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The Contained Self-Reactive Peripheral T Cell Repertoire: Size, Diversity, and Cellular Composition

David M. Richards,* Eliana Ruggiero,† Ann-Cathrin Hofer,* Julian P. Sefrin,* Manfred Schmidt,‡ Christof von Kalle,‡ and Markus Feuerer*

Individual self-reactive T cells have been discovered in both humans and mice. It is difficult to assess the entire contained self-reactive peripheral T cell repertoire in healthy individuals because regulatory T cells (Tregs) can render these cells anergic and, therefore, functionally indistinguishable. We addressed this issue by removing regulatory T cells, thereby allowing us to characterize the exposed self-reactive T cells. This resulted in activation of approximately 4% of both CD4+ and CD8+ T cells. Activation and division of these cells was not a bystander product of Ag-independent signals but required TCR stimulation. Analysis of TCR sequences showed that these responding cells were polyclonal and encompassed a broad range of structural TCR.

In addition, transfer of mature thymocytes showed that this response was an intrinsic T cell property and not a peripheral adaptation. Finally, we found that the unexpectedly strong contribution of the naive CD5low T cell pool was due to anergic T cell precursors. In contrast to mature T cells, these naive T cells are known to escape thymic selection and, therefore, functionally indistinguishable. We addressed this issue by removing regulatory T cells, thereby allowing us to characterize the exposed self-reactive T cells. This resulted in activation of approximately 4% of both CD4+ and CD8+ T cells. Activation and division of these cells was not a bystander product of Ag-independent signals but required TCR stimulation. Analysis of TCR sequences showed that these responding cells were polyclonal and encompassed a broad range of structural TCR.

Suppression by Foxp3+ regulatory T cells (Tregs) has been shown to be critical for maintaining immune homeostasis and bringing immune responses to a proper resolution. In fact, in mice and humans, absence of Tregs results in hyperimmune activation, autoimmune disease, and eventually death (5–11).

With increasing success of cancer immunotherapy via immune-checkpoint blockade inhibitors leading to durable responses in diverse types of advanced cancers, it becomes mandatory to understand the clinical implications ensuing from exposure of these potentially harmful self-reactive T cells (12–14). In fact, recent clinical trials with anti–CTLA-4 and anti-PD1 Abs uncovered that T cell reactivity against self is unleashed by these treatments. This now constitutes a major limitation for therapies that interfere with tolerance and immune homeostasis (15).

Currently, fundamental aspects of these hidden, potentially harmful self-reactive T cells are unknown, including their precursor frequency, TCR diversity, Ag specificity, and preactivation status. Individual self-reactive T cells have been identified in both mice and humans (16–18). These results showed that the frequency of T cells for a particular self-Ag was similar to that for viral Ags (18, 19). Although these approaches identify individual self-Ags, they are unsuitable for quantifying and characterizing the entire pool of self-reactive T cells. For example, is the true magnitude of the self-reactive peripheral T cell pool in the range of 1 in 10,000 T cells or 1 in 10 T cells? Recently, it was shown that Tregs are able to induce anergy in self-reactive CD8+ T cells (17). These anergic self-reactive T cells have a naive phenotype and are therefore difficult to identify, but they can be found in healthy individuals using MHC tetramers (17). These results suggest that the total self-reactive T cell repertoire has so far escaped our attention because it can only be accurately visualized in the absence of Treg-mediated control. In this study, we eliminated Tregs in healthy adult mice to characterize the entire contained self-reactive peripheral T cell repertoire including the size, the diversity, and the cellular composition.

Materials and Methods

**Mice**

Wild-type (WT) C57BL/6 (B6; CD45.2+CD90.2+), congenic B6.SJL-Ptprc<sup>−/−</sup>Pepcb<sup>−/−</sup>BoyCrl (CD45.1+; Charles River Strain Code: 494), congenic B6.PL-Thy1<sup>−/−</sup>CyJ (CD90.1+; Jackson stock no.: 000406), and Nr4a1-eGFP

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*Immune Tolerance, Tumor Immunology Program, German Cancer Research Center, 69120 Heidelberg, Germany; and †Division of Translational Oncology, German Cancer Research Center and National Center for Tumor Diseases, 69120 Heidelberg, Germany

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus under accession number GSE70154.

Address correspondence and reprint requests to Dr. Markus Feuerer, Immune Tolerance, Tumor Immunology Program, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. E-mail address: m.feuerer@dkfz-heidelberg.de

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Treg depletion

Tregs were depleted by i.p. injection of 40 ng/g body weight diphtheria toxin (DT)/Streptococcus pyogenes (S. pyogenes) into 200 μl PBS on 2 consecutive days. In the experiments using adoptive transfer, the first dose of DT was administered immediately after i.v. injection of T cells and the second dose 24 h later.

Preparation of cells

For enrichment and depletion, cells were labeled with biotin-conjugated Abs and Miltenyi anti-biotin microbeads. Magnetic cell separation was performed using an AutoMACS Pro Separator (Miltenyi). Samples FACS-sorted for adoptive transfer experiments were first depleted of CD19+ cells. For analysis after adoptive transfer of CD5 subsets, spleen samples were first enriched for donor CD90.1+ cells.

T cell adoptive transfer

MACS- or FACS-purified populations of T cells were injected i.v. into CD45.1+ or CD90.1+ recipient mice. Between 1 and 5 × 10^5 T cells were transferred into each recipient except for CD5 transfer experiments where only 5 × 10^5 T cells were transferred.

Tissue isolation and sample preparation

For tissue isolation, mice were killed, the spleen, thymus, and lymph nodes (LN) were removed, brains, and gonads were removed, and organs were processed by mashing and filtering to prepare single-cell suspensions. Ammonium chloride–potassium bicarbonate lysis buffer was used to lyse erythrocytes in spleen samples. The liver, lung, and pancreas were isolated, mechanically dispersed, and digested for 30 min at 37°C in DMEM (Life Technologies) plus 1 mg/ml collagenase type IV (C1538, Sigma), 0.5% (w/v) BSA (Sigma), and 20 μg/ml DNase I (Roche). Samples were enriched for lymphocytes by centrifugation through a Percoll gradient (40 and 80% solutions; GE Healthcare).

Flow cytometry and FACS

For flow cytometry, nonspecific binding of Abs to cells was blocked by incubation with rat and hamster serum [1% (v/v) each] and cells were labeled with the following Abs (clone) or streptavidin: CD4 (RM4-5 or GK1.5), CD8α (53-6.7), CD19 (6D5), IFN-γ (XM1.2), IL-4 (11B11), IL-10 (JES5-16E3), IL-17A (TC11-18H10.1), CD69 (H1.2F3), CD44 (IM7), CD62L (MEL-14), CD5 (53-7.3), CD24 (M1/69), CD45.1 (A20), CD90.1 (OX-7), TCRβ (H57-597), TCRVα2 (B20.1), TCRVβ6 (R4-7), TCRVα8.3 (B21.14), TCRVα11/11.2 (R8-1) (BD Pharmingen, Biologen, or eBioscience). Cells were analyzed using the BD Biosciences Canto II or LSR II. For FACS purification of cells, the BD Biosciences FACSaria II or III instruments were used. Ab quality was checked and gating was performed using isotype controls. Purity of cell sorts was assured through postsort analysis and postsort staining. Tree quality was checked and gating was performed using isotype controls. Purity of cells, the BD Biosciences FACSAria II or III instruments were used. Ab 11.2 (RR8-1) (BD Pharmingen, Biolegend, or eBioscience). Cells were an-

Intracellular cytokine expression

For intracellular cytokine staining, single-cell suspensions were made and the cells were stimulated with PMA (50 ng/ml; Sigma) and ionomycin (1 nM; Calbiochem) for 4 h at 37°C. We added GolgiStop (BD) to the culture at the recommended concentration during the last 3 h. We stained cells with mAbs specific for surface markers and then fixed and permeabilized them according to the manufacturer’s instructions (eBioscience), followed by intracellular staining for cytokines.

Cell counting

AccuCheck+ Counting beads (Invitrogen) were added to the sample before acquisition, and cell counts were determined according to manufacturer’s directions. For some experiments, cell count was determined by com-

FlowJo Proliferation Tool.

Analysis of TCR repertoire diversity

For the characterization of the TCR repertoire diversity, ∼5 × 10^5 FACS-purified cells from each of the indicated groups were analyzed. RNA was extracted from FACS-purified cells using the RNeasy Plus Micro Kit (Qiagen). DNase digestion was performed using the Turbo DNA-free kit (Ambion). cDNA was synthesized using the Superscript II (Invitrogen) and 100 ng was used for the analysis of the TCR diversity by an adapted nonrestrictive linear-amplification mediated PCR (nr/LAM-PCR) (25, 26). PCR amplicons were purified using the Agencourt Ampure beads (Beckman Coulter), and a fusion PCR was performed to add the 454 specific sequencing adaptors containing 6- to 10-bp barcodes. For the fusion PCR, 40 ng DNA was amplified with the following program: initial denaturation for 120 s at 95°C, 12 cycles at 95°C for 45 s, 58°C for 45 s, 72°C for 60 s, and final elongation for 300 s at 72°C. After sequencing, raw reads were sorted according to the individual barcodes used for each sample, then trimmed and aligned to TCR genes. CDR3 sequences were defined according to the ImMunoGeneTics nomenclature as those delimited by the last conserved cysteine of the V gene and the conserved phenylalanine in the FGGX motif of the J gene.

Diversity determination

To assess the diversity of the TCR repertoires in the analyzed samples, we calculated two diversity indices. On average, 2200 TCR sequences were recovered from each sample. The Shannon index is used to define the richness of a population (i.e., number of unique TCR clones), whereas the Simpson index defines the heterogeneity of the population (i.e., distribution unique TCR clones) (27, 28). Retrieval frequency for V β and J β gene usage was calculated for all samples and represented with heat maps. In addition, the quantitative contribution of individual CDR3 sequences was determined by counting identical sequences and is represented as a percentage of all TCR β-chain sequences.

RNA expression profiling

Naïve CD4+ CD5low, CD5+, and CD5hi T cell populations were FACS purified from three female and three male mice, to account for possible sex differences. RNA was extracted using the RNaseasy Plus Micro Kit (Qiagen). The DKFZ Genomics and Proteomics Core Facility amplified and hybridized material to the Illumina MouseWG-6 v2.0 Expression BeadChip. A total of 46,238 probes were further analyzed. To maintain an acceptable range of replicate variation, a coefficient of variation <0.8 was acceptable, which eliminated 45 probes from 46,238 probes total from the analysis.

Accession codes

Microarray data have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE70154.

Statistics

Statistical analysis of experimental data was performed using Prism software (GraphPad) or Excel (Microsoft). Employed statistical tests and corresponding parameters are mentioned in the figure legends. Results were considered statistically significant if p <0.05.
Results

**CD4**^+ and CD8**^+ T cells infiltrate peripheral tissue and produce inflammatory cytokines after transient Treg depletion**

To examine self-reactivity in a natural and polyclonal immune repertoire, we used a mouse model of DT receptor (DTR)–mediated Treg ablation (9). To better understand the development of this fatal autoimmune disease, we investigated T cell infiltration into peripheral tissues as well as inflammatory cytokine production after removal of Treg-mediated control. We treated recipients twice with DT and therefore only transiently depleted Tregs. Ten days after treatment, significantly more IFN-γ–producing CD4**^+** and CD8**^+** T cells were found in the spleens of DT-treated Foxp3–DTR**^+** (DTR**^+**) mice (Fig. 1A–D). In addition, there was an increase in the percentage of IL-4–producing but not IL-10– or IL-17A–producing CD4**^+** T cells (Supplemental Fig. 1A–D).

Treg depletion also resulted in an increase in CD4**^+** and CD8**^+** T cell infiltration in the lungs and livers of DTR**^+** mice treated with DT (Fig. 1E, IF). This infiltration corresponded with an increase in the percentage of T cells producing IFN-γ (Supplemental Fig. 1E, IF) and, more strikingly, a 20-fold increase in the number of CD8**^+** T cells expressing non-Tg TCRs (Fig. 3F–H). To determine whether this effect was specific for the TEa TCR-Tg or CD4**^+** T cells, we repeated these experiments with a second CD4**^+** TCR-Tg mouse model, as well as a CD8**^+** TCR-Tg mouse model (chicken OVA peptide–specific OT2 and OT1, respectively), and found similar results (Fig. 3I–K and data not shown).

These data indicated that these T cells of defined specificities were unresponsive because their stimulatory Ags were not present in the host environment and Ag-independent signals were not sufficient for activation, proliferation, and differentiation of these T cells despite the fact that the WT recipient T cells were vigorously activated. In contrast, TEa T cells responded dramatically to the absence of Treg-mediated control when their cognate Ag was provided by B16 tumors expressing the specific TEa Ag (Fig. 3L).

**Kinetics of the self-reactive T cell response after Treg depletion**

To delineate features of these self-reactive T cells within the heterogeneous peripheral T cell compartment, we performed a comprehensive analysis of T cell activation and differentiation at multiple time points after removal of Treg-mediated control. Within 24 h after DT treatment, there was a significant increase in the proportion of CD69CD4**^+** T cells in the spleens of DTR**^+** mice compared with WT B6 (or DTR**^-** ) mice (Fig. 2A, 2B; Supplemental Fig. 2A). This difference peaked on day 2 and decreased, but remained significant, by day 7. The activation of CD8**^+** T cells followed a very similar pattern (Fig. 2C, Supplemental Fig. 2B, 2C). In addition, throughout our investigation, we found no differences between DTR**^-** mice treated with DT and DTR**^+** mice treated with PBS (Supplemental Fig. 1B and data not shown).

By day 2, the responding CD4**^+** T cells had also begun to differentiate. The proportion of CD44**^high**CD62L**^low**CD4**^+** T cells in the spleens was significantly higher on day 2 and continued to increase through day 7 when >60% of the cells correspond to activated effector/memory cells (Fig. 2D, 2F; Supplemental Fig. 2D). The differentiation of CD8**^+** T cells also followed a very similar pattern (Fig. 2E, 2G; Supplemental Fig. 2E). Finally, similar findings were observed in other secondary lymphoid tissues including the skin draining LNs (Supplemental Fig. 2F, 2G, and data not shown).

**Responding self-reactive T cells have TCR-specific stimulatory signals**

T cells have the ability to respond to a number of Ag-independent signals in the microenvironment, including cytokines and space (29, 30). TCR-Tg cells have been extensively used to study this phenomenon. For example, TCR-Tg CD4**^+** “TEa” and CD8**^+** “OT1” T cells have been shown to vigorously proliferate and differentiate when transferred into irradiated or genetically lymphopenic recipients (31–33). To determine whether the activated and proliferating T cells in our system were responding to TCR-specific signals, we used a coadoptive transfer system to compare a polyclonal WT with a monoclonal defined specificity T cell repertoire.
These three independent assays revealed that an unexpected high number, about 4%, of CD4+ and CD8+ T cells are permanently controlled by Tregs.

The self-reactive T cell population is highly diverse

Our analysis of the precursor frequency of the self-reactive T cell pool suggested that a considerable number of T cells responded in the absence of Treg-mediated control. To obtain a more precise estimate of the size and diversity of this responding population, we FACS-purified CD4+ T cells from individual mice at multiple time points and subjected the CDR3 regions to deep sequencing using a modified PCR-based approach (25, 26). Conventional CD4+ T cells and Tregs from untreated mice were compared with early-activated cells (CD69+) from day 2, naive nonresponding cells (CD62LhighCD44low/int) from day 7, and responding effector/memory cells (CD62LlowCD44high) from day 7 after Treg depletion.

FIGURE 1. CD4+ and CD8+ T cells infiltrate peripheral tissue and produce inflammatory cytokines after transient Treg depletion. (A–D) Expression of IFN-γ in CD4+ (A and C) or CD8+ (B and D) T cells isolated from the spleens of DTR+ mice 10 d after treatment with DT or PBS. (E and F) Quantification of CD4+ (E) or CD8+ (F) T cell infiltrate in the pancreas, lungs, and livers of DTR+ mice 10 d after treatment with DT or PBS. (G and H) Expression of IFN-γ in CD4+ (G) or CD8+ (H) T cells isolated from the lungs of DTR+ mice 10 d after treatment with DT or PBS. (I and J) Quantification of IFN-γ+ CD4+ (I) or IFN-γ+CD8+ (J) T cell infiltrate in the pancreas, lungs, and livers of DTR+ mice 10 d after treatment with DT or PBS. Representative plots (A, B, G, and H) and data from two independent experiments (C–F, I, and J). Symbols represent individual mice and numbers indicate percentage of cells within the defined region. Horizontal lines indicate the mean value for each group. p values represent comparisons between DT and PBS samples using an unpaired t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
To assess the diversity of the TCR repertoires in the analyzed samples, we used the Shannon index to define the richness of the populations, that is, number of unique TCR clones, and the Simpson index was used to define the heterogeneity of the populations, that is, distribution of unique TCR clones. The calculated results failed to show major variations in either index, indicating comparable TCR repertoire diversity in all samples (Fig. 5A, 5B). The retrieval frequency for Vβ and Jβ gene usage, a more broad analysis, also failed to show any differences in repertoire diversity (Fig. 5C, 5D). In addition, calculating the quantitative contribution of individual CDR3 sequences showed that there were no differences between these groups (Fig. 5E). We also found no enrichment in the usage of

FIGURE 2. Kinetics of the self-reactive T cell response after Treg depletion. (A–C) Quantification of CD69 expression on CD4+ (A and B) or CD8+ (C) T cells isolated from the spleens of DTR+ or DTR− mice treated with DT between 0 and 7 d. (D–G) Expression of CD62L and CD44 on CD4+ (D and F) or CD8+ (E and G) T cells isolated from the spleens of DTR+ or DTR− mice treated with DT between 0 and 7 d. Representative plots from a single experiment covering the entire time course (A, D, and E) and data from two or more independent experiments at each time point (B, C, F, and G). Symbols represent individual mice and numbers indicate percentage of cells within the defined regions. Horizontal lines indicate the mean value for each group. The p values represent comparisons between DTR+ and DTR− samples at the same time point using an unpaired t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
FIGURE 3. Responding self-reactive T cells have TCR-specific stimulatory signals. (A) Cartoon depicting the congenic strategy for coadoptive transfer experiments. CD90.1 expression distinguishes recipient T cells from both donor populations. CD45.1 expression distinguishes TCR-Tg TEa T cells from WT T cells. (B–E) Expression of CFSE and CD44 by cotransferred CD4⁺ TEa and WT T cells isolated from spleens of DTR⁺ (Figure legend continues)
a second TCR α-chain after Treg depletion (Fig. 5F–H). Altogether, this analysis showed that neither the early-activated population at day 2 nor the responded population at day 7 was oligoclonal, indicating that TCRs of broad diversity are able to respond in the absence of Treg-mediated control.

**The naive T cell pool contains self-reactive T cell precursors**

Because the deep sequencing data did not reveal any dominant self-reactive T cell clones, we attempted to further define these cells. Naive T cell clones isolated from spleens of DTR+ mice 7 d after adoptive transfer and treatment with DT or PBS. Representative plots (outside of the R1 gate in mice 7 d after adoptive transfer and treatment with DT or PBS. (A) Expression of CD44 and CFSE, and CFSE-based “response index” calculated for transferred CD4+ (G and I) or CD8+ (H and J) T cells isolated from spleens of DTR+ mice 2 d after adoptive transfer and treatment with DT or PBS. (B) Quantification of CFSE (I) or unpaired t test. **p < 0.001, ***p < 0.0001.

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memory T cells was visible in PBS-treated recipients and, in general, the recovery of transferred effector/memory cells was not as efficient as the recovery of transferred naive cells (Supplemental Fig. 3B, 3E, 3F). This finding is presumably due to differences in survival and

FIGURE 5. The self-reactive T cell population is highly diverse. (A and B) Shannon (A) and Simpson (B) indices were calculated to assess the diversity of the β-chain TCR repertoires in FACS-purified conventional CD4+ T cells, Tregs, activated T cells isolated 2 d after DT treatment (CD69+; 2 d DT act), and naive (CD62LhighCD44low/int; 7 d DT naive) or effector/memory (CD62LlowCD44high; 7 d DT eff/mem) T cells isolated 7 d after DT treatment. Gates are defined in Fig. 2A and 2D. (C and D) Retrieval frequency (as a percentage of total sequences) for V gene (C) and J gene (D) usage was calculated for all samples and plotted as heat maps. (E) The quantitative contribution of individual CDR3 sequences, determined by counting identical sequences, is represented as a percentage of total sequences. Colored bars represent the 10 most frequent CDR3 sequences, and gray bars represent the remaining TCR sequences. Dotted lines (A, B, and E) indicate historical data depicting examples of reduced TCR repertoire diversity. (F–H) Analysis of dual TCR α-chain expression. Representative plots showing coexpression of TCR Va2 and Va11.1/11.2 (F) and quantification of dual TCR α-chain expression among indicated Va subsets (G and H) by CD4+ T cells isolated from spleens of DTR+ mice 7 d after treatment with DT or PBS. Symbols represent individual mice, and numbers indicate percentage of cells within the defined region.
migration into peripheral tissues. The naive pool of CD8⁺ T cells had a lower proportion of responsive cells as compared with the CD4⁺ naive pool (Supplemental Fig. 3C, 3D).

To determine whether this response was a peripheral adaptation or an intrinsic T cell property, we FACS-purified mature CD4 single-positive (SP) thymocytes and transferred them into DTR⁺ recipients. Seven days after treatment with PBS or DT, we found that the donor CD4⁺ SP thymocytes had the same ability to respond in the absence of Treg-mediated control as transferred naive peripheral CD4⁺ T cells (Fig. 6C–E).

Furthermore, we wanted to determine whether the self-reactive T cell response was stable and whether reintroduction of Tregs could regain control of this response. Therefore, we treated DTR⁺ recipients with DT, harvested responding and nonresponding cells, and retransferred them into second DTR⁺ recipients. Specifically, 7 d after treatment with DT, responding effector/memory (CD62Lₕ₁₉ CD44ₘ₉) and nonresponding naive (CD62Lₕ₁₉ CD44ₘ₉) T cells were FACS purified, CFSE labeled, and transferred into second DTR⁺ recipients receiving PBS or DT. On day 7 after Treg depletion in the second recipient, the majority of nonresponding CD4⁺ T cells had proliferated (Fig. 6F, 6G, Supplemental Fig. 3E). This suggested that not all of the self-reactive T cells were able to respond during the first round of Treg depletion. Interestingly, unlike the CD4⁺ T cells, very few nonresponding CD8⁺ T cells showed evidence of proliferation, suggesting that this population did not contain many more self-reactive T cells (Supplemental Fig. 3F–H). In addition, almost all of the responding CD4⁺ and CD8⁺ T cells proliferated during the second round of Treg depletion. In contrast, responding CD4⁺ and CD8⁺ T cells transferred into Treg-replete (PBS-treated) recipients did not proliferate as much, suggesting that Tregs were able to regain control of self-reactive cells.

Self-reactive T cells are present in both the CD5-low and the CD5-high naive T cell pools

To further characterize the self-reactive T cell population and determine whether there are subcompartments within the naive T cell pool that are more likely to respond in the absence of Treg-mediated control, we focused on CD5. CD5 is a membrane protein that is

**FIGURE 6.** The naive T cell pool contains self-reactive T cell precursors. (A and B) Representative plots (A) and quantification (B) of CFSE expression by transferred CD4⁺ T cells isolated from spleens of DTR⁺ mice 7 d after adoptive transfer and treatment with DT or PBS. Transferred (Ts) cells: naive and effector/memory (eff/mem) cells (defined in Supplemental Fig. 3A) were FACS purified from pooled LN and spleen (SPL) isolated from unmanipulated DTR⁺ animals. (C–E) Representative plots (C) and quantification (D and E) of CD44 and CFSE expression by transferred CD4⁺ T cells isolated from spleens of unmanipulated DTR⁺ mice 7 d after adoptive transfer and treatment with DT or PBS. Ts cells: mature CD4SP thymocytes were FACS purified from thymi isolated from unmanipulated DTR⁺ animals. (F and G) Representative plots (F) and quantification (G) of CFSE expression by transferred CD4⁺ T cells isolated from spleens of DTR⁺ mice 7 d after adoptive transfer and treatment with DT or PBS. Ts cells: naive and eff/mem T cells (defined in Supplemental Fig. 3A) were FACS purified from spleens isolated from DTR⁺ animals 7 d after treatment with DT. Representative plots (A, C, and F) and data from two or more independent experiments (B, D, E, and G). Symbols represent individual mice and numbers indicate percentage of cells within the defined region. Horizontal lines indicate the mean value for each group.
FIGURE 7. Self-reactive T cells are present in the CD5<sup>low</sup> naive T cell pool. (A) CD44 expression by CD5<sup>low</sup> and CD5<sup>high</sup> CD4<sup>+</sup> populations isolated from spleens of DTR<sup>+</sup> mice 7 d after treatment with DT or PBS. Analysis scheme and representative plots are shown in Supplemental Fig. 4A and 4B. (B) FACS purification scheme for 10% CD5<sup>low</sup>-, CD5<sup>int</sup>-, and CD5<sup>high</sup>-expressing naive CD4<sup>+</sup> populations. (C) Fold-change by p value plot of gene expression of CD5<sup>low</sup> versus CD5<sup>high</sup> populations defined in (B). (D) Heat map and hierarchical clustering of genes with significantly different expression comparing CD5<sup>low</sup>, CD5<sup>int</sup>, and CD5<sup>high</sup> CD4<sup>+</sup> populations (n = 6, three female and three male). Genes with greatest (red) or least (blue) transcript abundance in each group, presented as Pearson’s correlation (row normalized). (E) CD6 and Ly6C expression by CD5 populations (defined in B) (Figure legend continues).
associated with the TCR and its expression is correlated with higher signaling intensity, presumably because of higher TCR self-MHC/peptide affinity, during positive selection (1, 36). CD5 levels, hard-wired during thymic selection, correlate with higher basal CD3 \( \gamma \) chain phosphorylation and, therefore, CD5\textsuperscript{high} T cells might be more prone to respond against self (1, 36–38). We first compared the expression of CD5\textsuperscript{DTR\textsuperscript{low}} and effector/memory markers on T cells in the absence of Treg-mediated control. In all experiments, Tregs were carefully excluded from this analysis, because they are biased toward CD5\textsuperscript{DRO} expression, presumably because their TCRs have, on average, a higher affinity toward self-peptide. Four days after treatment of DTR\textsuperscript{+} recipients with PBS or DT, the CD4\textsuperscript{+} T cell population was divided into the bottom 20% and the top 20% based on CD5 expression and examined for signs of activation measured by upregulation of CD44 (Fig. 7A, Supplemental Fig. 4A, 4B). There was a significant increase in CD44 expression in both populations, demonstrating that CD5\textsuperscript{low} T cells also had the ability to respond in the absence of Treg-mediated control. However, the higher response in the CD5\textsuperscript{high} population is consistent with previous data (1, 36, 38).

We further characterized the CD5 subsets, based on global gene expression profiling and functional analysis. FACS was used to purify three distinct populations of naïve CD4\textsuperscript{+} T cells from the bottom, middle, and top 10% based on CD5 expression: CD5\textsuperscript{low}, CD5\textsuperscript{int}, and CD5\textsuperscript{high} (Fig. 7B, Supplemental Fig. 4C). CD4\textsuperscript{+} CD5 subsets were isolated from three female and three male mice, to account for possible sex differences. A fold-change comparison of CD5\textsuperscript{low} versus CD5\textsuperscript{high} revealed an overall very similar gene expression pattern with only a few genes being differentially expressed (Fig. 7C, 7D). These subtle differences were reproducible and common between male and female cells (Fig. 7D). Those differences included genes like Cd6 and Ly6c, which could be confirmed by flow cytometry and showed a perfect correlation between CD5 and CD6 expression (Fig. 7E, Supplemental Fig. 4D). Interestingly, the CD5\textsuperscript{low} population was truly intermediate with the expression of most of the differential expressed genes lying between the CD5\textsuperscript{low} and CD5\textsuperscript{high} T cell populations. Although no major differences were observed on the gene expression level, based on previous data, we tested whether significant differences existed on the functional level. After FACS purification of the three CD5 subsets from the naïve CD4\textsuperscript{+} T cell pool and adoptive transfer, we treated the mice with DT or PBS, waited for 4 d, and then subjected entire spleen samples to MACS enrichment to collect sufficient cells for analysis (Supplemental Fig. 4E, 4F). First, our results confirmed the stability of CD5 expression after adoptive transfer and Treg depletion (Supplemental Fig. 4G). In addition, all three populations responded by proliferating and upregulating CD44 expression (Fig. 7F, 7G, Supplemental Fig. 4H). In fact, CD5\textsuperscript{low} cells responded significantly more than CD5\textsuperscript{high} cells, whereas CD5\textsuperscript{int} cells had an intermediate phenotype. The unexpectedly strong contribution of the CD5\textsuperscript{low} T cell pool to the self-reactivity implies that the overall self-reactive response has not only a diverse polyclonal TCR repertoire but also comprises a broad range of affinities for self.

**Discussion**

The transient Treg depletion protocol described in this article activated a hidden population of self-reactive CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. We analyzed the magnitude, diversity, and cellular composition of this response.

It has been demonstrated that T cells have the ability to respond to a number of Ag-independent signals in the microenvironment. Collectively, this is called T cell homeostatic expansion (or proliferation) and can be triggered by lymphopenia (29, 30). DT treatment results in the elimination of 10–15% of the CD4\textsuperscript{+} T cell compartment (i.e., Tregs), and a recent report showed a transient lymphopenia associated with the Foxp3-DTR model (39). Therefore, we used adoptive transfer of multiple TCR-Tg T cells to examine this phenomenon. Our data proved that these T cells of defined specificities were unresponsive after DT treatment because: 1) their stimulatory Ags were not present in the host environment; and 2) Ag-independent signals were not sufficient for activation, proliferation, and differentiation of these T cells despite the fact that the polyclonal host T cells were vigorously activated.

We more precisely estimated the size of the self-reactive pool with three different methods. Activation was measured by CD69 expression, TCR signaling by Nr4a1 (Nur77) induction, and proliferation by CFSE. In each case, we back-calculated the percentage of responsive cells in the original population. Our data revealed that approximately 4% of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells are self-reactive and normally controlled by Tregs. In fact, this estimate is likely to be too conservative for CD4 T cells because not all self-reactive T cells responded after the first round of depletion as revealed by our retransfer experiments. Intriguingly, our data indicate that the size of the self-reactive peripheral T cell pool is in the range of the responses to allo-MHC complexes (1–10%) (24) or superantigens (5–20%) (40). This surprisingly high proportion of self-reactive T cells so far escaped our attention because the full population of self-reactive T cells can only be accurately visualized in the absence of Tregs. Previously, at the level of individual Ag-specific T cells, the frequency of CD8\textsuperscript{+} T cells for a particular self-Ag was shown to be similar to that for viral Ags, that is, in the range of <0.0001% as identified by specific peptide/MHC tetramers (18, 19). It should, however, be pointed out that frequency measurements by tetramer binding assays identify individual specificities, whereas our functional assays analyzed the entire contained pool of self-reactive T cells.

One week after the removal of Treg-mediated control, the majority of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the LN and spleen expressed effector/memory markers and had undergone at least six rounds of cell division. This is reminiscent of viral infections where up to 1–10% of CD8\textsuperscript{+} T cells can be virus specific on day 7. However, unlike viral infections where an initially broad TCR repertoire undergoes a dramatic contraction of diversity because of outgrowth of dominant clones (41), TCR sequencing analysis showed that the early responding T cells on day 2 were not oligoclonal, and the expanded population at day 7 did not converge to oligoclonality. This suggests that a broad spectrum of self-reactive TCRs exists, which can expand while still retaining its diversity. Our findings are in line with recently published observations showing that TCR diversity after CTLA-4 blockade in advanced melanoma patients was increased rather than reduced (42). This is not to say that self-reactive T cells infiltrating a particular organ retain the full scope of diversity.

Our data show that the hidden self-reactivity is not a peripheral adaptation but an intrinsic property of the postselection thymic T cell repertoire, because mature CD4 SP thymocytes already displayed this degree of self-reactivity. This raises the question of why such a high number of self-reactive T cells could pass the
quality-control step of negative selection, which is known to be highly specific, sensitive, and efficient. Is this a sign of incomplete negative selection, and thus potentially harmful, or is it possibly beneficial? It has been argued that too stringent negative selection would leave only an impaired repertoire. So those self-reactive T cells escaping negative selection are presumably useful in some context such as pathogen responses or tissue homeostasis. For example, it has been repeatedly reported that “physiological” (as opposed to pathological) self-reactive T cells play important roles in the homeostasis of processes as diverse as brain function (43, 44), hematopoiesis (45), and wound healing (46). In contrast, it is clear that self-reactive T cells can be harmful and, importantly, it has been shown that T cells with low avidity to self-Ags can escape thymic negative selection (47, 48) and can be restricted to ubiquitous self-Ags (16).

It has recently been proposed that the peripheral T cell repertoire could be subdivided into small “colonies” that share common nonstimulatory subthreshold ligands but have otherwise independent cognate ligands (49). The self-reactive T cell pool described in this article could conceivably share such subthreshold ligands that are derived from ubiquitous self-Ags. Some of the T cells responding after the removal of Treg-mediated control may actually be foreign-reactive, but vigorously respond to previously nonstimulatory subthreshold self-Ags because of changes in the activation status of APCs or the absence of direct suppressive effectors, like IL-10 or TGF-β. Indeed, it has been reported that the number and the activation status of DCs increased after Treg depletion, thus potentially providing stronger costimulation and a higher density of peptide-loaded MHC molecules (9). In addition, removal of Treg-mediated control could result in significant changes in the quality and/or quantity of Ags being presented by APCs. For example, the formation of immunoproteasomes could be induced in APCs, which, in turn, would generate altered peptide libraries that are presented on MHC molecules. The formation of immunoproteasome has been described after exposure to IFN-γ or TNF-α (50). Alternatively, the recently described anergic self-reactive T cells that can be identified in healthy individuals could be reactivated by the loss of Treg-mediated control (17).

Although we designated all T cells that became activated after Treg depletion as self-reactive because they require TCR interactions, they might still be heterogeneous with respect to the degree of self-reactivity. Although it is unclear whether all the activated T cells are capable of damaging tissues, we believe that being above the activation/proliferation threshold distinguishes these cells from the nonresponsive majority. The degree of self-reactivity of the nascent T cell repertoire is thought to be linked to the process of self-MHC recognition during positive selection in the thymus and to depend on the nature of the self-peptide(s) presented by cortical thymic epithelial cells (1). CD5 expression levels on T cells reflect the signaling strength of the TCR self-peptide/MHC interaction during positive selection, and these “tuned” levels become hardwired and thus maintained in the mature peripheral T cell population (37). Furthermore, it has been suggested that re-exposure of peripheral T cells to their positive-selecting ligand is essential for homeostasis through tonic TCR signals (51). CD5low T cells are thought to be less effective in homeostatic proliferation, and CD5high T cells were shown to have a head start in antipathogen responses (36, 38, 52). These findings could be caused by higher basal CD3 ε-chain phosphorylation levels found in CD5high T cells; therefore, they might be more prone to respond against self (1, 36). In a recent report describing the gene expression profile of naïve CD5high and CD5low CD8+ T cells, a small number of differentially expressed genes were identified that showed CD5high T cells would be better poised for initial activation than CD5low T cells (38). We analyzed whether the Treg-contained self-reactive T cell pool would show any bias with respect to this intrathymic tuning of TCR signal strength. The broad distribution of CD5 on the reactive T cell pool does not support the idea that CD5high T cells are more prone to self-reactivity. We rather found a stronger response in the CD5low T cell compartment. How these observations at the population level relate to findings reported for particular TCRs remains open.

So far, we can only speculate on the spectrum and identity of the self-Ags recognized by the self-reactive T cells and to which extent sets of tissue-specific self-Ags drive the observed T cell expansion in the various organs. One intriguing possibility is that some of the self-reactive T cells are specific for commensal Ags. Commensal products may be considered as part of the extended “immunological self” because uncontrolled immune responses against commensal products cause autoimmune-like pathology in the gut (53). Yet, because ablation of Tregs in germ-free mice also resulted in a systemic autoimmune syndrome with tissue inflammation comparable with those in specific pathogen-free housed mice, self-reactive T cells are unleashed regardless of the presence of commensal microbiota (9).

The recent success of immune-checkpoint blockade inhibitors in cancer treatment has shown that interfering with peripheral tolerance is a promising treatment strategy (12–14). Yet, the majority of cancer patients receiving immune-checkpoint blockade inhibitor therapy will develop autoimmune complications (15). In line with the findings reported in this article, these opportunistic autoimmune diseases seem to differ from classic organ-specific autoimmune diseases in their broader range of affected organs. Classic autoimmune diseases target specific tissue Ags such as pancreatic β cell Ags in type 1 diabetes or myelin sheath components in multiple sclerosis. These treatment-associated opportunistic autoimmune diseases are broader and more reminiscent of the breakdown of thymic tolerance as seen with AIRE deficiency and the corresponding disease autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (54). Interfering with TGF-β receptor signaling in T cells, as well as deficiencies of Foxp3, CTLA-4, or IL-2 signaling, also lead to systemic autoimmune diseases caused by self-reactive T cells, indicating that manipulation of these pathways can also unleash the heterogeneous and otherwise hidden self-reactive T cell pool (55–61). Encouragingly, our retransfer experiments showed that Tregs could regain control of the responding self-reactive T cells, pointing to a possible strategy to treat adverse immune episodes after immunotherapy.

In this study, we more precisely delineate the magnitude of the contained self-reactive peripheral T cell pool. Autoimmunity caused by these T cells is systemic and includes a diverse polyclonal TCR repertoire and a broad range of affinities for self. At least 4% of all T cells are potentially self-destructive, representing an unexpectedly high proportion of the T cell repertoire, which can be unleashed by interfering with peripheral tolerance. It will be important in the future to further define the term “self-reactivity” because, with intact peripheral tolerance, this high precursor frequency may serve important functions as “physiological” self-reactive T cells.

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**Disclosures**

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