored repertoire as TCR A, was largely absent from the tolerant peripheral repertoire, suggesting that it was a deleter TCR. To address whether these distinct cell fate-specifying properties correlated with TCR reactivity, we reexpressed the 4 public TCRs in CD4 T cell hybridoma cells carrying a GFP IL-2 reporter (40). All 4 hybridoma cell lines showed identical control responses to noncognate polyclonal stimulation with anti-CD3 antibody (Fig. 5*E*). Upon stimulation with titrated amounts of PLP peptide, the deleter TCR C displayed the highest functional avidity to cognate antigen stimulation, with an IC_{50} that was about 50-fold or 250-fold lower than that of the diverter TCR A or D, respectively. TCR B had the lowest responsiveness to antigen.

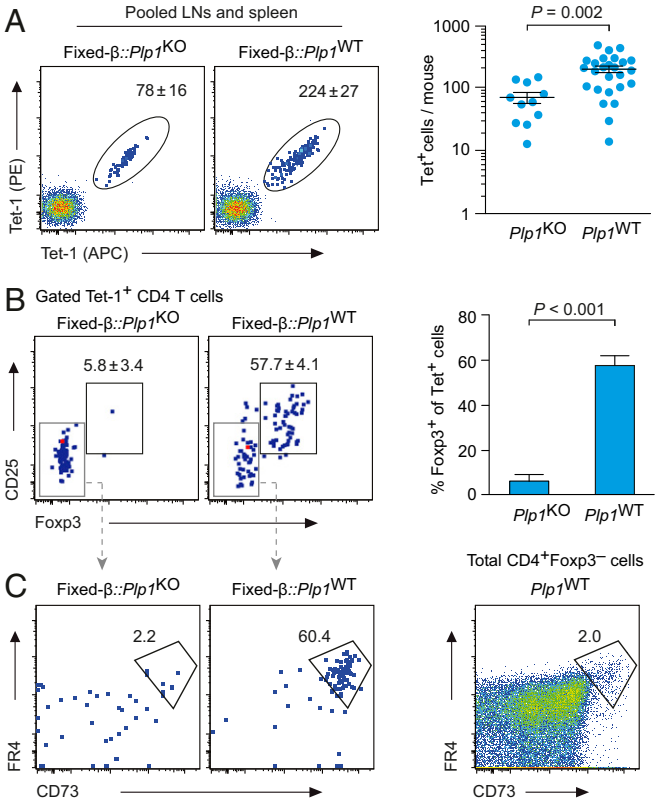

Fig. 3. Number and phenotype of $PLP_{11–19}$–specific peripheral CD4 T cells in *Plp1*^{KO} or *Plp1*^{WT} mice expressing a fixed TCRβ chain. (*A*) Flow cytometry of pooled spleen and lymph node cells after enrichment of $PLP_{11–19}$–specific CD4 T cells, gated on CD4$^+$CD8$^-$Dump$^-$ cells. The calculated mean number ± SEM of tetramer-positive cells/mouse is indicated ($n \geq 11$ each). The graph on the right shows a summary. Each data point represents an individual mouse. (*B*) Foxp3^{GFP} and CD25 expression in tetramer-positive cells from pooled spleen and lymph node cells. Numbers indicate the mean frequency ± SEM of Foxp3$^+$CD25$^+$ cells ($n \geq 11$ each). Data are summarized on the right. (*C*) FR4 and CD73 expression on gated Foxp3$^-$ $PLP_{11–19}$–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 ($n \geq 6$ each).

Discussion

Our findings corroborate the idea that CD4 T cell tolerance to TRAs may preferentially operate through T_{reg} 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 autorective T cell population (41). Moreover, our findings suggest that anergy is a third antigen-instructed cell fate option of TRA-specific CD4 T cells.

In line with a central prediction of the affinity model of clonal deletion versus T_{reg} cell induction (1, 10), the tip of PLP reactivity was absent from the tolerant repertoire. Although TCR C was still detectable among CD4 SP thymocytes from *Plp1*^{WT} mice, we deem 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 encountered 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 T_{reg} cell population reflected their relative antigen responsiveness rather than their frequency in the uncensored thymic precursor pool. One possible explanation is that mature T_{reg} cells of the highest

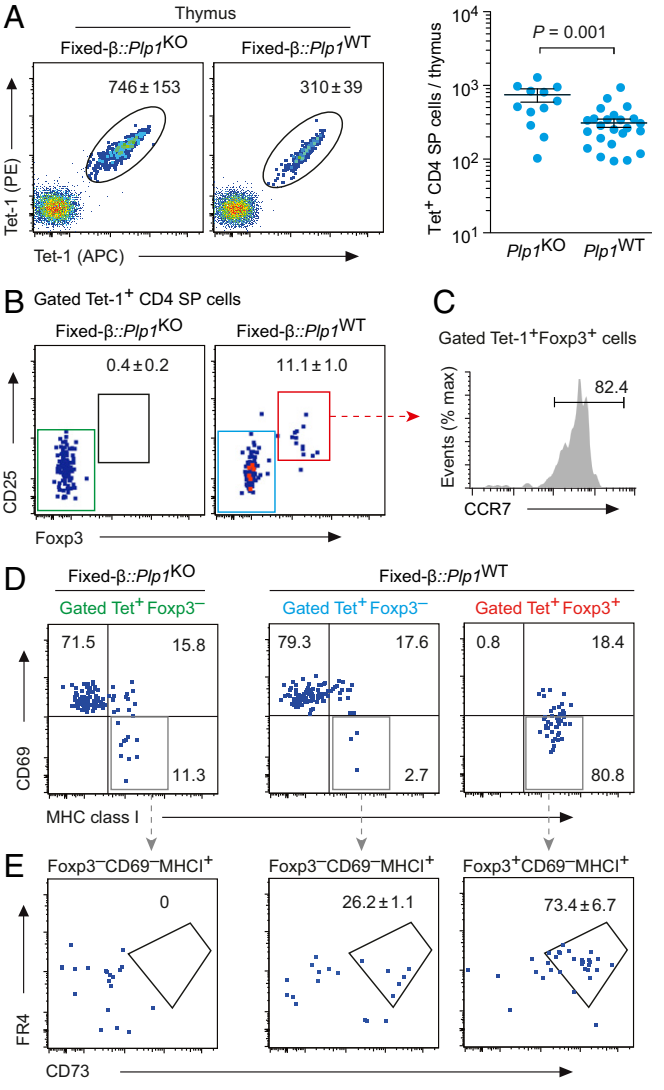

Fig. 4. Number and phenotype of $PLP_{11–19}$–specific CD4 SP thymocytes in *Plp1*^{KO} or *Plp1*^{WT} mice expressing a fixed TCRβ chain. (*A*) Flow cytometry of thymocytes after enrichment of $PLP_{11–19}$–specific cells, gated on Dump$^-$CD8$^-$CD4$^+$ cells. The calculated mean number ± SEM of tetramer-positive cells/thymus is indicated ($n \geq 12$ each). The graph on the right shows a summary. Each data point represents an individual mouse. (*B*) Foxp3^{GFP} and CD25 in Tet-1$^+$ CD4 SP cells. Mean ± SEM of $n \geq 12$ each. (*C*) CCR7 expression on gated Foxp3$^+$CD25$^+$ Tet$^+$ CD4 SP thymocytes. (*D*) CD69 and MHC class I (H-2K^b) on Tet-1$^+$ CD4 SP thymocytes from Fixed-β::*Plp1*^{KO} or Fixed-β::*Plp1*^{WT} mice. Colors refer to gating as in *B* (representative of $n \geq 6$ each). (*E*) FR4 and CD73 on mature (CD69$^-$MHCI$^+$) Tet-1$^+$ CD4 SP thymocytes from Fixed-β::*Plp1*^{KO} or Fixed-β::*Plp1*^{WT} mice, gated as in *D* (representative of $n \geq 5$ each).

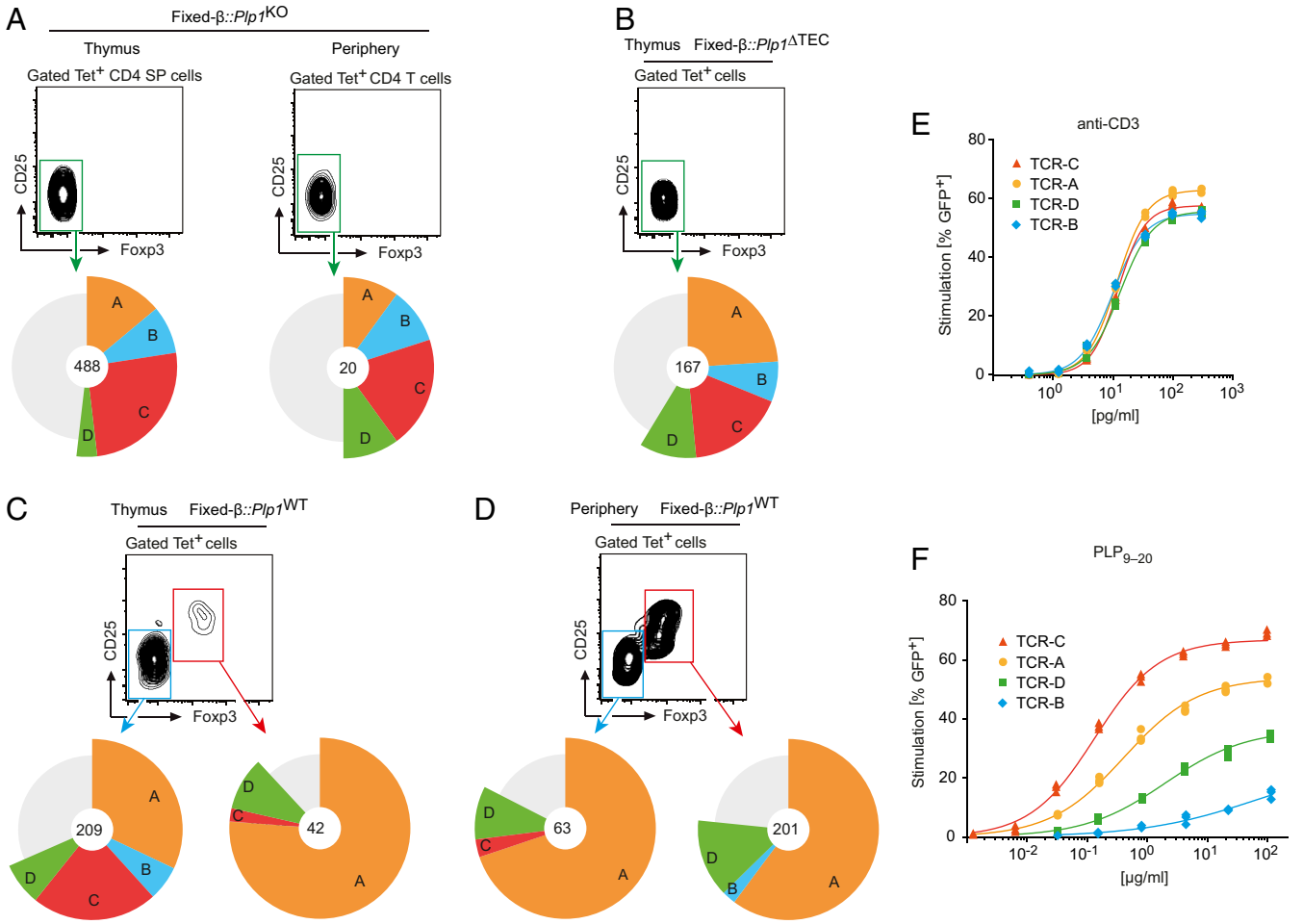

Fig. 5. TCR inventories of $PLP_{11–19}$–specific CD4 T cells from thymus or periphery of *Plp1*^{KO} and *Plp1*^{WT} mice expressing a fixed TCRβ chain. (*A*) Frequency of the 4 public TCRs A, B, C, and D in thymic or peripheral Tet-1$^+$ T cells of Fixed-β::*Plp1*^{KO} mice. The gray sector comprises all other TCR entities that were found at a frequency of >1%. Numbers in the center indicate the total number of cells that are represented in the respective pie chart. The graphs summarize data obtained from single cells from 111 mice analyzed in multiple pools. (*B*) Frequency of the TCRs A, B, C, and D in the thymic Tet-1$^+$ CD4 SP cell population of Fixed-β::*Plp1*$^{\Delta TEC}$ mice. Summary of data obtained from sorted single cells from 47 mice. (*C*) Frequency of the TCRs A, B, C, and D in Foxp3$^-$ or Foxp3$^+$ Tet-1$^+$ CD4 SP thymocytes from Fixed-β::*Plp1*^{WT} mice. Summary of data obtained from sorted single cells from 114 mice. (*D*) Frequency of the TCRs in peripheral Foxp3$^-$ or Foxp3$^+$ Tet-1$^+$ CD4 T cells from Fixed-β::*Plp1*^{WT} mice. Summary of cumulative data from sorted single cells from 132 mice. (*E*) Dose–response of CD4 T cell hybridoma cells expressing TCRs A, B, C, and D to stimulation with titrated amounts of anti-CD3 antibody. (*F*) Dose–response of CD4 T cell hybridoma cells expressing TCRs A, B, C, and D to stimulation with titrated amounts of cognate antigen. Data in *E* and *F* are representative of at least 3 experiments.

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 T_{reg} cells reflects a differential efficacy of T_{reg} lineage specification itself, as it is already evident in the nascent thymic T_{reg} cell repertoire. Consistent with this idea, the extent of T_{reg} 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 T_{reg} cell differentiation (24, 25). It will be interesting to address whether T_{reg} 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 T_{reg} cell fitness and function (44, 45). Hence, filling of the T_{reg} 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 T_{reg} cells of identical specificity that prevent their unwanted activation (10). Reminiscent of this buddy hypothesis, the distribution of TCRs among Foxp3-negative Tet$^+$ cells in the tolerant repertoire was essentially identical to that of T_{reg} cells. Importantly, though, rather than having escaped from tolerance induction, these Foxp3-negative cells mostly displayed phenotypic hallmarks of anergy. Where and how the anergic phenotype of peripheral PLP-specific cells is imprinted remains to be established. The presence of a considerable fraction of FR4$^+$CD73$^+$ cells among mature Foxp3$^-$ CD4 SP cells in PLP^{WT} thymi is consistent with a role of the thymus in anergy induction, and the strikingly similar TCR composition of Foxp3$^+$ and anergic Tet$^+$ cells raises the possibility that their developmental trajectories are interconnected (48). Downstream of the initial TCR stimulus, T_{reg} cell precursors compete for nonantigenic factors such as IL-2 to complete their differentiation (1). It is tempting to speculate that at least some of the primed T_{reg} 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 deleter TCRs are lost from the repertoire, and unraveling whether and which TRA-specific T_{reg} cells might be strategically positioned in the vicinity of their cognate autoantigen.

Materials and Methods

Animals. Foxp3^{GFP} reporter mice (DEREG) (49), *Tcra*$^{-/-}$ mice (50), and *Plp1*^{KO} (51) mice have been described previously. *Plp1*$^{\Delta TEC}$ mice (27) were obtained by crossing Foxn1-Cre mice (52) to mice carrying a conditional *Plp1* allele (*Plp1*^{fl}) (53). 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-A^b Tetramers. MHCII tetramers were produced as described previously (33). For details, see *SI Appendix*.

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 TCRα 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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