

TCR-based lineage tracing: no evidence for conversion of conventional into regulatory T cells in response to a natural self-antigen in pancreatic islets

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***Foxp3*-expressing regulatory T (T reg) cells derive primarily from selection in the thymus. Yet conversion of mature conventional CD4⁺ T (T conv) cell lymphocytes can be achieved in several conditions, such as transforming growth factor β treatment, homeostatic expansion, or chronic exposure to low-dose antigen. Such conversion might provide a means to generate peripheral tolerance by "converting" potentially damaging T cells that react to self-antigens. We tested this hypothesis in mice transgenic for the BDC2.5 T cell receptor (TCR), which is representative of a diabetogenic specificity that is naturally present in NOD mice and re-active against a pancreatic self-antigen. In the thymus, before any exposure to antigen, clonotype-positive T reg and T conv cells express a second TCR α chain derived from endogenous loci. High-throughput single-cell sequencing of secondary TCRs of the V α 2 family showed their joining CDR3 α regions to be very different in T reg and T conv cell thymocytes. These specific CDR3 α motifs, thus, provided a "tag" with which to test the actual impact of T conv to T reg cell conversion in response to peripheral self-antigen; should the autoreactive clonotypic TCR induce T conv to T reg cell conversion upon encounter of cognate antigen in the pancreas or draining lymph node, one would expect to detect tag CDR3 α motifs from T conv cells in the T reg cell populations. Sequencing large numbers of peripheral BDC⁺V α 2⁺ cells showed that little to no conversion occurs in response to this pancreatic autoantigen.**

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Abbreviations used: ACE, abundance-based coverage estimator; CDR, complementarity determining region; MH, Morisita-Horn; PLN, pancreas-draining LN; T conv, conventional CD4⁺ T cell; tg, transgenic.

Some potentially autoreactive T cells escape negative selection in the thymus and need to be kept under control by peripheral mechanisms of tolerance induction. Among these mechanisms is active regulation by a subset of CD4⁺ T cells, called regulatory T (T reg) cells, which are characterized by the transcription factor *Foxp3* and a distinct gene-expression signature (1). T reg cells dampen immune and inflammatory responses (2); deficiencies in *Foxp3* result in the lymphoproliferation and multiorgan autoimmunity of *scurfy* mutant mice and human immunodysregulation, polyendocrinopathy, enteropathy, X linked patients (3).

The T reg cell lineage seemingly originates in the thymus, like conventional CD4⁺ T (T conv) cells, which are derived from CD4⁺CD8⁺ thymocytes (4), encounter of self-antigens inducing, or favoring, differentiation into the regulatory lineage (5–8). This results in the selection of a

TCR repertoire that is quite distinct in T reg and T conv cells, as indicated by sequencing studies in transgenic systems that limit the possible TCR sequence variability (8–11).

On the other hand, the conversion of mature CD4⁺ T conv cells to a FoxP3⁺ T reg cell phenotype has been shown to occur subsequent to a variety of manipulations either in vitro or in vivo: by exposure to TGF β and IL-2 during activation (12), in conditions of lymphopenia-driven homeostatic expansion (13), or by chronic exposure to antigen delivered as peptide by an osmotic pump or by antibody-mediated targeting (14–17). It is not yet clear whether these converted cells acquire the full marker and regulatory characteristics of naive T reg cells. Yet, such a generation of regulatory cells outside of the thymus would represent a flexible and adaptable mode of establishing peripheral tolerance, particularly for self-antigens not encountered by T cells during thymocyte differentiation (18).

The online version of this article contains supplemental material.

We attempted to test the extent to which this conversion occurs in response to exposure to a natural self-antigen. To this end, we made use of BDC2.5/NOD TCR transgenic (tg) mice, which carry TCR $\alpha\beta$ transgenes encoding a receptor that recognizes a self-antigen specifically expressed in pancreatic islets and show a regulated development of autoimmunity (19–21). Normal populations of T conv and T reg cells are found in these mice (21–23), both of which express the islet-reactive clonotypic TCR. In addition, most CD4⁺ T cells in BDC2.5 mice display a second TCR α chain, which is generated by rearrangement of the endogenous *Tcr* α locus (20). As in other TCR transgenic systems, this second TCR α chain is essential for the selection of T reg cells, as they are absent when the BDC2.5 transgene is crossed onto a TCR α - or RAG-deficient background (21). This second TCR somehow allows the appearance of T reg cells in the BDC2.5 thymus, presumably because the clonotypic TCR is not compatible with positive selection into the T reg cell lineage. The second TCR α chain is not required for the differentiation or activity of T conv cells because such cells mature in BDC2.5 mice on a TCR α - or RAG-deficient background, and lead to very aggressive diabetes (20, 24). Previous studies have shown, however, that a sizeable proportion of T conv cells in BDC2.5 mice also express a second TCR α chain (25).

Thus, the BDC2.5/NOD system presents a self-contained context for examining the potential conversion of T conv to T reg cells in response to self-antigen. Essentially all CD4⁺ T cells express the same TCR, conferring reactivity to a self-antigen encountered specifically in the pancreas and its draining LN (26); if the repertoire differences between T reg and T conv cells observed in other systems (8–11) also apply for the BDC2.5 mice, the second TCR α chain should provide a lineage-tracing “tag,” allowing one to determine whether T reg cells observed in the pancreatic infiltrate emanate from the thymus-derived T reg cell lineage or from converted T conv cells. We thus determined the T conv and T reg cell repertoires for the endogenous TCR α by single-cell sequencing for individual cells whose lineage was also established by split-well RT-PCR for *Foxp3* transcripts (11).

RESULTS AND DISCUSSION

TCR α rearrangements containing V α 2 provide a mode of tracking prospective conversion

We began by investigating the surface expression of second TCR α chains in the T reg and T conv cell populations. We focused on the V α 2 family because this group of V α regions is used at fairly high frequency in CD4⁺ T cells (8–12% in different mouse strains), and can be detected with an effective mAb. Indeed, the V α 2 family has been exploited for such analyses in several previous repertoire studies (11, 27). We analyzed cells from BDC2.5/NOD mice bred to contain a heterozygous knockout mutation at the *Tcr* α locus to ensure that only a single, additional TCR α chain was expressed in each cell. The presence of the two TCRs on the cell surface was visualized by concomitant staining with a mAb detecting the BDC2.5 clonotype (V α 1V β 4) (22) and with the anti-V α 2 mAb. When

mature CD4⁺CD8⁻ T conv and T reg cell thymocytes (CD25⁻ and CD25⁺, respectively) from 3 10–14-wk-old BDC2.5/CD α ^{+/-} mice were analyzed, large numbers of dual-expressors were observed in both cell types (Fig. 1 A). The relative distributions of clonotype and V α 2 staining intensities were comparable (V α 2 mean fluorescence intensity = 6,449 and 9,723 and BDC2.5 mean fluorescence intensity = 248 and 206 for T reg and T conv cell populations, respectively).

We then investigated the amino acid sequences of the two populations' secondary TCR α s, on which the imprint of

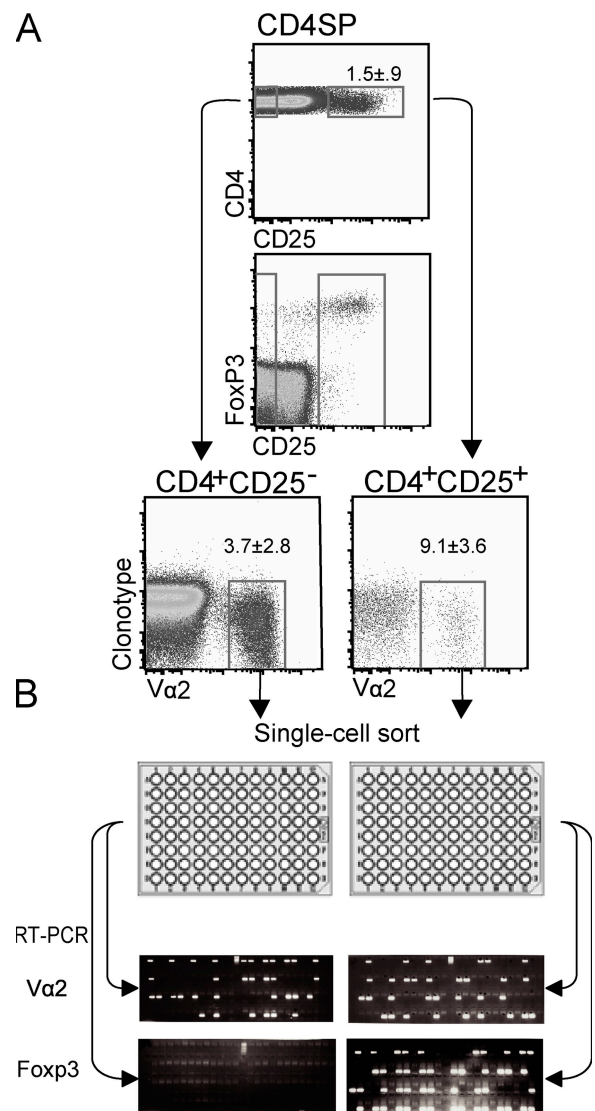


Figure 1. Concomitant expression of BDC2.5 and V α 2 TCR chains on both T reg and T conv cells. (A) Flow-cytometric analysis and sorting of CD4SP thymocytes from BDC2.5/TCR α ^{+/-} mice ($n = 3$). V α 2-expressing T reg and T conv cells were sorted according to the indicated gates. The CD25 versus FoxP3 intracellular staining depicts the frequency of FoxP3 positivity in each gate. (B) Cells were singly sorted into 96-well PCR plates, leaving every third well empty as a control. cDNA made from single cells were split into two PCR reactions for V α 2 and *Foxp3* transcripts, as depicted (bottom).

selection into either compartment should be visible. Individual CD4⁺CD8⁻ thymocytes were sorted according to surface marker expression (CD25⁺ and CD25⁻ for T reg and T conv cells, respectively) into wells of PCR plates (Fig. 1 B). Cells were lysed, and cDNA was produced by reverse transcription. The contents of each well were split into two, and parallel PCR amplifications were performed to detect *Vα2* and *Foxp3* transcripts, for concomitant TCR sequencing and phenotypic verification. *Foxp3* transcripts were frequent in samples from CD25⁺ cells (63.6%; Fig. 1 B, bottom right), which is comparable with our previous experience, where 84.5% of FoxP3⁺ cells were observed among CD25⁺ cells (11). FoxP3 transcripts were very rarely detected in the CD25⁻-sorted cells (0.4%; Fig. 1 B, bottom left), which is in accordance with the frequency shown by intracellular staining (Fig. 1 A, middle). *Vα2* transcripts were amplified with a success rate of 54.8 and 58.5% in CD25⁺ and CD25⁻ cells, respectively. These *Vα2* amplicons were sequenced to determine the CDR3 sequence and the *Vα2* family member, for 238 FoxP3⁺CD25⁺ T reg and 255 FoxP3⁻CD25⁻ cells derived from four individual mice. Stringent measures were taken to prevent false-positives arising from PCR contamination (such as leaving every third well empty and discarding plates with any sign of contamination).

A compendium of the TCR sequences obtained from BDC2.5 mice, and their occurrences in all organs and populations examined, can be found in Table S1 (available at <http://www.jem.org/cgi/content/full/jem.20070822/DC1>). The gross characteristics (CDR3 length and Jα usage) of the *Vα2* TCR sequences from T reg and T conv cell thymocytes were quite similar (Fig. S1 A). Interestingly, there was a differential distribution of charged amino acids in the sequences from these two populations: T reg cell thymocytes had an increased representation of positively charged CDR3α loops (Fig. S1 B). This finding reproduced an observation recently made in another TCR system (11), and it is compatible with the notion that positively charged CDR3α loops elicit a tighter interaction with MHC-self-peptide complexes more favorable to T reg cell differentiation.

The distribution between the six *Vα2* family members found in the NOD genome (nomenclature described in Table S2, available at <http://www.jem.org/cgi/content/full/jem.20070822/DC1>) was quite comparable in T conv and T reg cells (Fig. S1 C). The T reg and T conv cell repertoires were of similar breadth (total diversity of 588 and 317, respectively, using the abundance-based coverage estimator [ACE]), with a typical “power-law” distribution where approximately half of the sequences occurred only once, the remainder of the repertoire being comprised of CDR3α motifs observed repetitively, up to 36 times in the dataset. These “popular” sequences form the basis of the following analysis, their reproducible occurrence providing statistical reliability.

Most pertinent for the present study, the distribution of these secondary *Vα2* TCRs was very different for T reg and T conv cells (Fig. 2 A). T reg cells used a set of sequences that was seldom found among T conv cells; conversely, sequences

that dominated the T conv cell repertoire were hardly ever found in T reg cells (only 2.1% of T reg thymocytes used the set of T conv cell sequences defined in Fig. 2 A). Such a difference was previously observed in comparisons of primary repertoires (8–11), but it was interesting to find that this distinction extended to the secondary TCR sequences as well, in cells that otherwise shared the BDC2.5 clonotypic TCR. Quantitating the degree of sequence sharing using the Morisita–Horn (MH) technique for species overlap gave a very low index (0.03 on a

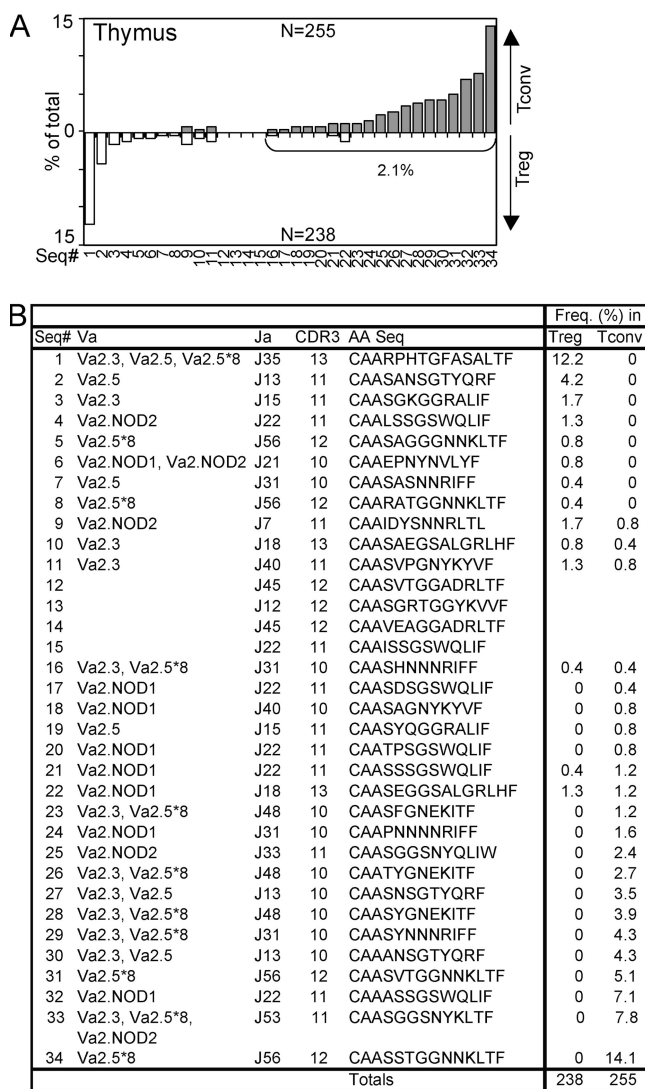


Figure 2. High-frequency *Vα2* TCR sequences from T reg and T conv cell thymocytes. (A) Frequency of individual TCR sequences. The bar histogram depicts the frequency in T conv and T reg cell repertoires (shaded bars above the midline and open bars below, respectively). Bracket represents the contribution of sequences #16–34 to the total T reg cell repertoire. (B) Tabulation of TCR sequences indicating *Vα* usage, *Jα* usage, CDR3 length (as defined by the amino acids that are bordered by the invariant C residue in TCRVα genes and the F/W-G-X-G *Jα* motif), and the percentage of TCR sequences within the T reg and T conv cell populations. Total sequences sampled are noted at the bottom.

scale from 0 to 1), which is lower than observed in previous T reg:T conv cell repertoire comparisons (8–11). A few CDR3 α motifs were represented at similar frequencies in both lineages, but that presence had little impact on the overall repertoires, each comprising <2% of either population.

Interestingly, the dominant CDR3 α motifs tended to be associated with only one or a restricted set of V α 2 family members (Fig. 2 B). Therefore, the preferential selection into the T reg or T conv cell lineages was dictated by the combination of CDR3 α with CDR1 α and CDR2 α . This combinatorial selection most likely reflects interactions with MHC–peptide ligands encountered on the thymic stroma, and it is consistent with our current understanding of TCR:MHC–peptide interactions derived from crystal structures (28).

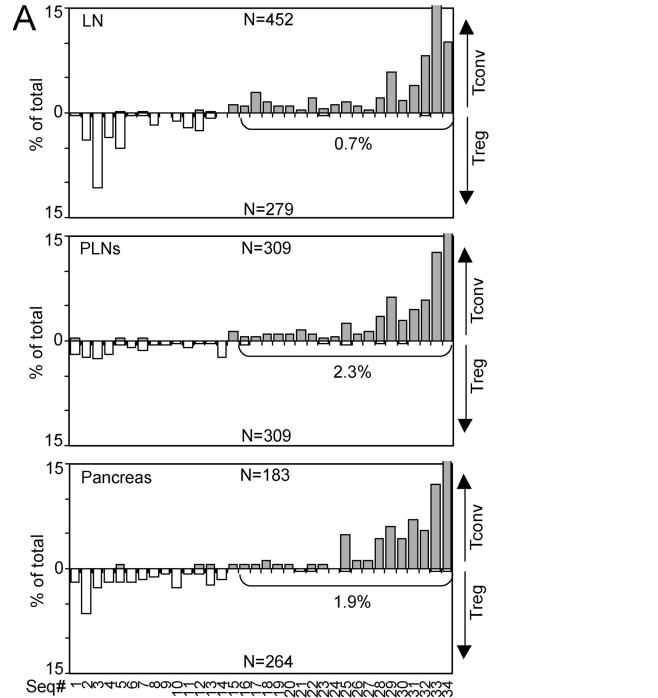
Thus, although all of them expressed the BDC2.5 TCR, immature thymocytes in BDC2.5/NOD mice were directed toward the T reg or T conv cell lineage largely as a function of the secondary TCR derived from rearrangement of the endogenous *Tcr* α locus. That T reg cells might show a restricted set of sequences in the secondary TCRs was somewhat expected because the original BDC2.5 TCR was derived from a nonregulatory T cell clone, and because we know these endogenous TCR α chains are required for selection into the T reg cell lineage to occur. According to current paradigms, these secondary TCRs would provide the high-affinity TCR interactions required, the resulting affinity/avidity for self-antigens on the thymic stroma being incompatible with selection into the T conv cell lineage. On the other hand, and as will be discussed later in the text, finding a restricted set of sequences in the T conv cell thymocytes was somewhat unexpected.

Whatever the underlying reason, the very distinct sets of secondary TCRs present in T conv and T reg cell thymocytes fully served our experimental plan: could we see, after export and migration into the sites where naive cells encounter the antigen recognized by the BDC2.5 TCR, any appearance in the T reg cell repertoire of sequences found solely in T conv cells in the thymus?

Peripheral T reg cell repertoires from disease-relevant and -irrelevant localities provide little evidence of conversion

T conv cells in BDC2.5 mice encounter their cognate antigen in the pancreas-draining LNs (PLNs), where they are first activated, and later in the pancreas itself, where they migrate upon activation (26). In contrast, BDC2.5 T conv cells in the subcutaneous LNs remain naive. To search for evidence of conversion, we examined the secondary TCRs in peripheral T reg and T conv CD4⁺ cells by single-cell analysis, before taking cells from the subcutaneous LNs, PLNs, and pancreases of 3–5 BDC2.5/TCR $\alpha^{+/-}$ mice. In total, 1,796 sequences were generated (Table S1). The sequences were obtained from mice between 10 and 14 wk of age at a time when insulinitis is well-established, ensuring that T conv cells were fully exposed to the BDC2.5 antigen in the PLNs and pancreas.

V α 2 sequences from the three peripheral tissues exhibited similar overall characteristics, much like those found in



B

Seq#	Va	Ja	CDR3 AA Seq	Freq. (%) in					
				LN		PLN		Panc	
				Treg	Tconv	Treg	Tconv	Treg	Tconv
1	Va2.3, Va2.5	J35	CAARPHGTGFASALTF	0.4	0	1.9	0.3	1.9	0
2	Va2.3, Va2.5	J13	CAASANSQTYGRF	3.9	0	2.3	0	6.4	0
3	Va2.3, Va2.5, Va2.NOD2	J15	CAASGKGRALIF	10.8	0	2.6	0	2.7	0
4	Va2.2, Va2.NOD2	J22	CAALSSGWSQLIF	3.6	0	1.9	0	1.9	0
5	Va2.5*8	J56	CAASAGGNKLTFF	5.0	0.2	0.6	0.3	1.9	0.5
6	Va2.NOD2	J21	CAEAPNPNVLYF	0.4	0	1.0	0	1.9	0
7	Va2.5	J31	CAASANNRIFF	0.4	0.2	1.3	0.3	1.5	0
8	Va2.5*8	J56	CAARATGGNNKLTFF	1.8	0	0.6	0	1.1	0
9	Va2.NOD2	J7	CAIDYSNNRRLTL	0.0	0	0.6	0	0.8	0
10	Va2.3	J18	CAASAEAGSALGRHLHF	1.1	0	0.3	0	2.7	0
11	Va2.3	J40	CAASVPGNYKYVF	2.2	0	1.0	0	0.8	0
12	Va2.3, Va2.4, Va2.5	J45	CAASVTGGADRLTF	2.5	0.4	0.3	0	0.8	0.5
13	Va2.NOD1	J12	CAASGRTGGYKVVFF	0.7	0.2	0.3	0	2.3	0.5
14	Va2.3	J45	CAAVEAGGADRLTF	0	0	2.3	0	1.5	0
15	Va2.2, Va2.NOD2	J22	CAAISSSGWSQLIF	0	1.1	0	1.3	0	0.5
16	Va2.3, Va2.5*8	J31	CAASHNNNRIFF	0	0.9	0.6	0.6	0	0.5
17	Va2.NOD1	J22	CAASDSSGWSQLIF	0	2.9	0	0.6	0	0.5
18	Va2.3, Va2.5, Va2.NOD1	J40	CAASAGNYKYVF	0	1.5	0	1.0	0	1.1
19	Va2.5	J15	CAASYGGRALIF	0	0.9	0	1.0	0	0.5
20	Va2.NOD1	J22	CAATPSSGWSQLIF	0	0.9	0	1.0	0	0.5
21	Va2.NOD1	J22	CAASSGWSQLIF	0	0.4	0	1.6	0.4	0
22	Va2.NOD1	J18	CAASEGSSALGRHLHF	0	2.2	0	1.0	0.4	0.5
23	Va2.3, Va2.5*8	J48	CAASFGNEKTF	0.4	0.7	0.3	0.3	0	0.5
24	Va2.NOD1	J31	CAAPNNNRIFF	0	1.1	0	0.6	0	0
25	Va2.5, Va2.NOD2	J33	CAASGSSNYQLIW	0	1.5	0.6	2.6	0.4	4.9
26	Va2.3, Va2.5*8	J48	CAATYGNKTF	0	0.9	0	1.0	0	1.1
27	Va2.3, Va2.5	J13	CAASNSGTYGRF	0	0.4	0	1.3	0	1.1
28	Va2.3, Va2.5*8	J48	CAASYNKTF	0	2.2	0.3	3.6	0	4.4
29	Va2.3, Va2.5, Va2.5*8	J31	CAASNNNRIFF	0	5.8	0	6.1	0	6.0
30	Va2.3, Va2.5	J13	CAANSQTYGRF	0	1.8	0.3	2.9	0	4.4
31	Va2.5*8	J56	CAASVTGGNNKLTFF	0	4.0	0	4.5	0	7.1
32	Va2.NOD1	J22	CAASGWSQLIF	0.4	8.2	0	5.8	0	5.5
33	Va2.3, Va2.5, Va2.5*8, Va2.NOD1, Va2.NOD2	J53	CAASGSSNYKLTFF	0	15.9	0	12.6	0.4	12.0
34	Va2.3, Va2.5*8	J56	CAASSTGGNNKLTFF	0	10.2	0	17.2	0.4	16.4
Totals				279	452	309	309	264	183

Figure 3. Paucity of T conv to T reg cell conversion in disease-relevant localities. (A) Bar histograms representing TCR sequences from LN, PLN, and pancreas (shaded bars above the midline and open bars below, respectively). Bracket represents the contribution of sequences #16–34 to the total T reg cell repertoire. (B) Table of TCR sequences indicating their frequency (as a percentage) of the indicated population and organ. Total sequences sampled are listed below.

thymocytes—power-law frequency distribution, diversity, CDR3 α length, and charge bias, V α usage, and J α usage (Fig. S2 and Fig. S3, available at <http://www.jem.org/cgi/content/>

full/jem.20070822/DC1). Importantly, the repertoires of the T conv and T reg cell populations from each organ exhibited the same TCR sequence polarity as had been seen in the repertoires of the thymocyte populations (Fig. 3). The same T reg-preferential sequences dominated the T reg cell repertoire, and the proportion of T reg cells using T conv cell-specific sequences, such as sequences #16–34, remained very low in all three peripheral lymphoid organs (0.7, 2.3, and 1.9%, in LN, PLN, and pancreas, respectively). Therefore, if conversion of self-reactive T conv cells to a T reg cell phenotype did occur in locations where they encountered the BDC2.5 antigen, it had a negligible impact on the T reg cell repertoire found at any of the peripheral locations.

Surprising consistency in the secondary TCR repertoires of T conv, but not T reg, cells

Examination of the distribution of sequences in T reg and T conv cells residing within different peripheral organs seemed to indicate a greater variability within the T reg than the T conv cell populations (Fig. 3). Greater divergence within the T reg pool could reflect organ-specific variation or more prosaically sampling fluctuation. To resolve this issue, we compared the sequence distribution in all organs of individual mice by constructing a matrix of MH similarity indices between every population in every mouse. Several points emerge from the representation of Fig. 4. First, this mouse-by-mouse display confirms that there was no conversion of T conv cells into T reg cells in locations where they were exposed to antigen; the repertoires of T reg cells in the PLN and pancreas were no more similar to T conv cells than were those of thymus T reg cell thymocytes. Second, the constancy found among the T conv cell repertoires of several organs was also reflected in a high degree of consistency between different animals (mean MH scores were 0.60, 0.63, 0.79, and 0.74 for the thymus, LN, PLN, and pancreas T conv cell samples, respectively). This constancy of the T conv cell repertoires of secondary V α 2 TCRs held between the individual samples for an organ, as well as between organs. In contrast, the T reg cell repertoires in each mouse, although also containing some high-frequency clones, showed less constancy between different organs or between different mice for the same organs (mean MH scores of 0.19, 0.39, 0.17, and 0.31 for the thymus, LN, PLN, and pancreas T reg cell samples, respectively), as if the T reg cell repertoires were allowed more flexibility.

Reproducibility of TCR repertoires between different individuals, particularly as concerns high-frequency “public” sequences, is well established from several studies of “primary” TCRs (8–11, 27). Mean MH scores of 0.42–0.74 between unique populations of inbred animals are commonly observed, and are comparable with the values found here for the secondary TCRs of T conv cells. What is somewhat baffling in this instance is the difference in consistency observed between the T reg and T conv cell repertoires. We expected little consistency for T conv cells, for which the clonotypic BDC2.5 TCR should suffice for positive selection and later

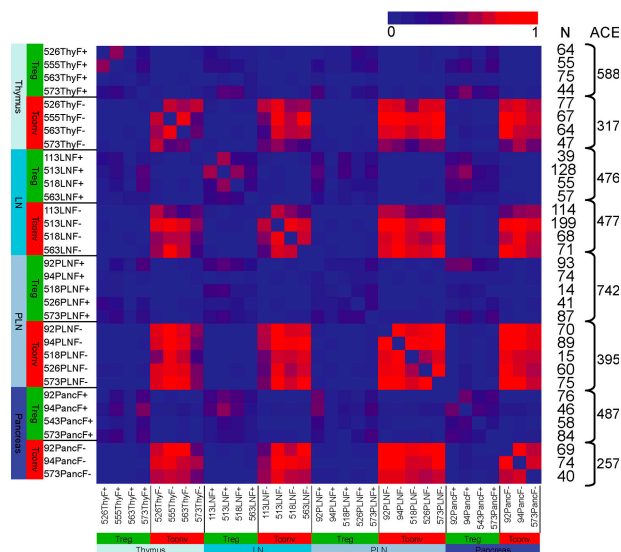


Figure 4. Mouse-to-mouse and organ-to-organ constancy of the T conv cell repertoire. Heat map of MH indices for each two-way comparison. Color scale indicated above. N represents the number of TCR sequences in the corresponding sample. ACE values estimate the number of unique sequences within the combined population sampled.

survival, and we anticipated that the secondary TCRs would encompass a random set of sequences, with little evidence of selection except for some imprint of negative selection against overly self-reactive TCRs. In contrast, we expected that the secondary TCRs of T reg cells would be more consistent between animals because these receptors are required for selection into the T reg cell lineage. Because the complete opposite was observed, we are led to reconsider the roles of the primary and secondary TCRs in this system. For T conv cells, although it remains possible that a very strong negative-selection imprint predicates the high conservation of secondary TCRs, one must also consider the possibility that positive selection requires some interplay between the primary and secondary TCRs. For T reg cells, the greater degree of diversification afforded the secondary TCRs may reflect a greater importance of the primary TCR than originally thought. Such considerations may relate to speculations on the role of secondary TCRs in selecting autoreactive repertoires (29, 30). T cells expressing two TCRs were suspected to elicit autoimmunity by using a benign specificity to dampen negative selection provoked by a second, self-reactive one (30). In this study, to select into the T conv cell lineage, secondary TCRs must not only facilitate evasion of negative selection, but also discourage T reg cell differentiation.

Conclusion

Whatever the origin of differential interanimal constancy of the T reg versus T conv cell repertoires, the answer to the primary question in this study was quite clear: secondary TCR sequences selected into the thymic T conv cell repertoire never ended up in the peripheral T reg cell repertoire as

a result of T conv to T reg cell conversion, in spite of the self-reactivity conferred by the primary BDC2.5 TCR. It may be worth stressing that the BDC2.5 TCR represents a spontaneous specificity present at high frequency in NOD mice and reactive against a natural self-antigen, and is thus more representative of self-reactive specificities than the TCRs elicited in responses to foreign antigens that have been used with neo-self peptides to model self-reactivity (14–16). This observation will need to be generalized to other TCR systems with natural reactivity against self-antigens, in case the affinity/avidity of the BDC2.5 TCR for its ligand fortuitously happened to be unfavorable for conversion. One might also object that the NOD background is refractory, given its issues with tolerance; we do know, however, that TGF β -induced conversion to FoxP3 positivity, as well as thymic induction of T reg cells by antigen, do occur efficiently on the NOD background (unpublished data). Finally, it will be interesting to test whether more conversion could be observed in conditions where the frequency of clonotype-positive cells is more limited than in the transgenic mouse.

The result does not invalidate hypotheses wherein T conv to T reg cell conversion can be important to avoid immune pathology in the context of long-term microbial exposure (e.g., for tolerance to commensal gut flora). But it does seriously question the notion that conversion of self-reactive T cells into T reg cells in the periphery plays much of a role in dominant tolerance to self.

MATERIALS AND METHODS

Mice. BDC2.5/TCR $\alpha^{+/-}$ mice on the NOD background have been previously described (20). Mice were maintained in the Joslin Diabetes Center barrier facility (protocol 99–19, 99–20 approved by the Joslin Diabetes Center's International Animal Care and Use Committee).

Single-cell sorting, RT-PCR, and V α 2 sequence analysis. Experiments were performed as detailed in Wong et al. (11). Lymphocytes from thymi, LNs, and PLNs were first sorted in bulk as BDC2.5 clonotype-expressing, V α 2⁺CD4⁺, and either CD25⁺ or CD25⁻, before re-sorting as individual cells into wells of 96-well PCR plates containing the RT reaction mix containing primer 5'-CCTCTTCTTGCAGAACTCAAATTCATC-TACG-3' for *Foxp3*-specific priming; lymphocytes from the pancreas were sorted directly into plates. The plates were incubated for 90 min at 37°C, and then heat inactivated for 10 min at 70°C. Plates were replicated by transferring 5 μ l of the cDNA into an empty plate. Nested PCR amplification was performed and contamination was monitored in the replicates for *Foxp3* or V α 2 transcripts, as previously described (11, 27). V α 2 amplifications were prepared for automated sequencing using Shrimp Alkaline Phosphatase (GE Healthcare) and Exonuclease I (New England Biolabs), as previously detailed (11). Products were subjected to automated sequencing (Dana-Farber/Harvard Cancer Center High-Throughput Sequencing Core). Raw sequencing files were filtered for sequence quality, and processed in an automated fashion. Data for each cell were tabulated in a database (Access; Microsoft), together with the cell's origin, surface phenotype, and *Foxp3* RT-PCR result.

Online supplemental material. Fig. S1 depicts the gross characteristics of the T reg and T conv cell thymocyte populations. Fig. S2 shows the TCR sequence distributions in peripheral localities. Fig. S3 shows high-frequency TCR sequences categorized by organ. Table S1 shows the complete listing of TCR CDR3 α sequences, along with their occurrences in each population

listed at the top of the chart. The totals are contained in the last line. Table S2 outlines the V α 2 nomenclature was applied throughout the text, including cross-references to GenBank accession nos. and IMGT classifications. The online version of this article is available at <http://www.jem.org/cgi/content/full/jem.20070822/DC1>.

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