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Frontline

High TCR diversity ensures optimal function and homeostasis of Foxp3$^+$ regulatory T cells

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Dominant tolerance to self-antigen requires the presence of sufficient numbers of CD4$^+$ Foxp3$^+$ Treg cells with matching antigen specificity. However, the size and role of TCR repertoire diversity for antigen-specific immuno-regulation through Treg cells is not clear. Here, we developed and applied a novel high-throughput (HT) TCR sequencing approach to analyze the TCR repertoire of Treg cells and revealed the importance of high diversity for Treg-cell homeostasis and function. We found that highly polyclonal Treg cells from WT mice vigorously expanded after adoptive transfer into non-lymphopenic TCR-transgenic recipients with low Treg-cell diversity. In that system, we identified specific Treg-cell TCR preferences in distinct anatomic locations such as the mesenteric LN indicating that Treg cells continuously compete for MHC class-II-presented self-, food-, or flora-antigen. Functionally, we showed that high TCR diversity was required for optimal suppressive function of Treg cells in experimental acute graft versus host disease (GvHD). In conclusion, we suggest that efficient immuno-regulation by Treg cells requires high TCR diversity. Thereby, continuous competition of peripheral Treg cells for limited self-antigen shapes an organ-optimized, yet highly diverse, local TCR repertoire.

Key words: Experimental GvHD · Foxp3 · High-throughput sequencing · TCR repertoire · Treg cells · Treg-homeostasis

See accompanying Commentary by Wing and Sakaguchi

Supporting Information available online

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Introduction

Polyclonal Treg cells establish and maintain unresponsiveness to self-antigen, regulate tolerance to food and flora antigen, and control T-cell-mediated inflammatory responses [1, 2]. It is believed that the repertoire of natural (thymic) Treg cells is selected by recognition of self-antigen in the thymus [3–9] and further shaped by self-antigen recognition in the periphery [10–13]. This involves TCR-MHC class II:peptide interactions with higher avidity than during positive selection of naïve $CD4^+$ T cells [3, 14]. However, due to intraclonal competition, only a limited number of thymocytes expressing the same TCR specificity are selected to develop into natural Treg cells, which ensures the generation of a highly diverse Treg-cell TCR repertoire [15, 16]. In addition to the well-established essential regulation of Treg-cell homeostasis by IL-2 [17–20], previous studies suggested that organ-specific self-antigen preferentially drives the survival and/or expansion of peripheral Treg-cell clones [11, 13, 21]. Further studies showed that transferred antigen-specific Treg cells were proliferating in target-organ draining lymph nodes [22] and, along the same line, that transferred Treg cells from target-organ draining lymph nodes were more efficient in suppressing autoimmune disease than those of non-draining lymph nodes [23–25]. Nishio et al. reported that unspecific TCR stimulation with anti-CD3 mAb surmounted intraclonal competition of Treg cells in lymph nodes and spleen and provided additional support for the notion that Treg-cell expansion in the periphery depends on self-antigen recognition [26]. However, new data showed that the Treg-cell pool can remain self-sustained over months [27]. Recently, comprehensive high throughput (HT) sequencing studies revealed a very high TCR diversity in human Treg cells, comparable to other T-cell subsets including naïve T cells [28]. This led us to the hypothesis that broad TCR diversity may be important for Treg-cell homeostasis and immuno-regulatory function. To address this, we compared highly diverse Treg cells from WT mice with less diverse Treg cells derived from Rag-sufficient TCR-transgenic (TCR-Tg) mice. In the latter, endogenous TCR rearrangements permit the generation of natural Treg cells with a polyclonal, albeit narrower, TCR repertoire compared with WT mice. Therefore, TCR-Tg mice turned out to be a valuable tool for analyzing the physiological impact of TCR diversity on Treg-cell function. In this system, we performed adoptive transfer experiments and revealed a robust homeostatic advantage of WT Treg cells in TCR-Tg recipients with a less complex Treg-cell repertoire. Such sustained survival and expansion of transferred Treg cells allowed us to recover sufficient numbers of WT Treg cells to correlate their TCR sequences and organ-specific distribution. Furthermore, we analyzed the influence of TCR repertoire size on in vitro suppressive capacity of Treg cells and compared these results with their ability to suppress allogeneic T-cell responses in an in vivo model of lethal acute GvHD. We conclude that, within the limitations of an IL-2-dependent homeostatic niche, TCR diversity is required for optimal Treg-cell homeostasis and suppressive function.

Results

Treg cells in TCR-Tg mice use non-clonotypic TCR rearrangements and have a polyclonal TCR-repertoire

In this study, we used Rag-sufficient OT-II TCR transgenic mice in which the TCR repertoire of Treg cells is limited to non-clonotypic 'escapees' that are selected on endogenous *Tcrb* and/or *Tcra* rearrangements. To monitor and sort $Foxp3^+$ Treg cells, we crossed male homozygous TCR-Tg and female *Foxp3-eGFP* reporter mice. Male F1-offspring are hemizygous for *Foxp3-eGFP* and carry the pre-rearranged TCR. GFP^+ Treg cells in TCR-Tg mice expressed no or only low levels of the clonotypic TCR and are selected for endogenous TCR rearrangements (Supporting Information Fig. 1) [29, 30]. These observations and previous studies of Treg cells with restricted TCR rearrangement options [7, 12, 31] supported the hypothesis that Treg-cell repertoires of TCR-Tg mice are diverse but narrower than those of congenic WT mice.

The polyclonal Treg-cell TCR repertoire diversity is constricted in TCR-Tg mice

HT sequencing has recently become available to comprehensively characterize TCR repertoires on the level of nucleotide sequences. We chose primers spanning the variable region between the constant Cα and 12 V-elements of the Vα8 (also TRAV12) family. A similar sequencing method was recently applied to describe and compare the TCR repertoire of human naïve and memory $CD8^+$ T cells [32]. After sequence analysis of several thousands of individual *Tcra* rearrangements, we used this information pars pro toto to characterize and compare TCR diversity in Treg cells sorted from *Foxp3-eGFP* (here used as WT) and *Foxp3-eGFP* × OT-II TCR-Tg. Figure 1A depicts 23 718 individual rearranged *Tcra* sequences from each WT and TCR-Tg Treg cells by size distribution. Both of these 'virtual Vα8-Cα spectratyping' plots showed similar strong bias for multiples of three nucleotides, reflecting a preference for in-frame VJ rearrangements. Among the 23 718 *Tcra* sequences of both Treg-cell populations, we found high numbers of unique sequences, namely 10 746 clones with one single copy (and 2139 clones with two copies) in WT Treg cells and 6377 clones with one single copy (and 1341 clones with two copies) in Treg cells from OT-II TCR-Tg mice (Fig. 1B). Of note, the most abundant sequence in WT Treg cells had 71 copies, whereas 15 sequences from the TCR-Tg Treg cells had more than 100 and up to 1254 copies (Fig. 1B). Total numbers of all individual sequences added up to 14 622 different sequences for Treg cells from WT and only 9275 for TCR-Tg Treg cells. Thus, Treg-cell diversity in the TCR-Tg mice was reduced to 63% of the WT (Fig. 1C). Subsequently, we compared all productive VJ rearrangements according to the international ImMunoGeneTics information system IMGT® [33]. Among the 23 718 sequences of each pool, 10 353 individual productive VJ rearrangements on the nucleotide level were found in WT and 5657 in TCR-Tg Treg

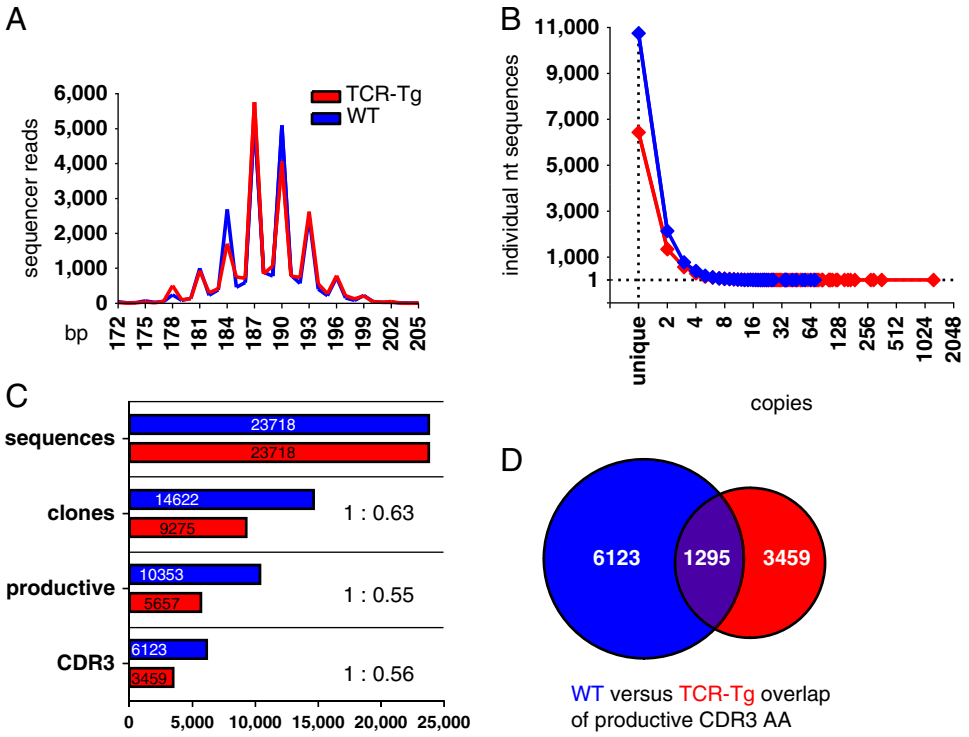

Figure 1. Treg cells in TCR-Tg mice have a narrow TCR-repertoire. 1×10^6 Foxp3$^+$ Treg cells were sorted from WT and TCR-Tg mice to a purity of >96% and Vα8-Cα amplicons were amplified from cDNA before high-throughput sequencing. (A) Length distribution of 23 718 CDR3α-spanning Vα-Cα sequences derived from WT (blue) and TCR-Tg (red) Treg cells. (B) Frequency of individual Vα-Cα sequences observed in WT (blue) and TCR-Tg (red) Treg cells. The copy number of Vα-Cα sequences is plotted against the number of individual nucleotide sequences. (C) Bars show summary of rearrangement analysis of 23 718 Vα-Cα sequences of WT (blue) and TCR-Tg (red) Treg cells using IMGT/HighV-QUEST. (D) Proportional Euler diagram shows amount of CDR3 regions with identical amino acid sequences from WT (blue) and TCR-Tg (red) Treg cells. Analysis of one of two independent sorting and sequencing experiments that gave similar results is shown.

cells (Fig. 1C). These encoded 6123 and 3459 distinct CDR3α respectively (Fig. 1C). These data suggested that on the amino acid level, the diversity of TCR antigen recognition in OT-II TCR-Tg Treg cells was reduced at least to 56% of WT. Qualitative comparison showed that 1295 of the CDR3α sequences from the TCR-Tg were identical to those from WT Treg cells (Fig. 1D). Collectively, our HT sequencing data showed that TCR-Tg Treg cells were essentially normal on a single cell basis but that their TCR repertoire was less diverse than that of WT Treg cells.

Treg cells with a broad TCR repertoire expand in hosts with a narrow TCR repertoire

To investigate how TCR diversity would affect their homeostasis, we performed adoptive cell transfers. In former studies, Treg cells adoptively transferred into WT mice have been followed for up to several wks, although recovery rates were generally very low [34, 35]. Here, purified Foxp3$^+$ WT Treg cells with a broad TCR repertoire showed a robust and continuous expansion when transferred into TCR-Tg hosts with restricted Treg-cell TCR diversity (Fig. 2A and B). After 2 months, donor Treg cells constituted approximately 20% of all Treg cells in the recipient blood and peripheral lymph nodes (pLNs). Conversely, this phenomenon was not observed when TCR-Tg Treg cells with a narrow TCR repertoire were transferred into WT hosts (Fig. 2B, left panel). Moreover, transfer of CD4$^+$ Foxp3$^-$ WT cells led neither to a considerable expansion of CD45.1$^-$ donor cells nor to a significant conversion into Foxp3$^+$ WT Treg cells (Fig. 2B, right panel). These results could be confirmed in a different experimental system by employing luciferase-expressing Treg cells [36] with a WT TCR repertoire. Monitoring Foxp3-specific light emission clearly showed similar WT Treg-cell expansion in OT-II TCR-Tg hosts over time (Fig. 2C). In addition, the same effect of efficient competition with the less diverse endogenous host Treg cells was observed after transfer into a different strain of TCR-Tg hosts, namely OT-I (Fig. 2C). Furthermore, WT donor Treg-cell frequency in TCR-Tg hosts correlated to the input dose (Fig. 2D) and their expansion was associated with higher proliferation rates and an activated phenotype (Fig. 2E and F). Re-analysis of recovered donor Treg cells 2 months after transfer revealed a reduced but still highly diverse TCR repertoire when compared with similar numbers of the control group (Supporting Information Fig. 2). Therefore, sustained survival and expansion was not restricted to a small number of clones but included a broad set of donor Treg

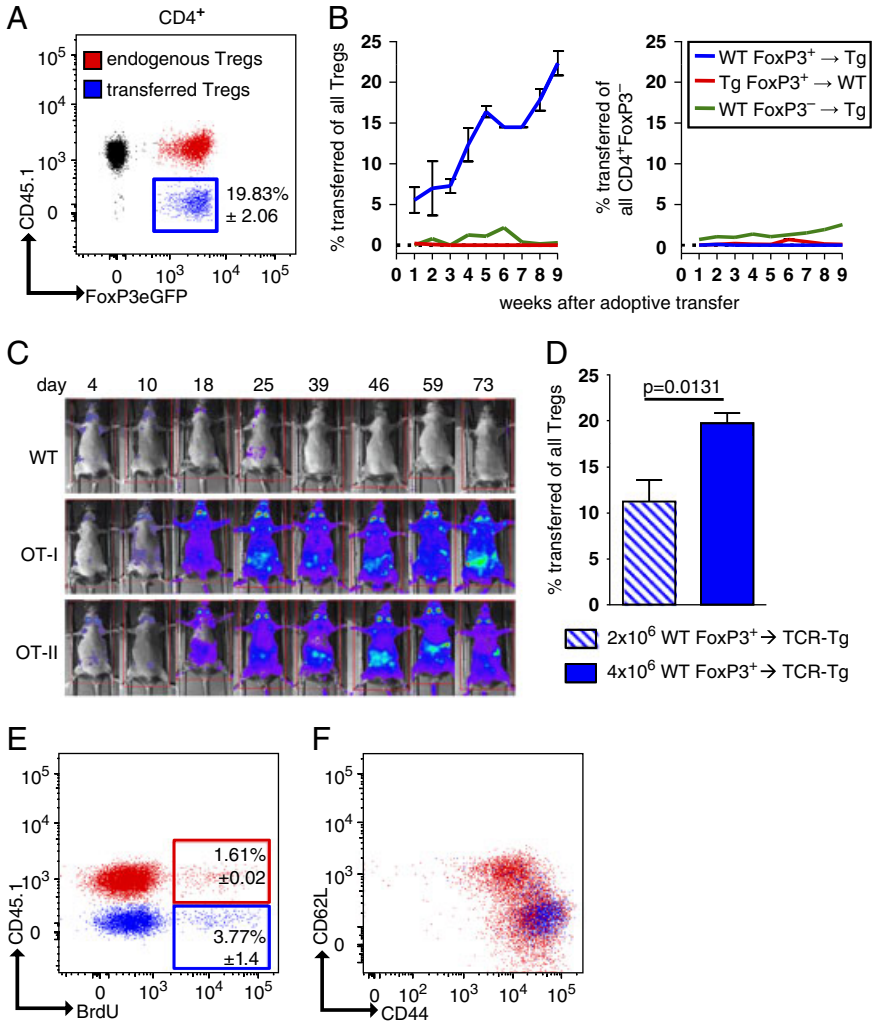

Figure 2. Treg cells with a broad TCR repertoire expand in hosts with a narrow TCR repertoire. Adoptive transfers of FACS-sorted WT Treg cells or $CD4^+Foxp3^-$ into WT or TCR-Tg recipients and vice versa. (A) Analysis of WT Treg cells in pLNs (inguinal, brachial) of TCR-Tg recipients 9 wk after transfer of 4×10^6 WT Treg cells. Numbers next to $CD45.1^-GFP^+$ gate show mean frequency ±SD, $n = 6$, of $CD45.1^-$ WT Treg cells (blue) of all $Foxp3^+$ cells. (B) Lines indicate proportion of the indicated donor T cells of all $CD4^+Foxp3^+$ (left panel) and $CD4^+Foxp3^-$ (right panel) in blood samples of recipients ($n = 1$–2). (C) 1.1×10^7 enriched $CD4^+$ cells from *Foxp3.LuciDTR-4* donors were injected into WT, OT-I, and OT-II recipients ($n = 2$). Mice were repeatedly imaged 5 min after injection of D-luciferin at the indicated time points. (D) Bar graphs show mean frequency + SEM of WT Treg cells in TCR-Tg recipients gated as in (A) 9 wk after transfer of either 2×10^6 (hatched blue) or 4×10^6 (blue) FACS-sorted WT Treg cells. *P*-value calculated by two-tailed unpaired *t*-test. (E) Dot plot showing at 20 h after BrdU injection the mean frequency ±SD, $n = 3$, of $BrdU^+$ endogenous Treg cells (red) and transferred WT Treg cells (blue) in TCR-Tg recipients 9 wk after transfer of 4×10^6 FACS-sorted WT Treg cells gated as in (A). (F) Representative dot plot showing expression of CD62L and CD44 of endogenous Treg cells (red) and transferred WT Treg cells (blue) in TCR-Tg recipients gated as in (A) 9 wk after transfer. Data shown are representative of three independent experiments.

cells. This suggests that TCR diversity and continuous self-antigen recognition control the total size of the peripheral Treg-cell pool independently of homeostatic factors such as IL-2. Of note, exogenous administration of recombinant IL-2 increased Treg-cell proliferation and absolute numbers in both WT and TCR-Tg mice (Supporting Information Fig. 3). Taken together, these adoptive transfer experiments revealed a hitherto unappreciated role for TCR diversity in Treg-cell homeostasis and imply that it is probably a combination of TCR specificity and TCR-independent factors that determine on the one hand the competitive/homeostatic fitness of Treg cells and on the other hand the total pool/niche size.

Treg cells from WT and TCR-Tg mice are not intrinsically different

In principle, endogenous rearrangements in TCR-Tg mice were able to produce any potential TCR chain combination and thus there were no distinct gaps in their Treg-cell repertoire. However, we still observed a few qualitative differences on the Treg-cell population level. Jα-usage of the analyzed Vα8 family sequences in Treg cells from TCR-Tg mice showed an increased proportion of the elements TRAJ5*01 and TRAJ34*02 (Supporting Information Fig. 4), while Jα-element usage was consistent in independent experiments for both types of Treg cells (Supporting

Information Fig. 4). It is likely that this biased Jα-usage reflects preferential selection of *Tcra* rearrangements that can efficiently pair with the clonotypic *Tcrb* chain. Furthermore, productive VJ rearrangements in TCR-Tg mice included on average more non-templated N-nucleotides compared with WT Treg cells (6.679±0.079 versus 5.89±0.050 N-nucleotides; $p<0.0001$). Also, we found lower isoelectric point (p*I*) values of pH 9.289±0.029 for the Treg cells from TCR-Tg mice versus pH 9.473±0.021 ($p<0.0001$) in WT. This is in line with a report showing that CDR3α regions of Treg cells have a relatively high p*I* [12]. Because of these significant, albeit subtle, differences, we wondered whether individual Treg cells derived from TCR-Tg mice were intrinsically less competitive than WT Treg cells. For that reason, we generated mixed BM chimeras of WT and TCR-Tg mice and compared thymic and peripheral Treg-cell levels. When a 1:1 ratio of both donors was used to reconstitute lethally irradiated recipients, we found only a marginal contribution of TCR-Tg precursors to the generation of the thymic and peripheral Treg-cell pool (Fig. 3). This is consistent with the assumption that only a few T-cell precursors in TCR-Tg mice are able to rearrange proper endogenous TCR chains prior to positive selection by the transgenic TCR. However, in chimeras derived from 20 parts TCR-Tg to 1 part WT BM, approximately 15% of thymic Treg cells were from the TCR-Tg donor as defined by the congenic markers Thy.1.1 and Thy1.2 (Fig. 3). This frequency did not decrease in the periphery, indicating that TCR-Tg donor-derived Treg cells showed similar fitness to compete for peripheral Treg-cell niches once successfully developed in competition with WT Treg cells. We cannot rule out that the repertoire of TCR-Tg donor-derived Treg cells may be skewed in a competitive environment. However, we can conclude that rearrangement of endogenous TCR chains in OT-II TCR-Tg mice generates Treg cells that individually are as fit as Treg cells in WT mice.

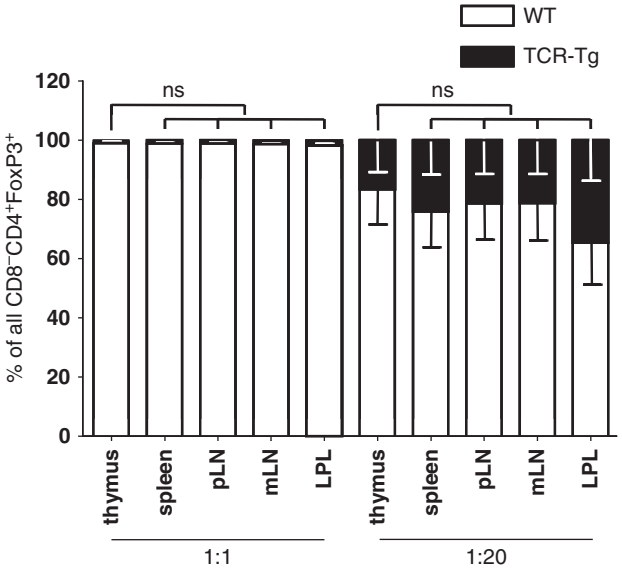

Figure 3. Frequency and fitness of WT and TCR-Tg donor-derived Treg cells in mixed BM chimeras. Lethally irradiated C57BL/6 WT mice received BM transplantation of either a 1:1 or 1:20 mixture of Thy1.2$^-$*Foxp3-eGFP* WT to Thy-1.2$^+$*Foxp3-eGFP-OT-II*, respectively. Bar graphs show mean proportion–SEM of Treg cells that developed from Thy1.2$^-$ (WT = white) and Thy1.2$^+$ (TCR-Tg = black) precursors among all CD4$^+$Foxp3$^+$ Treg cells in different organs as analyzed by FACS 8 wk after reconstitution. Data are representative of two independent experiments with $n = 4$ mice for each condition.

Organ-specific maintenance and expansion of transferred Treg cells

A recent study suggested that the Treg-cell repertoire varies by anatomical location [13]. However, it was so far difficult to address the influence of TCR specificity on Treg-cell homing in adoptive transfer experiments because recovery rates were not sufficient. Here, 9 wk after adoptive transfer, the distribution of WT Treg cells into TCR-Tg hosts showed a clear preference for pLN and spleen over mesenteric lymph nodes (mLNs) (Fig. 4A). Input Treg cells were pooled from spleens and all lymph nodes, comprising approximately 15–20% mLN-derived Treg cells. In contrast, one would likely need to perform a very high number of experiments in order to decide whether significant organ-specific homing might occur after transfer into WT mice because recovery rates were approximately 100-fold lower (Fig. 4B). It is possible that dissimilar expression of gut-associated lymphoid tissue (GALT) homing receptors of the donor Treg cells additionally influenced their migration in the host. When comparing Treg cells from spleen, pLN, and mLN of WT and OT-II TCR-Tg mice, we found that the frequency of double-positive cells for the GALT homing markers CCR9 [37] and of the homing/activation marker CD103 [38] was increased in mLNs compared with that in pLNs (Fig. 4C). However, we largely observed only minor differences in the expression of CCR9 and CD103 (Fig. 4C). Hence, it is plausible that antigen specificity and expression of a distinct homing signature correlate with each other and are not mutually exclusive determinants of the observed organ-specific Treg-cell distribution. In mLNs, available MHC class II presented antigen may also comprise considerable proportions of intestinal antigen derived from food and bacterial flora. Therefore, we investigated the TCR sequence overlap of re-isolated donor Treg cells from spleen, pLN, mLN, and LPL (lamina propria lymphocytes) 9 wk after adoptive transfer of WT Treg cells as described for Fig. 2. We were able to analyze several thousands of recovered Treg cells and revealed strikingly overlapping *Tcra* rearrangements in mLN and intestinal LPL (Fig. 5A). Comparing the 25 most abundant CDR3 sequences from each tissue, we found that mLN and LPL samples shared 14 out of 25 identical AA sequences, whereas only one was similar between pLN and mLN or pLN and LPL (Fig. 5B and Table 1). Next, we asked to what extent such organ-specific expansion would be specific for Treg cells as compared with Foxp3$^-$ T cells. Therefore, we performed adoptive transfers of either pLN or mLN whole lymphocyte suspensions from CD45.1$^-$ WT mice into CD45.1$^+$ TCR-Tg recipients (Fig. 6A). The percentage of input Foxp3$^+$ Treg cells among all CD4$^+$-gated T cells was similar in both cell suspensions. Nine wks after transfer of pLN cells, the frequency of

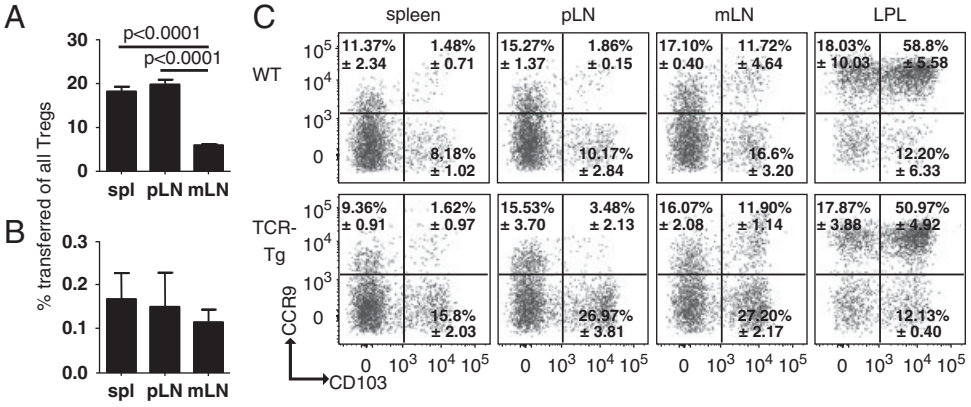

Figure 4. Preferential expansion of transferred Treg cells in peripheral LN and spleen as opposed to GALT. Comparison of donor Treg cell frequency in different lymphoid organs. (A, B) Bar graphs show mean frequency + SD of WT donor Treg cells 9 wk after transfer in (A) TCR-Tg recipients or (B) WT recipients gated as described in Fig. 2A in spleen, pLN, and mLN. *P*-values calculated by two-tailed unpaired *t*-test. (C) Dot plots show expression of CCR9 and CD103 on Treg cells from WT (upper panel) or TCR-Tg mice (lower panel) in spleen, pLN, mLN and LPL. Numbers in plots show mean frequency ± SD of the corresponding gate; FACS plots are representative of three independent experiments.

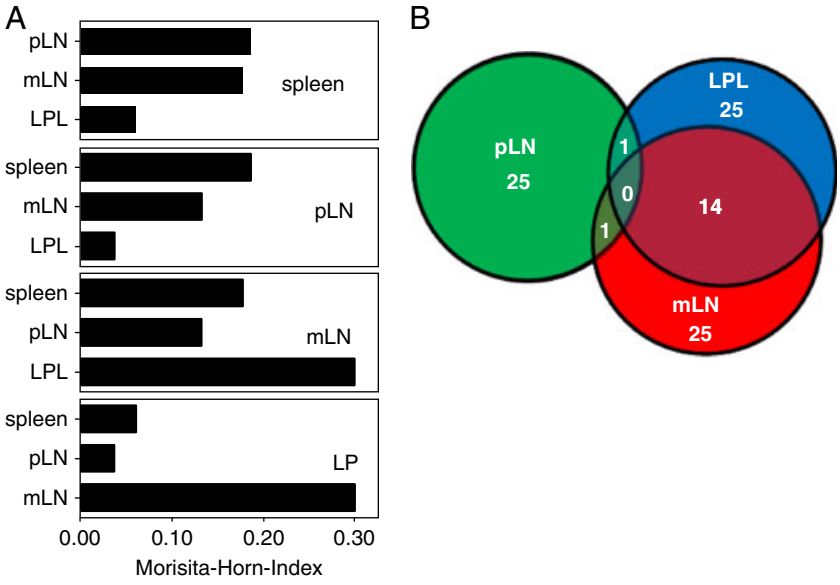

Figure 5. Biased yet diverse organ-specific TCR repertoires reveal overlapping antigen-specificities of Treg cells in mLN and intestinal lamina propria. (A, B) Statistical analysis of TCR repertoire similarity of transferred WT Treg cells between organs 9 wk after transfer into TCR-Tg recipients. (A) Morisita-Horn indices are calculated to compare similarity of CDR3 amino acid sequences of recovered Treg cells between indicated organs. (B) Proportional Euler diagram shows overlap of the 25 most abundant CDR3 regions in Treg cell samples recovered from pLN (green), mLN (red), and LPL (blue) 9 wk after transfer into TCR-Tg recipients. Data were pooled from $n = 3$ mice, comprising 5979, 4798, and 1938 functionally rearranged clones from pLN, mLN, and LPL respectively. Detailed sequence information is shown in Table 1.

Treg cells among all CD45.1$^-$CD4$^+$ input T cells was assessed. It had increased in spleen, pLN, and mLN (Fig. 6A and B), which is in line with the Treg-cell expansion after transfer of purified Treg cells shown above. A decreased proportion among LPL may reflect antigen-specific expansion of Foxp3$^-$CD4$^+$ T cells. At the same time, transfer of mLN cells resulted in stable proportions of Treg cells in LPL and elevated frequencies in both mLN and pLN (Fig. 6A and B). Interestingly, expansion of mLN-derived Treg cells was similar in pLN and mLN, although lower than the expansion after transfer of pLN suspensions (Fig. 6B). In conclusion, these results suggested that, besides homing receptor cues, organ-specific TCR shaping created distinct, highly diverse but still overlapping TCR repertoires in pLNs and mLNs. After transfer, such locally optimized TCR repertoires supported the maintenance of donor Treg cells in their respective organs of origin.

A broad TCR-repertoire is required for Treg-cell mediated suppression of GvHD

Next, we investigated the impact of Treg-cell repertoire diversity on their genuine function, i.e. their capacity to suppress T-cell

Table 1. Top CDR3 sequences of transferred WT Treg cells

Sequecnes	pLN (5979[a])	mLN (4798)	LPL (1938)
ALSGNNNNAPR	0[b]	54	67
ALSFSSNTNKVV	0	27	11
ALSGGSNYKLT	0	26	54
ALSWSGGSNAKLT	0	20	14
ALSDTGQGGSAKLI	0	16	0
ALREGTGGYKVV	0	16	0
ALLTASLGKLQ	0	12	15
ALSKSGSWQLI	0	3	15
ALSEGNYNQGKLI	0	0	13
ALLNSNNRIF	0	1	11
ALLSNTNKVV	1	33	22
ALSDTNAYKVI	1	25	15
ALSAMNYNQGKLI	1	15	29
ALRAGNTGKLI	1	18	0
ALNNNNAPR	1	17	5
ALSETSSGQKLG	1	2	25
ALRDQGGSAKLI	1	0	21
ALRATGYQNFY	1	2	15
ALSASPSSNTNKVV	1	4	13
ALRPSSNTNKVV	2	44	18
ALSASGGSNAKLT	2	14	12
ALSDRTGNTGKLI	3	43	84
ALSATSSGQKLV	3	41	98
ALSRNNNNAPR	3	26	4
ALSDPGYQNFY	4	21	6
ALRTGGYKVV	4	19	0
ALRSSNTNKVV	8	43	14
ALSETSSGQKLV	8	83	223
ALSDRSSGSWQLI	10	23	0
ALSEGTSGGNYKPT	11	56	41
ALSPITGNTGKLI	11	15	0
ALITGNTGKLI	11	11	31
ALSEITGSGGKLT	17	22	26
ALRPSNTNKVV	23	15	0
ALSEDYSNNRLT	40	1	0
ALRPGTGSNRLT	40	0	0
ALSEGAGNKLT	41	15	3
ALSQGTGGYKVV	42	2	0
ALRNNYAQGLT	43	7	0
ALSLTTASLGKLQ	44	3	0
ALRDSGYNKLT	45	0	5
ALSVSGGSNAKLT	48	3	0
ALRGTGGNNKLT	51	1	0
AVQVVGQLT	51	4	0
ALRPNTNKVV	52	0	0
ALKQGTGSKLS	52	1	3
ALSDRGSGGNYKPT	58	7	0
ALNAGGYKVV	59	5	0
ALGSALGRLH	59	3	0
ALSGRNNNNAPR	65	10	4
ALSDWTGNTGKLI	69	1	0
ALKSGSFNKLT	70	0	0
ALLRPSGSWQLI	72	1	1
ALSEGSNYNVLY	72	2	0
ALSPNSGTYQR	90	8	1
ASDTNAYKVI	91	6	1
ALRSGSFNKLT	124	5	0
ALASSSFSKLV	126	4	13
ALSDLGFASALT	134	2	1

The 25 most abundant CDR3 sequences among all recovered transferred WT Treg cells were chosen from all productive clones of pLN (5979), mLN (4798), and LPL (1938) and highlighted in green, red, and blue respectively.

[a] Numbers in brackets indicate for each tissue the total amount of clones with productive *Tcra* rearrangements (IMGT) of donor Treg cells recovered from TCR-Tg mice 9 wk after transfer of WT Treg cells.

[b] Number of clones that code for the corresponding CDR3 AA sequence in the left column.

activation. In an in vitro system based on T-cell activation with anti-CD3 mAb, Treg cells from TCR-Tg mice were equally efficient as Treg cells from WT mice in suppressing the proliferation of $CD8^+$ and of $CD4^+$ T cells (Fig. 7A and B). In contrast, in an experimental model of acute GvHD [35] less diverse Treg cells were less efficient than WT Treg cells in preventing the lethal disease (Fig. 7C and D). Co-transfer of allogeneic Treg cells derived from OT-II TCR-Tg mice showed only alleviation of the disease but not protection from GvHD (Fig. 7C and D). Taken together, these results suggest that the impact of TCR diversity on Treg-cell function is context dependent. Limited diversity is not per se affecting the capability of Treg cells to suppress unspecific T-cell proliferation in vitro, but it is an essential prerequisite for effective suppression of allogeneic T-cell activation in vivo in a MHC-mismatched GvHD model.

Discussion

Here, we present evidence that TCR diversity is an essential aspect of $Foxp3^+$ Treg-cell homeostasis and function. Treg cells with a broader TCR repertoire exhibited sustained survival and expansion in hosts with less diverse Treg cells, which likely reflected their advantage in competition for self peptides and other peptides presented by MHC class II. Adoptive transfer experiments revealed that the TCR repertoire of Treg-cell populations varied by anatomical location. Functionally, our data strongly suggest that TCR diversity is a critical factor for efficient Treg-cell mediated suppression of experimental acute GvHD.

If not crossed to a Rag-deficient background, TCR-Tg mice contain functional Treg cells that develop through thymic selection of endogenous, non-clonotypic TCR rearrangements [14, 39, 40]. Only in rare exceptions, e.g. in AND- or HA- TCR-Tg mice [41, 42], a limited number of clonotypic thymocytes was shown to develop into $Foxp3^+$ Treg cells [15, 16, 43]. Here, the use of broadly available OT-II TCR-Tg as Treg-cell recipients allowed efficient in vivo expansion of adoptively transferred WT Treg cells with a broader TCR repertoire. Moreover, congenic markers in combination with the eGFP-reporter in the Foxp3 locus assured unambiguous detection of Treg cells after adoptive transfer. To the best of our knowledge, such a robust expansion of adoptively transferred Treg cells as described here is unprecedented in non-lymphopenic mice.

Several studies in humans and mice have implied that TCR diversity is an important feature of Treg cells. A comprehensive study on one single human T-cell repertoire recently concluded that Treg cells were the most diverse T cells [28]. The authors predicted 89 920 TCRα CDR3 sequences in Treg cells (defined as $CD4^+CD25^+$) compared with 58 325 in all other naive and transitional $CD45RA^+$ non-Treg cells. This is in line with former data obtained by spectratyping of human Treg-cell CDR3 regions [44, 45]. Furthermore, earlier studies using classical sequencing approaches also found at least similar diversity in mouse Treg cells [6, 7]. Our study demonstrated that the TCR repertoire of WT mouse Treg cells was indeed very broad, however, at least TCR-Vα8 CDR3 diversity was found to be even higher in WT $Foxp3^-CD4^+$ T cells than in Treg cells (Supporting Information Fig. 2). Recent studies suggested that thymic intra- and inter-clonal competition for limited antigen presented on MHC class II may be an important mechanism to generate Treg cells with a broad TCR spectrum [15, 16, 46]. This was specific for natural Treg cells but not for $Foxp3^-CD4^+$ T cells and thus led to the conclusion that TCRs from Treg cells may on average have higher affinity for self-peptide-MHC. Here, our findings suggest

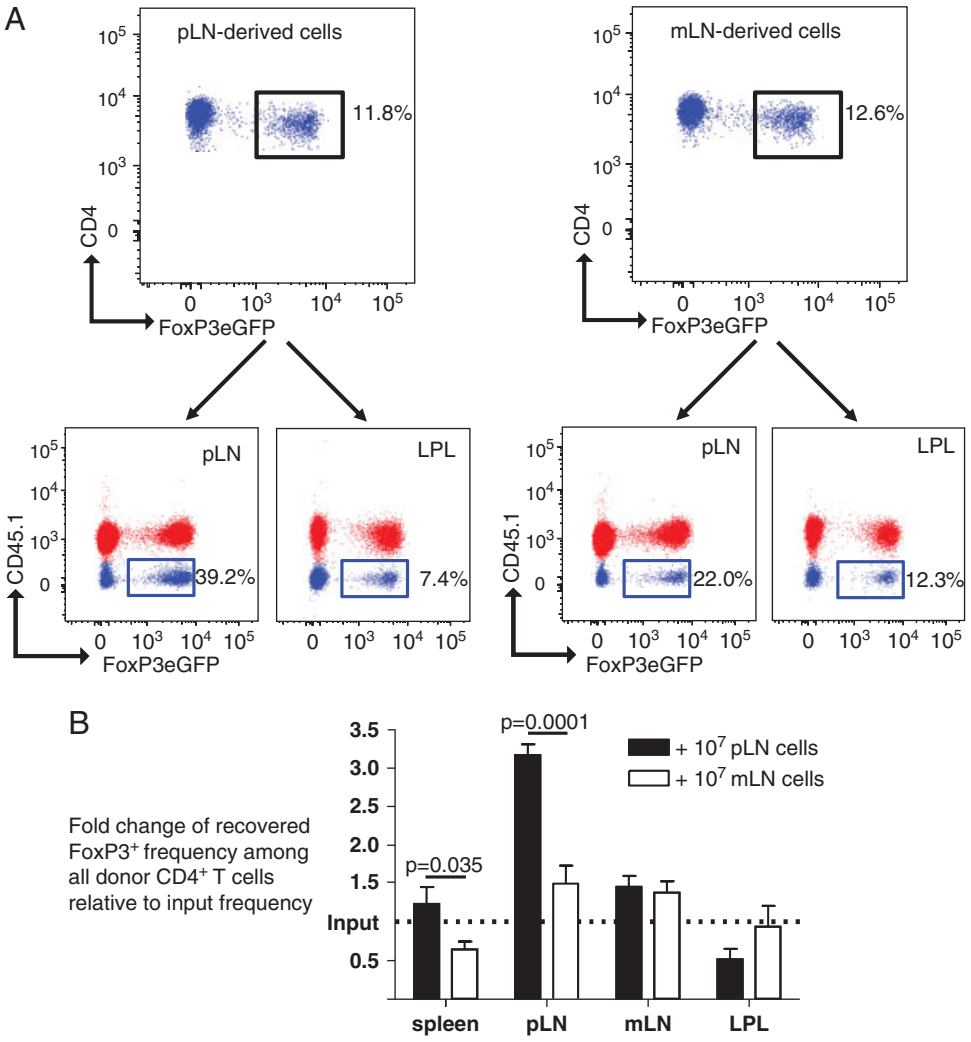

Figure 6. Expansion after transfer is Treg-cell specific and depends on the origin of donor cells. 1×10^7 lymphocytes from either pLN (A, left side; B, black) or mLN (A, right side; B, white) of WT donors were adoptively transferred into TCR-Tg recipients. (A) Upper dot plots show the input frequency of GFP^+ Treg cells among all $CD4^+$ T cells and lower dot plots show the frequency of GFP^+ Treg cells among all transferred $CD4^+$ T cells in pLN and LPL of recipients 9 wk after transfer. (B) Input frequency of GFP^+ Treg cells was defined as 1, bar graphs show fold change of GFP^+ Treg cells 9 wk after transfer. Data were combined from three independent experiments with two mice per group each. *p*-Values calculated by two-tailed unpaired *t*-test.

that Treg cells continue to compete in a similar way for limited matching self-peptide-MHC complexes after egress from the thymus, which in turn ensures an optimal organ-specific but diverse repertoire of relevant self-reactive TCR in the periphery.

The impact of TCR repertoire diversity on Treg-cell function is controversial. Regarding the prevention of autoimmune disease, previous studies on the effective suppression of EAE through Treg cells with limited TCR repertoires came to divergent conclusions [47, 48]. A recent study by Adeegbe et al. found that limited TCR diversity of transferred Treg cells was a risk factor for autoimmune disease in IL-2Rbeta$^{-/-}$ mice [49]. Intriguingly, non-obese diabetic mice were recently shown to select a low diversity Treg-cell TCR repertoire [50].

Understanding the parameters that govern Treg-cell homeostasis will be critical for the design of future Treg-cell-based intervention strategies. Sufficient availability of organ-specific antigen must be considered in translational attempts to manipulate organ-specific autoimmunity with engineered Treg cells of known self-peptide specificity. Otherwise, exogenous therapeutic Treg cells may be lost quickly after transfer. Previous studies suggested that organ-specific self-antigen preferentially drives the survival and/or expansion of organ-specific Treg-cell clones [11, 13, 21, 22]. Our results also support the view that the antigen specificity of Treg cells changes by anatomical location, although TCR sequences of recovered Treg cells from pLNs and mLNs were largely overlapping. This may be the result of two possible scenarios. Either Treg cells recirculate less than naïve T cells or differences are due to selective local survival.

Importantly, our study infers that Treg-cell diversity is connected to diversity and availability of specific self- and foreign-antigen and thus the amount of DCs presenting it on

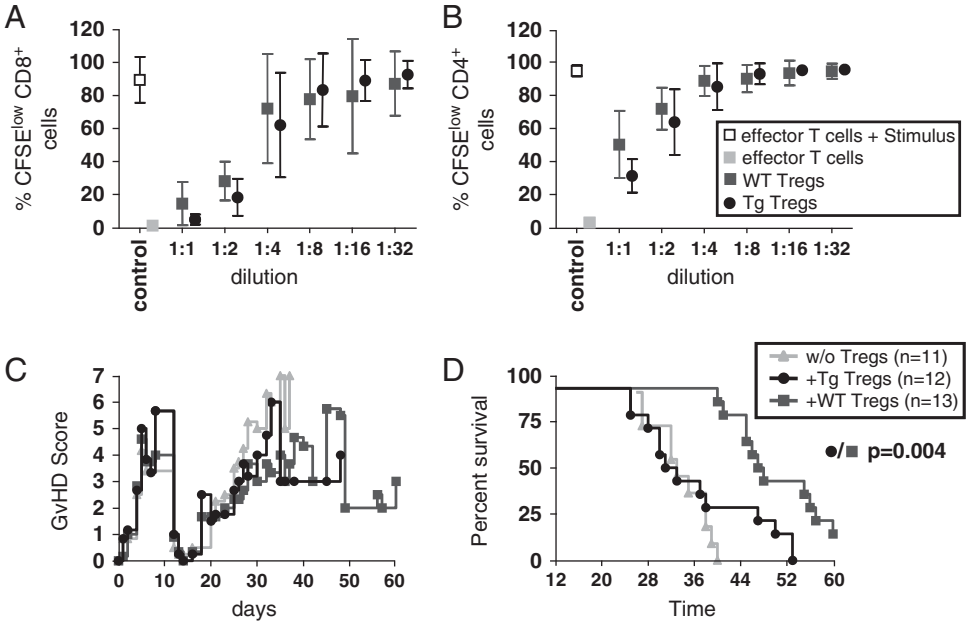

Figure 7. TCR-repertoire size compromises Treg-cell function in vivo in experimental GvHD but not in vitro. (A, B) In vitro suppression by WT and TCR-Tg Treg cells. Treg cells were co-cultured with CFSE-labeled Thy1.2$^+$ T cells in the presence of anti-CD3/anti-CD28 beads. Frequency of proliferated CFSE^{low} (A) CD8$^+$ and (B) CD4$^+$ Thy1.2$^+$ T cells are shown for the indicated ratios of Treg cells to effector cells. Data are representative of three independent experiments with four replicates each. (C, D) In vivo suppression of experimental GvHD. (C) Score and (D) survival of lethally irradiated BALB/c mice after transplantation of allogeneic BM and effector T cells alone or together with equal numbers of WT or TCR-Tg Treg cells as indicated. Data are pooled from three independent experiments.

MHC class II. In accord, it was recently shown that DC ablation reduced Treg-cell frequencies [51, 52], whereas an increase of DC numbers by FLT3L treatment led to expansion of peripheral naturally occurring Treg cells [52, 53]. However, in the latter report, it was concluded that Treg-cell proliferation was mainly IL-2 dependent. In our study, we also recognized IL-2 as a master regulator that controls the absolute size of the Treg-cell pool. We propose that an optimal and maximally broad organ-specific Treg-cell TCR repertoire is continuously shaped by inter- and intraclonal competition for diverse antigen. Within a peripheral Treg-cell niche, sufficient population diversity seems to be crucial for proper Treg-cell function. Hence, in future studies, HT-sequencing analysis of Treg-cell diversity may be suitable to predict the relative risk of T-cell-mediated diseases.

Materials and methods

Animals

C57BL/6-*Foxp3eGFP* (here: WT) [54], C57BL/6-*Foxp3.LuciDTR-4* [36], and C57BL/6-Tg*(TcraTcrb)*425Cbn/J (here: OT-II/TCR-Tg) [55] mice have been described. The Thy1.1 allele of the C57BL/6-*Foxp3eGFP* mice was derived from the original Bruce 4 ES cells [35]. C57BL/6J, BALB/cJ, C57BL/6-Tg*(TcraTcrb)*1100Mjb/J (here: OT-I), and C57BL/6.SJL-*Ptprc*^{a} (CD45.1) mice were obtained from Charles River (Germany). Mice were bred and housed under specific pathogen free (SPF) conditions in the central animal facility of Hannover Medical School (Germany) and used at 6–12 wk of age. All experiments were approved by the Local Institutional Animal Care and Research Advisory committee and authorized by the local government. This study was conducted in accordance with the German Animal Welfare Law and with the European Communities Council Directive 86/609/EEC for the protection of animals used for experimental purposes.

Antibodies and reagents

Anti-CD4-PacificOrange (RmCD4-2), anti-CD4-PacificBlue (GK1.5), anti-CD4-Cy5 (RmCD4-2), anti-CD8β-PacificOrange, anti-CD8β-biotin (RmCD8), and anti-CD62L-PacificOrange (MEL-14) were purified from hybridoma supernatants and conjugated in house. Anti-CD44-PacificBlue (IM7), anti-TCRβ-allophycocyanin-Alexa750 (H57-597), anti-Thy1.2-PE (MMT1), and anti-CD62L-allophycocyanin-AlexaFluor780 (MEL-14) were obtained from eBioscience. Anti-CD25-PerCP-Cy5.5 (PC61), anti-BrdU-Alexa647 (mglG1k), anti-Thy1.1-biotin (HIS51), anti-CD45.1-Alexa405 (A20), anti-CD103-PE (M290), anti CD8α-allophycocyanin-Cy7 (53-6.7), anti-Vα2-PE (B20.1), anti-Vβ3-PE (KJ25), anti-Vβ4-PE (KT4), anti-Vβ5-biotin (MR9-4), anti-Vβ6-PE (RR4-7), anti-Vβ7-PE (TR310), anti-Vβ8-PE (F23.1), anti-Vβ11-PE (RR3-15), and Streptavidin coupled to PE-Cy7 or PerCP were purchased from BD Bioscience. CCR9 staining with rat anti-mouse CCR9 (7E7-1-1) was performed as described [56]. Human rIL-2 (Roche) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Cell isolation

Lymph nodes and spleens were mashed through a 100-μM nylon gauze and washed with PBS/3% FCS (PAA). Spleen and blood samples were treated with erythrocyte-lysis buffer. For isolation of LPL, gut content and Peyer's patches were removed before intestines were opened longitudinally, washed twice in cold PBS/3% FCS, and incubated 3×15 min in HBSS (Gibco) with 10% FCS and 2 nM EDTA at 37°C. After each incubation step, tubes were shaken for 10 s and the supernatant was discarded. Intestines were washed once in PBS, incubated 2×45 min in RPMI 1640 (Gibco) containing 10% FCS, 0.24 mg/mL collagenase A (Roche), and 40 U/mL DNase I (Roche) at 37°C, then tubes were shaken for 10 s, and cell suspensions pooled, resuspended in 40% Percoll (Amersham) in RPMI 1640/PBS, overlaid onto 70% Percoll in RPMI 1640/PBS, and centrifuged at 2000 rpm for 20 min at room temperature. LPL were recovered from the interphase and washed with PBS/3% FCS.

Flow cytometry and BrdU labeling

To assess BrdU incorporation, mice received 2 mg BrdU in PBS i.p. and were sacrificed after 20 h. Before staining, cell suspensions were incubated at 4°C for 5 min with Fc block (mAb 2.4G2). For multicolor staining, cells were incubated with antibodies for 15 min at 4°C, washed with PBS/3% FCS, and, if required, incubated with secondary reagent SAv-PerCP or SAv-PE-Cy7 for 10 min at 4°C and washed with PBS/3% FCS. BrdU staining was performed with the APC BrdU Flow Kit (BD Biosciences) according to manufacturer's protocol. Flow cytometric analysis was performed on an LSR II cytometer (BD Bioscience) equipped with the BD FACSDiva software. Post acquisition analyses were conducted using the FlowJo software (Treestar).

TCR amplification and HT sequencing

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) from 6×10^5 FACS-sorted GFP^+ Treg cells, purity >95%, from either 2 WT or 2 OT-II donors per experiment. cDNA templates were synthesized using SuperScript® II reverse transcriptase (Invitrogen) according to manufacturer's recommendation. To generate template libraries of rearranged TCR CDR3 regions from Treg-cell cDNA for the Genome Sequencer FLX System (454 sequencing, Roche), we used primers spanning the variable region between constant Cα and V elements of the Vα8 family (comprising TRAV12-1*01, TRAV12-1*03, TRAV12-1*04, TRAV12-1*05, TRAV12D-2*01, TRAV12D-2*02, TRAV12D-2*03, TRAV12D-2*04, TRAV12D-2*05, TRAV12D-3*01, TRAV12D-3*02, and TRAV12D-3*03). (For primers and PCR conditions please see Supporting Information Table 1.) Forward and reverse primers contained at their 5′ ends the universal adapter sequences and a multiplex identifier (MID) respectively. Amplicons were purified by agarose gel electrophoresis and QIAquick Gel Extraction Kit (Qiagen), and quantified by Quant-iT™ dsDNA HS Assay Kit (Invitrogen). Single PCR amplicon molecules were immobilized onto DNA Capture Beads within an oil–water emulsion to enable clonal amplification in a second PCR process with universal primers. The emulsion was then disrupted and isolated beads were loaded onto PicoTiterPlates. Sequencing reactions were performed by ultra-deep 454 pyrosequencing on the Genome Sequencer FLX system (Roche Applied Sciences). Productive rearrangements and CDR3α regions were defined by comparing nucleotide sequences to the reference sequences from IMGT®, the international ImMunoGeneTics information system® (http://www.imgt.org) [33]. Rearrangements were analyzed and CDR3α regions were defined using IMGT/HighV-QUEST [57].

Adoptive cell transfer

For transfers of purified cell populations, suspensions from pooled spleens and lymph nodes (inguinal, brachial, axillary, submandibular, and mesenteric) were enriched by magnetic beads ($CD4^+$ T Cell Isolation Kit, MiltenyiBiotec) and subsequently sorted into $Foxp3^+$ and $Foxp3^-$ cells by FACS. 4×10^6 or 2×10^6 of either $Foxp3^+$ or $Foxp3^-$ sorted cells, 1×10^7 unpurified pLN or mLN cell suspensions, or 1.1×10^7 enriched $CD4^+$ cells from *Foxp3.LuciDTR-4* donors were resuspended in 150 μL PBS and injected into the lateral tail vein of indicated recipient mice. After 9 wk, mice were sacrificed and pLN, mLN, spleen, and the small intestine were taken to recover and analyze transferred Treg cells identified by congenic markers and GFP reporter fluorescence.

Bioluminescence imaging

Mice were imaged 5 min after i.p. injection of 4.5 mg D-luciferin (SynChem, Elk Grove Village, IL, USA) using the IVIS 100 imaging system (Xenogen, Hopkinton, MA, USA) as described [36]. Analysis was performed with the Living Image software (v2.50, Xenogen).

Generation of BM chimeras

Lethally irradiated (9 Gy) *C57BL/6* WT recipients received adoptive transfer of a total number of 1×10^7 BM cells that were either a 1:1 or a 1:20 mixture of $Thy1.2^-$*Foxp3-eGFP* WT to $Thy1.2^+$*Foxp3-eGFP* OT-II, respectively. Chimeric mice were analyzed at 8–10 wk after transfer.

Experimental acute GvHD model

The *C57BL/6* (H-2b) into *BALB/c* (H-2d) acute GvHD model was performed as described elsewhere [35]. In addition, some groups received 0.5×10^6 sorted Treg cells from WT or OT-II mice with a purity of >95%.

In vitro suppression assay

Thy1.1$^+$ Treg cells from either WT or OT-II donors were co-cultivated in round-bottom 96-well plates with MACS-enriched CFSE-labeled Thy1.2$^+$ T cells at the indicated ratios under stimulatory conditions applying RPMI 1640 supplemented with 10% FCS, 2 mM glutamine and antibiotics, 100 IU/mL rh-IL2, and 1.5 μL T-cell expander beads (anti-CD3/anti-CD28, Dynal). After 4 days of co-culture, proliferation was assessed by flow cytometry determining CFSE dilution on live Thy1.2$^+$ T cells. Dead cells were identified by counterstaining with 4′,6-diamidino-2-phenylindol.

Statistical analysis

Averages and SD or SEM were calculated with Graphpad Prism®. Group data were compared with the two-tailed unpaired *t*-test. Similarity between two sequenced TCR repertoires was statistically measured by the Morisita-Horn index [58]. This index ranges between 0 (complete dissimilar) and 1 (identical) and is comparatively resistant to sample size. Proportional Euler diagrams were generated using the program VennMaster, which is available at http://www.informatik.uni-ulm.de/ni/staff/HKestler/vennm/doc.html.

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References

1 **Zheng, Y. and Rudensky, A. Y.,** Foxp3 in control of the regulatory T cell lineage. *Nat. Immunol.* 2007. **8**: 457–462.

2 **Sakaguchi, S., Yamaguchi, T., Nomura, T. and Ono, M.,** Regulatory T cells and immune tolerance. *Cell* 2008. **133**: 775–787.

3 **Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Holenbeck, A. E., Lerman, M. A., Naji, A. and Caton, A. J.,** Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2001. **2**: 301–306.

4 **Apostolou, I., Sarukhan, A., Klein, L. and von Boehmer, H.,** Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 2002. **3**: 756–763.

5 **van Santen, H. M., Benoist, C. and Mathis, D.,** Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J. Exp. Med.* 2004. **200**: 1221–1230.

6 **Hsieh, C. S., Liang, Y., Tyznik, A. J., Self, S. G., Liggitt, D. and Rudensky, A. Y.,** Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity* 2004. **21**: 267–277.

7 **Pacholczyk, R., Ignatowicz, H., Kraj, P. and Ignatowicz, L.,** Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity* 2006. **25**: 249–259.

8 **Hsieh, C. S., Zheng, Y., Liang, Y., Fontenot, J. D. and Rudensky, A. Y.,** An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat. Immunol.* 2006. **7**: 401–410.

9 **Ribot, J., Enault, G., Pilipenko, S., Huchenq, A., Calise, M., Hudrisier, D., Romagnoli, P. and van Meerwijk, J. P.,** Shaping of the autoreactive regulatory T cell repertoire by thymic cortical positive selection. *J. Immunol.* 2007. **179**: 6741–6748.

10 **Romagnoli, P., Hudrisier, D. and van Meerwijk, J. P.,** Preferential recognition of self antigens despite normal thymic deletion of CD4(+)CD25(+) regulatory T cells. *J. Immunol.* 2002. **168**: 1644–1648.

11 **Fisson, S., Darrasse-Jeze, G., Litvinova, E., Septier, F., Klatzmann, D., Liblau, R. and Salomon, B. L.,** Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. *J. Exp. Med.* 2003. **198**: 737–746.

12 **Wong, J., Obst, R., Correia-Neves, M., Losyev, G., Mathis, D. and Benoist, C.,** Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4+ T cells. *J. Immunol.* 2007. **178**: 7032–7041.

13 **Lathrop, S. K., Santacruz, N. A., Pham, D., Luo, J. and Hsieh, C. S.,** Antigen-specific peripheral shaping of the natural regulatory T cell population. *J. Exp. Med.* 2008. **205**: 3105–3117.

14 **Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F. and Sakaguchi, S.,** Thymus and autoimmunity: production of CD25+ CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J. Immunol.* 1999. **162**: 5317–5326.

15 **Bautista, J. L., Lio, C. W., Lathrop, S. K., Forbush, K., Liang, Y., Luo, J., Rudensky, A. Y. and Hsieh, C. S.,** Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nat. Immunol.* 2009. **10**: 610–617.

16 **Leung, M. W., Shen, S. and Lafaille, J. J.,** TCR-dependent differentiation of thymic Foxp3+ cells is limited to small clonal sizes. *J. Exp. Med.* 2009. **206**: 2121–2130.

17 **Malek, T. R. and Bayer, A. L.,** Tolerance, not immunity, crucially depends on IL-2. *Nat. Rev. Immunol.* 2004. **4**: 665–674.

18 **Malek, T. R.,** The main function of IL-2 is to promote the development of T regulatory cells. *J. Leukoc. Biol.* 2003. **74**: 961–965.

19 **Setoguchi, R., Hori, S., Takahashi, T. and Sakaguchi, S.,** Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 2005. **201**: 723–735.

20 **Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. and Rudensky, A. Y.,** A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 2005. **6**: 1142–1151.

21 **Taguchi, O., Kontani, K., Ikeda, H., Kezuka, T., Takeuchi, M., Takahashi, T. and Takahashi, T.,** Tissue-specific suppressor T cells involved in self-tolerance are activated extrathymically by self-antigens. *Immunology* 1994. **82**: 365–369.

22 **Walker, L. S., Chodos, A., Eggena, M., Dooms, H. and Abbas, A. K.,** Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J. Exp. Med.* 2003. **198**: 249–258.

23 **Green, E. A., Choi, Y. and Flavell, R. A.,** Pancreatic lymph node-derived CD4(+)CD25(+) Treg cells: highly potent regulators of diabetes that require TRANCE-RANK signals. *Immunity* 2002. **16**: 183–191.

24 **Samy, E. T., Parker, L. A., Sharp, C. P. and Tung, K. S.,** Continuous control of autoimmune disease by antigen-dependent polyclonal CD4+CD25+ regulatory T cells in the regional lymph node. *J. Exp. Med.* 2005. **202**: 771–781.

25 **Wheeler, K. M., Samy, E. T. and Tung, K. S.,** Cutting edge: normal regional lymph node enrichment of antigen-specific regulatory T cells with autoimmune disease-suppressive capacity. *J. Immunol.* 2009. **183**: 7635–7638.

26 **Nishio, J., Feuerer, M., Wong, J., Mathis, D. and Benoist, C.,** Anti-CD3 therapy permits regulatory T cells to surmount T cell receptor-specified peripheral niche constraints. *J. Exp. Med.* 2010. **207**: 1879–1889.

27 **Rubtsov, Y. P., Niec, R. E., Josefowicz, S., Li, L., Darce, J., Mathis, D., Benoist, C. and Rudensky,A. Y.,** Stability of the regulatory T cell lineage in vivo. *Science* 2010. **329**: 1667–1671.

28 **Wang, C., Sanders, C. M., Yang, Q., Schroeder, H. W., Jr., Wang, E., Babrzadeh, F., Gharizadeh, B. et al.,** High throughput sequencing reveals a complex pattern of dynamic interrelationships among human T cell subsets. *Proc. Natl. Acad. Sci. USA* 2010. **107**: 1518–1523.

29 **Sharma, R., Ju, A. C., Kung, J. T., Fu, S. M. and Ju, S. T.,** Rapid and selective expansion of nonclonotypic T cells in regulatory T cell-deficient, foreign antigen-specific TCR-transgenic scurfy mice: antigen-dependent expansion and TCR analysis. *J. Immunol.* 2008. **181**: 6934–6941.

30 **Zheng, L., Sharma, R., Kung, J. T., Deshmukh, U. S., Jarjour, W. N., Fu, S. M. and Ju, S. T.,** Pervasive and stochastic changes in the TCR repertoire of regulatory T-cell-deficient mice. *Int. Immunol.* 2008. **20**: 517–523.

31 **Correia-Neves, M., Waltzinger, C., Mathis, D. and Benoist, C.,** The shaping of the T cell repertoire. *Immunity* 2001. **14**: 21–32.

32 **Venturi, V., Quigley, M. F., Greenaway, H. Y., Ng, P. C., Ende, Z. S., McIntosh, T., Asher, T. E. et al.,** A mechanism for TCR sharing between T cell subsets and individuals revealed by pyrosequencing. *J. Immunol.* 2011. **186**: 4285–4294.

33 **Lefranc, M. P., Giudicelli, V., Ginestoux, C., Jabado-Michaloud, J., Folch, G., Bellahcene, F., Wu, Y. et al.,** IMGT, the international ImMunoGeneTics information system. *Nucleic Acids Res.* 2009. **37**: D1006–D1012.

34 **Komatsu, N., Mariotti-Ferrandiz, M. E., Wang, Y., Malissen, B., Waldmann, H. and Hori, S.,** Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc. Natl. Acad. Sci. USA* 2009. **106**: 1903–1908.

35 **Koenecke, C., Czeloth, N., Bubke, A., Schmitz, S., Kissenpfennig, A., Malissen, B., Huehn, J. et al.,** Alloantigen-specific de novo-induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD. *Eur. J. Immunol.* 2009. **39**: 3091–3096.

36 **Suffner, J., Hochweller, K., Kuhnle, M. C., Li, X., Kroczek, R. A., Garbi, N. and Hammerling,G. J.,** Dendritic cells support homeostatic expansion of Foxp3+ regulatory T cells in Foxp3.LuciDTR mice. *J. Immunol.* 2010. **184**: 1810–1820.

37 **Kang, S. G., Wang, C., Matsumoto, S. and Kim, C. H.,** High and low vitamin A therapies induce distinct FoxP3$^+$ T-cell subsets and effectively control intestinal inflammation. *Gastroenterology* 2009. **137**: 1391–1402 e1391–1396.

38 **Siewert, C., Lauer, U., Cording, S., Bopp, T., Schmitt, E., Hamann, A. and Huehn, J.,** Experience-driven development: effector/memory-like alphaE+Foxp3+ regulatory T cells originate from both naive T cells and naturally occurring naive-like regulatory T cells. *J. Immunol.* 2008. **180**: 146–155.

39 **Olivares-Villagomez, D., Wang, Y. and Lafaille, J. J.,** Regulatory CD4(+) T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis. *J. Exp. Med.* 1998. **188**: 1883–1894.

40 **Van de Keere, F. and Tonegawa, S.,** CD4(+) T cells prevent spontaneous experimental autoimmune encephalomyelitis in anti-myelin basic protein T cell receptor transgenic mice. *J. Exp. Med.* 1998. **188**: 1875–1882.

41 **Szymczak-Workman, A. L., Workman, C. J. and Vignali, D. A.,** Cutting edge: regulatory T cells do not require stimulation through their TCR to suppress. *J. Immunol.* 2009. **182**: 5188–5192.

42 **Hinterberger, M., Aichinger, M., da Costa, O. P., Voehringer, D., Hoffmann, R. and Klein, L.,** Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. *Nat. Immunol.* 2010. **11**: 512–519.

43 **DiPaolo, R. J. and Shevach, E. M.,** CD4+ T-cell development in a mouse expressing a transgenic TCR derived from a Treg. *Eur. J. Immunol.* 2009. **39**: 234–240.

44 **Kasow, K. A., Chen, X., Knowles, J., Wichlan, D., Handgretinger, R. and Riberdy, J. M.,** Human CD4+CD25+ regulatory T cells share equally complex and comparable repertoires with CD4+CD25- counterparts. *J. Immunol.* 2004. **172**: 6123–6128.

45 **Fujishima, M., Hirokawa, M., Fujishima, N. and Sawada, K.,** TCRalpha-beta repertoire diversity of human naturally occurring CD4+CD25+ regulatory T cells. *Immunol. Lett.* 2005. **99**: 193–197.

46 **Singh, Y., Ferreira, C., Chan, A. C., Dyson, J. and Garden, O. A.,** Restricted TCR-alpha CDR3 diversity disadvantages natural regulatory T cell development in the B6.2.16 beta-chain transgenic mouse. *J. Immunol.* 2010. **185**: 3408–3416.

47 **Olivares-Villagomez, D., Wensky, A. K., Wang, Y. and Lafaille, J. J.,** Repertoire requirements of CD4+ T cells that prevent spontaneous autoimmune encephalomyelitis. *J. Immunol.* 2000. **164**: 5499–5507.

48 **Hori, S., Haury, M., Coutinho, A. and Demengeot, J.,** Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 2002. **99**: 8213–8218.

49 **Adeegbe, D., Matsutani, T., Yang, J., Altman, N. H. and Malek, T. R.,** CD4(+) CD25(+) Foxp3(+) T regulatory cells with limited TCR diversity in control of autoimmunity. *J. Immunol.* 2010. **184**: 56–66.

50 **Ferreira, C., Singh, Y., Furmanski, A. L., Wong, F. S., Garden, O. A. and Dyson, J.,** Non-obese diabetic mice select a low-diversity repertoire of natural regulatory T cells. *Proc. Natl. Acad. Sci. USA* 2009. **106**: 8320–8325.

51 **Ohnmacht, C., Pullner, A., King, S. B., Drexler, I., Meier, S., Brocker, T. and Voehringer, D.,** Constitutive ablation of dendritic cells breaks self-tolerance of CD4T cells and results in spontaneous fatal autoimmunity. *J. Exp. Med.* 2009. **206**: 549–559.

52 **Darrasse-Jeze, G., Deroubaix, S., Mouquet, H., Victora, G. D., Eisenreich, T., Yao, K. H., Masilamani, R. F. et al.,** Feedback control of regulatory

T cell homeostasis by dendritic cells in vivo. *J. Exp. Med.* 2009. **206**: 1853–1862.

53 **Swee, L. K., Bosco, N., Malissen, B., Ceredig, R. and Rolink, A.,** Expansion of peripheral naturally occurring T regulatory cells by Fms-like tyrosine kinase 3 ligand treatment. *Blood* 2009. **113**: 6277–6287.

54 **Wang, Y., Kissenpfennig, A., Mingueneau, M., Richelme, S., Perrin, P., Chevrier, S., Genton, C. et al.,** Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of Foxp3+ regulatory T cells. *J. Immunol.* 2008. **180**: 1565–1575.

55 **Barnden, M. J., Allison, J., Heath, W. R. and Carbone, F. R.,** Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 1998. **76**: 34–40.

56 **Haas, J. D., Gonzalez, F. H., Schmitz, S., Chennupati, V., Fohse, L., Kremmer, E., Forster, R. and Prinz, I.,** CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells. *Eur. J. Immunol.* 2009. **39**: 3488–3497.

57 **Brochet, X., Lefranc, M. P. and Giudicelli, V.,** IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res.* 2008. **36**: W503–W508.

58 **Magurran, A. E.,** *Ecological Diversity and Its Measurement*, Princeton University Press, NJ, USA 1988, p. 192.

Abbreviations: **HT:** high throughput · **LPL:** lamina propria lymphocytes · **pLN:** peripheral lymph nodes

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