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The TCR Repertoires of Regulatory and Conventional T Cells Specific for the Same Foreign Antigen Are Distinct

Lance M. Relland,* Jason B. Williams,* Gwendolyn N. Relland,* Dipica Haribhai,* Jennifer Ziegelbauer,* Maryam Yassai,† Jack Gorski,† and Calvin B. Williams*

The relationship between the TCR repertoires of natural regulatory T cells (nTregs) and conventional CD4+ T cells (Tconv) capable of responding to the same antigenic epitope is unknown. In this study, we used TCRβ-chain transgenic mice to generate polyclonal nTreg and Tconv populations specific for a foreign Ag. CD4+ T cells from immunized 3.12β^{nτ−} TCRα^{nτ−} Foxp3^{EGFP} mice were restimulated in culture to yield nTregs (EGFP+) and Tconv (EGFP−) defined by their antigenic reactivity. Relative to Tconv, nTreg expansion was delayed, although a higher proportion of viable nTregs had divided after 72 h. Spectratype analysis revealed that both the nTreg and Tconv responses were different and characterized by skewed distributions of CDR3 lengths. CDR3 sequences derived from nTregs displayed a divergent pattern of J usage, minimal CDR3 overlap (3.4%), and less diversity than did CDR3 sequences derived from Tconv. These data indicate that foreign Ag-specific nTregs and Tconv are clonally distinct and that foreign Ag-specific nTreg populations are constrained by a limited TCR repertoire. The Journal of Immunology, 2012, 189: 000–000.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ACE, abundance coverage estimation; Hb, hemoglobin; iTreg, induced regulatory T cell; MCC, moth cytochrome C; MHI, Morisita–Horn index; nTreg, natural regulatory T cell; Tconv, conventional CD4+ T cell; Treg, regulatory T cell; WT, wild-type.

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Materials and Methods

**Mice**

All mice used in this study were bred onto the B6.AKR background for >14 generations and analyzed between 6 and 12 wk of age. The transgenic mice that express the β-chain of the 3.1L2 TCR (3.1L2B) were generated in the transgenic facility at the Medical College of Wisconsin with the same construct used to make 3.1L2 μb TCR transgenic mice (16). The creation and characterization of Foxp3EGFP mice are described elsewhere (17); TCRα−/− mice were purchased from The Jackson Laboratory. All mice were housed in the animal facility at the Medical College of Wisconsin and handled in accordance with institutional guidelines. The institutional review committee approved all animal studies.

**Analytical flow cytometry and cell sorting**

Single-cell suspensions with up to 5 × 10^5 cells/sample were stained for cell surface markers, as described (3). Data were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar). Cells were sorted using a FACSAria cell sorter (BD Biosciences). Peripheral tissues were pooled before Ab staining and sorting. After culture, T cells were sorted to isolate nTreg and Tconv populations that were either unresponsive or reactive to Hb(64–76) peptide. The average purity of cell sorting was 98% for both the nTreg and Tconv populations.

**Abs, peptides, and cytokines**

The Abs used were obtained from BD Pharmingen or Caltag and included PE-Cy5–conjugated anti-mouse CD3 (145-2C11), Pacific Blue–conjugated anti-mouse CD4 (RM4-5), Pacific Orange–conjugated anti-mouse CD8 (53-6.7), PE-Cy7–conjugated anti-mouse CD25 (PC61), allophycocyanin–conjugated anti-mouse CD44 (IM7), PE-Texas Red–conjugated anti-mouse CD62L (MEL14), allophycocyanin-conjugated anti-mouse TCRβ (H57), and PE-conjugated anti-mouse Vβ8.3 (IB3.3). In cases where CD4 staining was done prior to sorting cells for culture, a different clone and fluorochrome-conjugate was used for CD4 staining in subsequent sorting procedures.

The peptides were synthesized, purified, and analyzed, as previously described (18). The peptide sequences, in single-letter amino acid code, are Hb(64–76) – GKKVITAFNEGLK (abbreviated “N72” for the Asn at position 72, the primary T cell contact residue) and moth cytochrome C Hb(64–76) – GKKVITAFNEGLK (abbreviated “N72” for the Asn at position 72).

**Immunization**

To create a source of activated APCs for cell culture, B6.AKR mice were immunized s.c. with CFA emulsified 1:1 with PBS (100 μl/mouse). Trigenic 3.1L2B Foxp3EGFP TCRα−/− mice were similarly immunized s.c., but 1 d later and with CFA emulsified 1:1 with PBS containing 400 μg Hb (64–76) peptide for a final peptide concentration of 200 μM (20 nmol/100 μl/mouse). One week after each respective immunization, single-cell suspensions were derived from the draining lymph nodes and spleen and stained for cell sorting.

**PKH26 and CellTracker staining**

The PKH26 mixture was prepared freshly per the manufacturer’s recommendations and used at a final concentration of 1 μM. The diluted PKH26 dye solution was combined 1:1 with sorted CD3+CD4+ T cells that were resuspended at a concentration of 2–4 million cells/ml. Cells were stained for 3 min at room temperature before stopping the reaction with calf se- rum. Violet CellTracker (Invitrogen) was used following the manufac- turer’s recommendations.

**Cell culture**

All cell cultures were carried out at 37°C and 5% CO2 in a final volume of 200 μl/well in a 96-well plate. CD3+ CD4+ APC populations were initially plated in 100 μl of the preceding the addition of PKH26-stained CD3+CD4+ T cells in another 100 μl media. APCs and T cells were cultured for three more days before analysis. A total of 4.5 × 10^3 and 5 × 10^4 APCs and T cells was cultured/well, respectively. All peptide/cytokine treatments were kept constant throughout the entire duration of the culture. All samples were treated with 50 U/ml human IL-2 and 1 μg/ml anti-CD28. Experimental samples were supplemented with 10 μM exogenous Hb(64– 76) peptide, whereas control samples included those cultured in media with or without 10 μM of exogenously added MCC peptide.

**RNA and cDNA isolation**

For sorted cell yields > 2.5 × 10^5 cells, total RNA was isolated using an RNeasy Mini kit (QIAGEN), following the manufacturer’s protocol. An 8- μl aliquot of RNA was reverse-transcribed using a Superscript First-strand Synthesis kit (Invitrogen), also following the manufacturer’s protocol. For lower sorted cell yields, cDNA was isolated directly using Superscript III CellsDirect cDNA Synthesis System (Invitrogen), following the manufac- turer’s protocol and upscaling the volumes as needed. All cDNA samples were either used immediately or stored at −20°C.

**TCRα spectratyping**

Murine TCRα variable (Vα) gene primers were developed as listed in Unit 10.28 of Current Protocols in Immunology (19). Each Vα region primer was used with a FAM-labeled or unlabeled constant (Cα) region primer to generate PCR products for subsequent TCRα spectratyping and cloning. FAM-labeled PCR products were dyed using GeneMapper software to determine the distribution and proportion of CDR3 lengths. Individual bands were separated via PAGE using a Bio-Rad sequencing gel apparatus. The resultant bands were then visualized using a Typhoon scanner and purified using a QIAEX II DNA Extraction Kit (QIAGEN), following the manufacturer’s protocol.

The various spectratypes were compared by generating an overall skew value for each experimental population relative to the CD4+ peripheral T celld control data. Spectratype data were collected as fluorescent peak areas from GeneMapper software and were converted to relative frequencies by dividing each fluorescent peak area by the total peak fluorescence. This compensates for small changes in the dataset due to unequal sample size. For generating the control distribution, splenocytes from two or three untreated mice were analyzed and, when possible, an average normal distribution was assigned. The skew was calculated for each CD3+CD4+ population. This can be envisioned as an analog equivalent to a Hanning distance calculation, in which 100 intensity markers are distributed among N bins, and the skew counts how many changes have to be made in the experimental distribution of markers to arrive at the control distribution. The minimum skew would be 0, and the maximum is 2. Some Vα families were poorly represented in the 3.1L2 TCRα mice, and a control distribution could not be calculated. Nevertheless, stimulation resulted in expansion of T cells that used these families (e.g., Vα17). For a few Vα families, the length distribution differed between the experimental mice, in which case the skew were calculated on a mouse-by-mouse basis (e.g., Vα3, 6, and 13).

**TA-TOPO cloning and sequencing**

To improve the cloning efficiency, PCR products were reamplified for an additional five cycles using unlabeled primers to dilute out the FAM label. Each reamplified sample was combined with the pCR4-TOPO vector, incubated at room temperature for 30 min, and then added to One Shot TOP10 chemically competent Escherichia coli. The bacteria were plated on Luria–Bertani agar plates containing ampicillin, and colonies were randomly picked, grown for 16 h, and shipped to Agencourt Bioscience (Beverly, MA) for further processing and CDR3 sequencing.

**Statistics**

Two-tailed Student *t* tests were used to determine significance between two given groups. One-way ANOVA and the Tukey test were applied for analyses containing more than two groups. The abundance coverage esti- mator and Morisita–Horn Index (MHI) were calculated using the Esti- mateS 8.0.0 software package (http://vicerey.eeb.uconn.edu/EstimateS/).

**Results**

**Characterization of 3.1L2B−/− X TCRα−/− mice**

We first compared B6.AKR TCRα−/− Foxp3EGFP mice (wild-type [WT]) with 3.1L2B−/− TCRα−/− Foxp3EGFP mice that expressed the 3.1L2 TCRβ-chain (3.1L2B). In peripheral lymph nodes, there was no significant difference in the percent and number of CD4+ Tconv or nTreg cells between the two strains (Fig. 1A). In 3.1L2B transgenic mice, nearly all (98%) CD4+ T cells expressed Vβ8.3. Many nTregs from these mice also had high levels of CD25 and CD44, while CD62L expression was relatively low (Fig. 1B), consistent with the nTreg cell surface phenotype (20). Tconv from
3.1Lβ−/− mice had low levels of CD25 and CD44, with high levels of CD62L, consistent with naive Tconv phenotype. When we stimulated unprimed splenocytes from 3.1Lβ mice with HB(64–76) peptide (N72) in cell culture, we detected a dose-dependent proliferative response. This indicated a higher frequency of N72-reactive T cells following immunization (17). Overall, this approach of “fixing” the TCR repertoire of Tconv from CD90.2+ 3.L2 mice resulted in little to no proliferation and demonstrated the specificity of the response for the N72 (Fig. 2A, Supplemental Fig. 1). A more in-depth assessment of this response revealed that the proportion of divided Tconv showed a significant increase after 48 h in culture, whereas a similarly significant increase did not occur in the nTregs until after 72 h (Fig. 2B). Because the frequency of Ag-specific precursors in the larger Tconv subset could impact the proportional representation of dividing nTregs, we plotted the number of divided cells within each population as a percentage of their respective 72-h end points (Fig. 2C, dashed lines). The relative lag in nTreg division after 48 h was also evident in this analysis, consistent with the delay in nTreg proliferation observed in the draining lymph nodes of immunized mice and with the requirement for high levels of exogenous IL-2 for nTreg division (17, 23). Despite these differences, the proportion of divided nTregs (PKH26low EGFP⁺) after 72 h was significantly greater than that found among the Tconv at this time point (Fig. 2C, bars).

Next, we gauged the capacity of Tconv to express Foxp3 and the stability of Foxp3 expression among nTregs using similar in vitro experiments that started with sorted EGFP⁺ T cells and EGFP⁻ T cells, respectively. With an initial EGFP⁺ T cell input, 0.3% of the CD4⁺ T cells expressed EGFP after 72 h (Fig. 2D, upper panel), indicating that iTregs were not produced under these culture conditions. In the reciprocal experiments starting with EGFP⁺ cells, we noted an early loss of EGFP expression. After 24 h, 14.1% of the surviving CD4⁺ T cells lost EGFP expression, although only 7.6% of these were PKH26low EGFP⁺ (Fig. 2D, lower panel). This indicated that a fraction of the EGFP⁺ T cells had unstable Foxp3 expression that resulted in its loss before cell division. After 72 h, 21.3% of the surviving CD4⁺ T cells were EGFP⁺, and 57.9% of these had divided (Fig. 2D, E). These data indicated that the “unstable” fraction of cells was capable of cell division upon restimulation and that the 79.2% of CD4⁺ T cells that remained EGFP⁺ were enriched for the “stable” nTreg phenotype. Cell size and granularity analysis of the stable versus unstable fractions showed phenotypic differences between these two populations. Although all nondivided T cells showed similar profiles (Fig. 2E, solid lines), the proliferating EGFP⁺ T cells were noticeably larger and more granular than those divided nTregs that retained EGFP expression (Fig. 2E, dotted lines). This difference may be related to their presumably different function.
nTREGS HAVE LIMITED TCR REPERTOIRES FOR FOREIGN Ag

In these experiments, TCR variability is constrained by rearrangements at a single TCRα-chain locus in the context of a transgenic TCRβ-chain. This allowed us to evaluate TCR repertoires using a series of previously designed Vα primers to amplify the CDR3 and flanking segments of the TCRα sequences for each sorted N72-specific T cell population (19). For the purposes of this study, we enumerated Vα and Jα regions based on the nomenclature in *Current Protocols of Immunology* (19), the former of which differs from the T cell receptor variable domain designations in the International Immunogenetics Information System database (24). Resolving the PCR products by length indicated that there were a number of Vα families for which either the Tconv or nTregs were skewed away from the normal length distribution associated with peripheral CD4+ T cells (Table I). The average peripheral length was established by examining the splenocytes from two or three control animals. The fluorescent peak areas corresponding to the different CDR3 lengths were converted to relative frequencies for each length and peaks compared (Supplemental Fig. 2A). Downstream analyses focused on select Vα samples (Vα2, Vα3, Vα5, Vα6, Vα13, Vα15, Vα17, Vα18) that deviated from the typical Gaussian distribution of CDR3 lengths. Within each cell type, CDR3 spectra for most Vα products were similar between mice (Fig. 3), which allowed us to pool the data accordingly. These data show that both nTreg and Tconv populations are highly skewed relative to controls (p < 0.0005 for both nTregs and Tconv), which indicates that they are highly selected. Compared with each other, the overall skewes for the nTreg and Tconv populations were not significantly different (p = 0.35), suggesting a similar level of skewing in each repertoire.

For TCR repertoire analysis, we cloned and sequenced the amplified nTreg and Tconv TCRα CDR3 regions from each mouse. From the eight Vα families analyzed, we recovered 819 in-frame nucleotide sequences corresponding to 326 unique amino acid sequences (222 Tconv, 93 nTreg, 11 overlapping sequences). Ln-rank versus ln-rank frequency plots for the Tconv and nTreg amino acid repertoires revealed an excellent fit for a power law-like relationship. This indicates similar results at all scales of measurement and is consistent with adequate sampling of the repertoires (Supplemental Fig. 2B). An overview of the sequencing data is shown in Fig. 3. Within a Vα family, each unique CDR3 amino acid sequence was considered its own distinct “species” and is represented proportionally by slices in the pie charts. For those samples that shared spectratype peaks at common CDR3 lengths (Vα2, Vα3, Vα5, Vα13, Vα17, Vα18, Fig. 3A), we analyzed sequences from the same-sized products from the respective Tconv and nTreg TCR repertoires. Two of these samples (Vα5 and Vα18) also contained a notable nTreg peak that was one codon shorter than the dominant Tconv peak, which we included in the analysis (Fig. 3B). Analysis of the Vα18+ Tconv population at this shorter length was not informative because of a lack of CDR3 sequences (data not shown). For Vα15-containing nTreg and Tconv clones, a distinguishing feature of their spectratypes is that each was largely restricted to a dominant CDR3 length, with the nTreg pool expressing CDR3 regions that were

<table>
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<tr>
<th>Vα Region</th>
<th>Tconv</th>
<th>nTreg</th>
<th>CD4+ T Cells</th>
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<tbody>
<tr>
<td>Vα2</td>
<td>1.20</td>
<td>1.08</td>
<td>0.03</td>
</tr>
<tr>
<td>Vα3</td>
<td>0.30</td>
<td>1.09</td>
<td>1.15, 0.49</td>
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<tr>
<td>Vα5</td>
<td>0.38</td>
<td>1.10</td>
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<td>Vα6</td>
<td>1.15</td>
<td>1.36</td>
<td>1.17</td>
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<tr>
<td>Vα13</td>
<td>0.79</td>
<td>1.12</td>
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<td>Vα15</td>
<td>1.10</td>
<td>1.31</td>
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<td>Vα18</td>
<td>1.22</td>
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The peripheral control skew was calculated by comparing the spectratypes from the individual animals used to generate the control data. The relatively high value for Vα6 most likely reflects the effect of the transgenic β-chain on the selection process. Multiple values are shown for individual mice in those cases in which the spectratypes were different for each animal. The maximum skew possible is 2.
predominantly one amino acid shorter than the Tconv (Fig. 3C). The Vα6+ samples demonstrated little consistency between mice, although each spectratype contained a single dominant CDR3 length. Therefore, we considered the entire range of CDR3 lengths for Vα6+ clones in a pooled analysis (Fig. 3D). Consistent with the skewed spectratypes and fewer CDR3 species, the nTreg pool used fewer unique Jα regions and exhibited a different pattern of Jα usage than did the corresponding Tconv population for each of these Vα analyses (Supplemental Fig. 3). Furthermore, Jα segments in common between the two populations showed a bias in Jα usage toward one of the given T cell subsets. The similar spectratype skewing with a reduced number of CDR3 species indicates that the N72-specific nTreg TCR repertoire is less diverse than the corresponding N72-specific Tconv TCR repertoire.

Next, we examined the “public” and “private” composition of the repertoires. We considered any CDR3 amino acid sequence to be public if it was recovered from two or more mice. Most Tconv and nTreg sequences at the dominant CDR3 length were private (Fig. 3A, open slices, 85 and 89% private, respectively). Thus Ag-specific nTreg and Tconv TCR repertoires are largely private. In apparent contrast, the Vα15+ Tconv and nTreg populations were mostly public (Fig. 3C, filled slices). These data indicate that the public versus private nature of the repertoires correlates with use of certain V segments and is not closely associated with T cell function.

**TCR repertoire comparisons of Ag-specific nTregs and Tconv**

To estimate the total size of the two repertoires and the clonal relationships between them, we compared the nTreg TCR repertoire with that of Tconv using statistical estimation of species richness (number of different CDR3 sequences) and shared species (overlap between CDR3 repertoires). Previous studies applied these methods (8, 25), which include the abundance coverage estimator (ACE) and MHI. ACE is a nonparametric statistical tool that uses the number of rare CDR3 sequences (<10 occurrences) and the number of singletons to estimate the number of “unseen” CDR3 sequences and, therefore, the total number of CDR3 sequences present in the population. MHI is an abundance-based similarity index that calculates the overlap between two populations, with values ranging from 0 (no overlap) to 1 (complete overlap). MHI is relatively resistant to undersampling, because the most abundant CDR3 sequences dominate the probability of overlap and are always present in the sample.

In every case, the nTreg ACE values were lower than those for the Tconv (Fig. 4). This result indicated less diversity among the nTreg TCRs, in agreement with the spectratype data. Of the seven Vα groups that allowed comparison of equivalent CDR3 lengths between the two T cell populations, four of them showed no overlap (Fig. 4A). Of the remaining three Vα groups, the percentage of overlap appeared to be greatest in the Vα13 sample (Fig. 4B), with 17% of unique CDR3 sequences or 34% of the total number of Vα13+ samples. However, taking into consideration the biodiversity of each Vα13+ population yielded an MHI of only 0.103 (Fig. 4B). In fact, this was less than the maximal MHI value of 0.119 that we observed in the Vα2+ sample, which also showed comparatively less overlap (i.e., 6% of unique CDR3 sequences or 18% of the total number of samples). Nevertheless, although some overlap existed between the Tconv and nTreg TCR repertoires, it remained minimal and amounted to only 3.4% of all of the unique sequences that we considered in our study.

Interestingly, the overlapping sequences that we observed were consistently more public than were those sequences that were unique to either the Tconv or nTreg populations (Fig. 4B). Publicity ranged from 75 to 100% of overlapping TCR amino acid sequences, whereas 0–38% of the nonoverlapping TCRs within these Vα groups were public (Fig. 4B). These data demonstrate that, among those Tconv and nTreg that respond to the same epitope, shared TCRs are more likely to be public.

**TCRα CDR3 amino acid sequences**

Thus far, our data support the notion that, when considering foreign Ag-specific responses, the TCR repertoire of nTregs is relatively limited and distinct from that of Tconv. The bulk of each CDR3α sequence is made up of the Jα region. A closer look at the primary sequences within the Vα groups (excluding Vα6) that showed no CDR3 overlap revealed that most involved N- and/or P-nucleotide additions (Fig. 5A–C). Each of these groups used certain Jα regions multiple times, in which case variability within CDR3s that used a common Jα region was limited to the intersection.
between the variable and junctional regions (Fig. 5A–C, boxed amino acids). CDR3 variability in most of these Tconv subsets (Vα3, Vα5, Vα17) was high, as demonstrated by each of these Vα groups being populated most frequently by singletons (44–69%). The notable exception was the Vα18 group, in which the most frequently recovered sequence was that of the original 3.L2 TCR (line #5). The Vα6 samples also showed no overlap between the Tconv and nTreg populations (Fig. 5B), although the dominant CDR3 lengths were inconsistent between mice.

The Vα samples that did show some overlap between the Tconv and nTreg populations also consisted largely of CDR3s that included N- and/or P-nucleotides (Fig. 5C). Additionally, most of the overlapping sequences showed variability in upstream sequences, which suggested that these TCRs were more likely to use different Vα subfamily members. A number of amino acids was each encoded by more than one codon, and this occurred more often among the overlapping sequences compared with those that showed no overlap. All overlapping Vα2* and Vα13* CDR3s used the Jα52 and Jα27 regions, respectively, whereas the overlapping Vα15* CDR3s used a wider variety of Jα regions. Next, we considered the possibility that each shared CDR3 sequence may be derived from a unique clone. If this were true, we would expect the respective Tconv and nTreg nucleotide sequences from any given mouse to be identical in their entirety. Instead, we found that 65% (49/76) and 30% (23/76) were specific to the Tconv and nTreg populations, respectively (Fig. 5D). Only ~5% of the unique nucleotide sequences that made up the pool of overlapping CDR3 amino acid sequences were contained within both T cell populations. This indicated that, within an individual, overlapping sequences were not clonally related. In fact, there was a bias toward the use of certain Jα regions, which resulted in separate Vα groups sharing a common CDR3 amino acid sequence (Supplemental Fig. 4). Similarities in the Jα27 versus Jα52 primary sequence also allowed one of the overlapping sequences (CAAGANTGKLTF) to have incidences of both of these Jα segments.

**FIGURE 4.** Overlap between the nTreg N72-specific TCR repertoire and its Tconv counterpart. (A) Statistical data are shown for those select Vα samples (Vα3, Vα5, Vα17, Vα18) that had no apparent overlap (MHI = 0.00) between the nTreg and Tconv repertoires. (B) For those Vα repertoires that exhibited some degree of overlap (Vα2, Vα13, Vα15), the Venn diagrams show the proportion and number of unique CDR3 sequences that were found solely in the Tconv population (gray), solely in the nTreg population (black), or in both populations (striped). The pie charts and bars below the Venn diagrams represent the publicity data. In the pie charts, filled slices represent public sequences, and notches show the number and proportion of unique CDR3 amino acid sequences. The underlying bars and numbers represent the percentage distribution of public sequences recovered from the Tconv (left) and nTreg (right) populations. For each given Vα analysis, CDR3 amino acid sequences are considered public if retrieved from two or more mice. All publicity data are presented in the pie charts and bars (B) or in tabular format (A) as a percentage, based either on giving equal weight to each unique CDR3 amino acid sequence or on the number of samples in each group, as indicated. Biodiversity estimations include MHI (boxed data) and ACE. These data are representative of three independent experiments, with a total of three mice/group. Sample sizes are as follows: Vα2 = 89, Vα3 = 84, Vα5 = 89, Vα13 = 109, Vα15 = 161, Vα17 = 98, and Vα18 = 110.

**Discussion**

Our study compared a foreign Ag-specific TCR repertoire of nTregs with the corresponding TCRs of a Tconv population that responded to the same epitope. This type of analysis is particularly significant, because good protection from pathogens involves participation of both the Tconv and nTreg subsets to keep the response adequate, yet well controlled. In this context, we found the nTreg TCR repertoire to be distinct, restricted, and less diverse than that of the Tconv.

A restriction in nTreg foreign Ag-specific responses may originate during thymic selection. In the thymus, nTreg precursors have different affinity requirements for their maturation compared with developing Tconv thymocytes (1, 2). Induction of Foxp3 requires interaction with an agonist self-peptide, and the frequency of Foxp3+ cells is directly related to the strength of the agonist (3). Consequently, the differences in affinity-based selection should result in populations of Tconv and nTregs with discrete TCR repertoires. In combination with the autoreactivity of the nTreg population (7, 26), these data support the idea that the recognition of foreign Ags by nTregs relies on polyspecificity (27). However, some studies showed that nTreg precursors are subject to the process of negative selection (26, 28, 29), which functions to eliminate cells with cross-reactive TCRs (30). It follows that the autospecific nTreg population should be relatively deficient in foreign Ag recognition. Our data are in agreement with this interpretation. For each Vα analysis, the nTreg population was consistently less diverse, as determined by the lower ACE value. Taken together, these data support a model in which negative selection acts to shape the nTreg TCR repertoire. This conclusion leads us to propose that if foreign Ag recognition by nTregs depends on polyspecificity, it may be important to limit the extent of this cross-reactivity within the nTreg compartment to allow for the generation of effective immune responses to foreign Ags. This hypothesis was not directly addressed in this study.

Our findings stand in apparent contrast to previous analyses of naïve polyclonal nTreg populations, which show that nTreg and...
Tconv TCR repertoires are at least equally diverse (7, 9). Such unselected repertoire analyses estimate the total diversity potential contained within an individual. In this report, we examined a TCR repertoire that was defined by its functional capacity to respond to a specific foreign Ag. Thus, the relatively restricted nTreg TCR repertoire described in this article is a reflection of an Ag-driven response.

**FIGURE 5.** TCRs CDR3 amino acid sequences from N72-specific nTreg and Tconv clones. (A–C) CDR3 sequences found two or more times are shown using a single-letter amino acid code. Singleton sequences are pooled. The frequency of each CDR3 amino acid sequence is given in the “Treg” and “Tconv” columns. Asterisks in the “V” column indicate the use of two or more V-region subfamilies, and the number of mice containing the sequence is listed in the “M” column if the sequence was recovered from more than one mouse. The “Nuc” column indicates the use of N- (N), P- (P), or both N- and P-nucleotides (NP) in one or more samples within each of these respective sequences. Lines with sequences found in both nTreg and Tconv populations are contained within gray boxes. At given CDR3 lengths, common Jα usages and the corresponding amino acid positions that show variability in sequence are in bold type and contained within black boxes. Sample sizes are listed in the header of each respective Vα analysis. Conserved amino acids that have more than one underlying codon sequence are also in bold type and shaded light gray, dark gray, or black for 2, 3, or 4+ unique codons, respectively. Line numbers (“#” column) are provided for organizational purposes. (A) Analysis of nonoverlapping Vα groups based on CDR3 length-selected clones. (B) Analysis of the nonoverlapping Vα6 group, in which the CDR3 regions were not preselected for sequencing based on their length. (C) Analysis of CDR3 size-selected Vα groups that show some overlap between the nTreg and Tconv populations. (D) Venn diagrams show the number of unique clones (n = 76) that were found solely in the Tconv population (gray; n = 49), solely in the nTreg population (black; n = 23), or in both populations (striped; n = 4). To be considered clonal, PCR products needed to share the same sequence and come from the same mouse. The bars and numbers are colored similarly and represent the percentage distribution of samples recovered from the Tconv (left) and nTreg (right) populations. These data are representative of three independent experiments, with a total of three mice/group.
selection process in the periphery rather than an estimate of the theoretical potential for nTreg diversity generated by developmental processes in the thymus. The smaller size of the resultant nTreg TCR repertoire also was not due to less cell division after antigenic stimulation. Although the nTregs showed a relative lag in the kinetics of their response, the extent to which they proliferated was greater than that of Tconv. Alterations in the proximal TCR signaling of nTregs could have contributed to their delay in proliferation (31, 32). Additionally, nTregs have relatively reduced levels of CD4, which may, in fact, require them to have higher TCR ligand affinity for activation compared with their Tconv counterparts. Consistent with this hypothesis, elimination of CD4 in Tconv increases the affinity threshold for T cell selection in the thymus (33). For mature nTregs, such an increase in the activation threshold would tend to limit the repertoire of clones capable of responding to any given Ag. Thus, although the respective TCR diversity potentials of the two populations are equivalent, the number of nTreg TCRs responding to a specific Ag may also be constrained by cell-intrinsic criteria unrelated to the origin (self versus foreign) of that Ag.

The Ag-specific nTreg responses in our system generally did not share CDR3a amino acid sequences with the corresponding Tconv pool (11 of 326 clones; 3.4%). This observation was more pronounced when considering these sequences at the nucleic acid level. Only 7.1% of the DNA sequences that composed these overlapping amino acid sequences were identical between the nTreg and Tconv populations isolated from the same mouse (i.e., overlapping CDR3a amino acid sequences were rare and were derived from different clones). These data are consistent with our previous studies that found a limited overlap between nTregs and iTregs, the latter of which arises from the Tconv population and can make up 10–15% of the peripheral Treg pool (12). In the current study, we also found that 14% of EGFβ T cells lost Foxp3 expression within the first 24 h following restimulation in culture. Taken together, these data suggest that the loss of Foxp3 expression described in this study might be attributable to the down-regulation of Foxp3 in iTregs. Indeed, if iTregs were maintained during in vitro restimulation, they would be scored as nTregs and would increase the overlap between nTregs and Tconv. As an alternative explanation, it was shown that nTregs may also lose Foxp3 expression and become “ex-Foxp3” effector/memory cells alternatively explanation, it was shown that nTregs may also lose Foxp3 expression and become “ex-Foxp3” effector/memory cells. When the nTreg repertoire is insufficient to control the inflammation supports transiently the production of iTregs proportional to the needs of the host. Therefore, our data are consistent with the more fundamental hypothesis that iTregs broaden the diversity of regulatory responses to foreign Ags (12).

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References


