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Chronic Modulation of the TCR Repertoire in the Lymphoid Periphery

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Using TCR Vβ transgenic mice as a model system, we demonstrate that the induction of peripheral tolerance can mold the TCR repertoire throughout adult life. In these mice, three distinct populations of peripheral T cells are affected by chronic selective events in the lymphoid periphery. First, CD4+Vβ5+ T cells are deleted in the lymphoid periphery by superantigens encoded by mouse mammary tumor viruses-8 and -9 in an MHC class II-dependent manner. Second, mature CD8+Vβ5+ T cells transit through a CD8highVβlow deleterional intermediate during tolerance induction by a process that depends upon neither mouse mammary tumor virus-encoded superantigens nor MHC class II expression. Third, a population of CD4+CD8-Vβ5+ T cells arises in the lymphoid periphery in an age-dependent manner. We analyzed the TCR Vα repertoire of each of these cellular compartments in both Vβ5 transgenic and nontransgenic C57BL/6 mice as a function of age. This analysis revealed age-related changes in the expression of Vα families among different cellular compartments, highlighting the dynamic state of the peripheral immune repertoire. Our work indicates that the chronic processes maintaining peripheral T cell tolerance can dramatically shape the available TCR repertoire. The Journal of Immunology, 1999, 162: 3131–3140.

Throughout life, the immune system must maintain a balance between the need to respond to the universe of foreign Ags and the need to maintain tolerance to self-Ags. Among T cells, this balance is maintained by both thymic and peripheral selective events. In the thymus, developing thymocytes are subject to both positive and negative selection, processes which together tailor the population of mature self-tolerant T cells to recognize foreign peptides in the context of self-MHC molecules (1, 2). Once mature T cells have left the thymus, a variety of peripheral events continue to modulate the T cell repertoire. For example, viral infection can cause a dramatic expansion of Ag-specific T cells, resulting in over-representation of a few TCR specificities (3, 4). Tolerance induction in the lymphoid periphery can also shape the T cell repertoire through a variety of mechanisms, including clonal deletion (5, 6), clonal diversion (7, 8), clonal exhaustion (9, 10), and clonal anergy with or without an associated down-regulation of the TCR and/or accessory molecule expression on autoreactive cells (11–18).

Our studies of the induction of peripheral tolerance use as a model system C57BL/6 (B6)Vβ1 mice (H-2b, I-Eb-) transgenic (Tg) for a rearranged TCR Vβ5.2 chain (17, 19, 20). An advantage of this strain is that Vβ5+ T cells can be readily followed in vivo during responses to superantigens (SAgs), which interact with T cells largely through the TCR Vβ-chain (for review see Ref. 21). In addition, the limited diversity of the TCR repertoire in Vβ5 Tg mice permits us to study perturbations in the immune repertoire within a relatively homogeneous population of cells. Previous studies of tolerance induction among Vβ5+ T cells have demonstrated that Vβ5+ T cells in MHC class II I-Eb+ mice are deleted intrathymically by vSAG9, a SAg encoded by the endogenous mouse mammary tumor virus (Mtv)-9 (19, 22–25). In MHC class II I-Eb-Vβ5 Tg mice, mature CD4+ and CD8+ T cells escape the thymus, but are selected against in the lymphoid periphery by endogenous self-Ags (17, 19, 20). Peripheral CD4+Vβ5+ T cells are activated and rendered anergic before their deletion (20), while the chronic and incomplete deletion of peripheral CD8+Vβ5+ T cells correlates with the formation of CD8highVβlow cells, defined as deleterional intermediates (17). We have now evaluated how the distinct tolerance pathways taken by these cells influence the TCR Vα repertoire. While the thymus is responsible for molding the preimmune TCR repertoire, age-related changes in TCR Vα expression among different cellular compartments in both Vβ5 Tg and non-Tg B6 mice emphasize that postthymic events can also modify the TCR repertoire. Our studies show that, even after thymic involution, poorly expressed, weak tolerogens in the lymphoid periphery can induce dramatic and long-term alterations in the immune repertoire.

Materials and Methods

Mice
B6 Vβ5 and B6.OT-1 TCR Tg mice were derived to express either the Vβ5 chain only (Vβ5 Tg), or the Vα2 and Vγ7 chain (OT-1 Tg) from a CD8+ CTL clone specific for chicken OVA and H-2Kb and have been described previously (19, 26). These mice carry the endogenous proviral integrants Mtv-8, -9, -17, and -30. Tg mice were maintained as heterozygotes by crossing Vβ5 Tg or B6.OT-1 Tg mice with B6 mice purchased from The Jackson Laboratory (Bar Harbor, ME) and by screening for Vβ5 expression by flow cytometry. Non-Tg mice were offspring from the same matings. Vβ5 Tg B6 × BXD15 mice (Mtv-1, -6, -8, -9, -11, -13, -17, and -30).
were obtained by crossing B6.Tg B6 mice to the H-2\(^b\), I-E\(^b\)-B6.D1 recombinant inbred line (The Jackson Laboratory). An Mtv \(^b\) line of B6.Tg mice and B6.Tg mice carrying single Mtv-integrants were derived by intercross/backcross breeding of B6.Tg mice with a male WLC-0 mouse generously provided by Dr. D. Morris (University of California at Irvine, CA). WLC-0 mice were originally wild-derived and are I-E\(^b\) and Mtv\(^b\) by stringent molecular analyses (27–29). Tg offspring were screened for the presence of Mtvs by Southern blot using a probe that cross-hybridizes with all endogenous Mtvs, as described (20). B6.Tg Mtv class II\(^+\) mice were obtained by crossing the B6.Tg transgene onto MHC class II\(^+\) mice (30) purchased from The Jackson Laboratory and backcrossed to the B6 background through the 12th generation. Offspring were screened for the presence of Mtvs by Southern blot using a probe that cross-hybridizes with all endogenous Mtvs, as described (20).

**Reagents**

Phycoerythrin (PE)-conjugated-anti-CD8\(^{a,b}\) mAbs, biotin-conjugated-anti-Thy-1.2 (30-H12) and -anti-CD4 (RM-1) mAbs, and anti-CD8\(^{a,b}\) mAbs are obtained from ascites or tissue culture supernatants. Purified goat anti-rabbit Ig and Tricolor (TC)-conjugated streptavidin were purchased from Caltag Laboratories (South San Francisco, CA). Guinea pig complement and PCR primers were purchased from Life Technologies (Grand Island, NY).

**Flow cytometry**

Unless otherwise noted, lymph node (LN) cells were isolated from pooled inguinal, axillary, brachial, cervical, and mesenteric LNs. PBL were obtained by water lysis of whole heparinized blood. Cells were stained as described previously (19) and analyzed on a FACScan using CellQuest software (Becton Dickinson, San Jose, CA). Unless otherwise noted, dead cells were excluded on the basis of forward and side scatter profiles, and a minimum of 10\(^6\) live gated events were collected. Cell sorting was performed on a FACStar\textsuperscript{Plus} with LYSYS II software (Becton Dickinson).

**Purification of cell populations**

CD4\(^+\) and CD8\(^+\) T cells from young (8–10 wk), middle aged (30 wk), and old (60–65 wk) B6 TgB6.Tg and non-Tg mice were enriched from pooled spleen and LN cells by Ab plus complement-mediated depletion of CD8\(^+\) and CD4\(^+\) T cells, respectively. Tg populations were stimulated for 4 days in anti-V\(\beta\)5-BSA-coated flasks in the presence of 100 U/ml IL-2 (Perkin-Elmer Cetus Corporation, Emeryville, CA), and transferred to uncoated flasks for 2 days to allow dissociation of bound anti-V\(\beta\)5 mAb. Non-Tg cells were stimulated with ConA at 3 \(\mu\)g/ml (Calbiochem, San Diego, CA) for 3 days. CD4\(^+\) and CD8\(^+\) T cell blasts were then positively selected by panning on anti-CD4- or anti-CD8-coated plates. The purity of the populations was assessed by flow cytometry using anti-CD4 and anti-CD8 mAbs. The nonadherent cells (DN) were removed and their purity assessed by flow cytometry. CD8\(_{low}\) and CD8\(_{high}\) T cells were purified by flow cytometric sorting using nylon wool nonadherent splenocytes from 15- to 18-wk-old B6.Tg B6 \(\times\) B6.D15 mice.

**RT-PCR**

Total RNA was extracted from purified cell populations with guanidinium thiocyanate/phenol (32) and reverse transcribed to cDNA with avian myeloblastosis virus reverse transcriptase (Life Technologies) and random hexamer primers (Pharmacia, Piscataway, NJ). To quantitate cDNAs, threefold serial dilutions of the cDNA reactions were subjected to PCR using primers specific for the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) (33) for 30–35 cycles of 94°C, 1 min, 60°C, 1 min, and 72°C, 1 min on a DNA ThermalCycler 480 (Perkin-Elmer Cetus). Beginning with similar amounts of cDNA, threefold serial dilutions of cDNA were then subjected to PCR using a conserved C3′-specific primer paired with a V\(\alpha\) family-specific primer (Table I) for 30–35 cycles of 94°C, 1 min, 55°C or 60°C (as noted in Table I), 1 min, and 72°C, 1 min. Because the V\(\alpha\)4 primer cannot recognize V\(\alpha\)4.4, we designed a separate V\(\alpha\)4.4 primer. The specificity of the primer pairs was determined empirically by their ability to amplify cDNA from hybridomas expressing known V\(\alpha\) genes. PCR reaction products were electrophoresed on a 2% agarose

Table I. **PCR primers used**

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<tr>
<th>Primer</th>
<th>Sequence and Source (5′→3′)</th>
<th>Temperature (°C)</th>
<th>Product Size (bp)</th>
<th>Number of Family Members(^b)</th>
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<td>V(\alpha)2</td>
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<tr>
<td>V(\alpha)4</td>
<td>CAGATTACCGGAGAAGGTC</td>
<td>55</td>
<td>384</td>
<td>12 (not V(\alpha)4.4)(^e)</td>
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<tr>
<td>V(\alpha)4.4</td>
<td>CAATACTCGGAGAAGGTC</td>
<td>55</td>
<td>384</td>
<td>4.4 only(^f)</td>
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<td>15</td>
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<tr>
<td>C(\epsilon)e</td>
<td>CAGTCGACACAGGTTCGAT</td>
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</tbody>
</table>

\(^a\) Annealing temperature during PCR.  
\(^b\) Arden et al. (34).  
\(^c\) Casanova et al. (35).  
\(^d\) New sequence.  
\(^e\) McElligott et al. (36).  
\(^f\) Harrington et al. (37).  
\(^g\) A separate V\(\alpha\)4.4 primer was designed, because the V\(\alpha\)4 primer hybridizes with all members of the V\(\alpha\)4 family except V\(\alpha\)4.4.
were analyzed.

To investigate which SAg drives the deletion of CD4⁺T cells, B6.OT-1 Tg mice share the same Vβ5.2 transgene with the Vβ5 Tg mice, but develop CD8⁺ T cells poorly (Fig. 4A). In fact, the few CD8⁺ cells that do develop in B6.OT-1 Tg mice overcome the normally tight TCR α-chain allelic exclusion seen with this transgene and are enriched for cells that have down-regulated Va2 expression, while CD8⁺ cells maintain high levels of Va2 expression (Fig. 4B). Taken together, these data indicate that the formation of CD8⁺ cells in this system occurs in the absence of MHC class II molecules and vSAGs, but does depend on the expression of particular TCR α-chains.

The TCR Va repertoire changes during aging and during the chronic induction of tolerance in Vβ5 Tg and non-Tg B6 mice

To determine whether the TCR α-chains expressed in Vβ5 Tg mice play an important role in tolerance induction, we analyzed the peripheral Va repertoire in both Tg and non-Tg B6 mice at three distinct ages: young (8–10 wk) before maximal CD4⁺ deletion or CD8⁺ formation has occurred, middle-aged (30 wk) at the peak of CD4⁺ T cell deletion, and old (60–65 wk) when CD4⁺ T cell numbers recover slightly. Despite very low frequencies of some cell subsets in middle-aged and old mice, we were able to isolate highly purified cell populations (Table II) by enriching for CD4⁺ cells. Southern blotted under alkaline conditions to zeta-Probe GT membrane (Bio-Rad, Hercules, CA), and detected with either an HPRT- or Co-specific probe. Bands were quantitated on a Phosphorimager 425 using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To normalize Vα expression levels to HPRT levels, the integrated volume of the HPRT product was divided by the integrated volume of the HPRT product for each dilution. Because a different primer was used for each Vα family, no attempt was made to compare the frequencies of different Vα families. Instead, relative intensities of each Vα family between the cell subsets were analyzed.

Results

SAg(s) delete peripheral CD4⁺Vβ5⁺ T cells in both Vβ5 Tg and non-Tg B6 cells

Our previous studies of the induction of tolerance among mature peripheral T cells have characterized the chronic selection against both CD4⁺ and CD8⁺ T cells in TCR Vβ5 B6 mice (17, 19, 20). Age-dependent deletion of CD4⁺ T cells and relatively stable numbers of CD8⁺ T cells in Vβ5 Tg mice combine to drive the inversion of the CD4⁺:CD8⁺ ratio among peripheral T cells (19, 20). This decline in CD4⁺Vβ5⁺ T cells is also evident in non-Tg B6 mice, and is therefore not an idiosyncrasy of the transgene. Thus, in both Vβ5 Tg and non-Tg B6 mice, CD4⁺Vβ5⁺ PBL are deleted, leading to a decline in the CD4⁺:CD8⁺ ratio among Vβ5⁺ T cells from 3.1 at 5 wk of age to <0.2:1 at 30 wk of age (Fig. 1). During this time frame, the CD4⁺:CD8⁺ ratio among total T cells in non-Tg B6 mice declines only slightly. This correlation between deletion and expression of a defined Vβ element suggests that the tolerogen driving CD4⁺Vβ5⁺ T cell deletion is a SAg.

vSAG8 and vSAG9 both drive tolerance induction among mature CD4⁺Vβ5⁺ T cells

To investigate which SAg drives the deletion of CD4⁺Vβ5⁺ T cells, we generated lines of Mtv⁻ Tg and non-Tg mice (H-2b, I-E⁻) and strains carrying each of the Mtv genes found in B6 mice (Mtv-8, -9, -17, and -30) singly or in combination. At various ages, we examined PBL for evidence of CD4⁺Vβ5⁺ T cell deletion, which is seen in the original B6 Vβ5 Tg line as a dramatic decline in the CD4⁺:CD8⁺ ratio followed by a slight recovery after 1 year of age (Fig. 2A) and as a loss of Vβ5 expression among peripheral CD4⁺ T cells (Fig. 2B). Unlike B6 Tg mice, Mtv⁻ Tg mice neither invert their CD4⁺:CD8⁺ ratio (Fig. 2A) nor lose Vβ5 expression among peripheral CD4⁺ T cells (Fig. 2B), indicating that a SAg is required for tolerance induction among CD4⁺Vβ5⁺ T cells. Tg mice carrying either Mtv-17, Mtv-30, or both did not differ in their phenotype from Mtv⁻ Tg mice, indicating that these vSAGs do not drive deletion of CD4⁺Vβ5⁺ T cells (data not shown). However, vSAG8 and vSAG9 can independently drive the deletion of CD4⁺Vβ5⁺ T cells, because mice carrying Mtv-8, Mtv-9, or both recapitulate the original B6 Tg phenotype (Fig. 2).

MHC class II⁺ bone marrow-derived cells are required for the deletion of CD4⁺Vβ5⁺ peripheral T cells

We explored the potential contribution of MHC class II A molecules to Vβ5⁺ T cell tolerance by generating radiation chimeras in which bone marrow cells from Vβ5 Tg MHC class II⁺/⁺ (WT) or Vβ5 Tg Class II⁻/- mice were injected into irradiated B6.Thy1.1 mice. At each time point after reconstitution, Class II⁻/- → WT chimeras had significantly greater numbers of CD4⁺ T cells (Fig. 3, left, and data not shown) and a significantly greater CD4⁺:CD8⁺ ratio (Fig. 3, middle, and data not shown) than did WT → WT chimeras. This indicates that the expression of MHC class II molecules on bone marrow-derived cells is required for CD4⁺ T cell deletion in Vβ5 Tg mice.

The formation of CD8⁻ low cells in Vβ5 Tg mice is MHC class II⁺ and Mtv-independent but TCR-dependent

In Vβ5 Tg mice, CD8⁻ T cells transit through a well-defined CD8⁻ low Vβ5⁻ compartment during their deletion (17). Although CD8⁻ low cells are more frequent among Vβ5⁺ Tg, CD8⁻ low cells can also develop among Vβ5⁺ cells in non-Tg B6 mice (data not shown), indicating that the correlation between Vβ5 expression and tolerance induction is not as tight among CD8⁺ T cells as among CD4⁺ T cells in B6 mice. The percentage of CD8⁻ low cells in the PBL of Class II⁺/⁺ → WT chimeras did not significantly differ from that in WT → WT chimeras (Fig. 3, right), demonstrating that CD8⁻ low formation does not require MHC class II expression on bone marrow-derived cells. In addition, CD8⁻ low formation does not require vSAG expression, because CD8⁻ low cells develop in both Mtv⁻ and Mtv-8⁺⁻ Tg mice (Fig. 4A) and in Tg mice carrying other Mtvs (data not shown), though their formation may be slower in Mtv⁻ mice. These data demonstrate that the tolerogen triggering CD8⁻ low formation differs from that driving CD4⁺ T cell deletion.

The formation of CD8⁻ low cells is dependent upon the TCR expressed by the CD8⁻ low Vβ5⁻ T cell. B6.OT-1 Tg mice share the same Vβ5.2 transgene with the Vβ5 Tg mice, but develop CD8⁻ low cells poorly (Fig. 4A). In fact, the few CD8⁻ low cells that do develop in B6.OT-1 Tg mice overcome the normally tight TCR α-chain allelic exclusion seen with this transgene and are enriched for cells that have down-regulated Va2 expression, while CD8⁺ cells maintain high levels of Va2 expression (Fig. 4B). Taken together, these data indicate that the formation of CD8⁻ low cells in this system occurs in the absence of MHC class II molecules and vSAGs, but does depend on the expression of particular TCR α-chains.

FIGURE 1. The CD4⁺:CD8⁺ ratio inverts with age among Vβ5⁺ T cells in both Tg and non-Tg mice. PBL from Tg and non-Tg mice aged 6–45 wk and spleen cells from mice aged 0–5 wk were stained with FITC-anti-Vβ5 and PE-anti-CD4 or PE-anti-CD8α and analyzed by flow cytometry. The CD4⁺:CD8⁺ ratio and SEs among total T cells and Vβ5⁺ T cells were calculated for 3–18 mice per age group.

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and CD8+ T cells both before and after stimulation with either anti-Vb5 Abs (for cells from Tg animals) or ConA (for cells from non-Tg animals). We then compared the expression levels of Vα families between cell subsets (Fig. 5). The expression levels of Vα2, Vα4, Vα8, and Vα11 increase with age in both Tg and non-Tg animals (Fig. 5A), while Vα5, Vα7, Vα9/10, Vα13, and Vα18 expression levels are highest in young or middle-aged mice (Fig. 5). Vα1 is unusual in its poor expression among CD8+ T cells from both Tg and non-Tg B6 mice and therefore was not quantitated. This analysis illustrates significant age-related changes in Vα expression in both Vβ5 Tg and non-Tg B6 mice.

To determine how tolerance induction affected the TCR repertoire, we compared Vα expression levels between Tg and non-Tg animals within each cell population. In young animals, a number of Vα families are selectively up-regulated in Tg relative to non-Tg animals, including Vα3 and Vα13 among CD4+ T cells and Vα2, Vα7, Vα13, and Vα18 among CD8+ T cells (Table III). When compared with young Tg mice, young non-Tg mice express more Vα8 on CD4+ T cells and more Vα4.4 and Vα11 on CD8+ T cells (Table III). Middle-aged Tg animals have generally lower expression of many Vα family members than do age-matched non-Tg mice. Many families (Vα1, Vα9/10, Vα13, and Vα18) are poorly expressed in CD4+ T cells from old Tg animals (Table III). The nearly undetectable Vα7 expression in CD8+ T cells from old}

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**FIGURE 2.** Both Mtv-8 and Mtv-9 drive the deletion of CD4+ T cells in Vβ5 Tg mice. A, Mtv-8 and Mtv-9 are responsible for the inversion of the CD4:CD8 ratio in Vβ5 Tg mice. PBL from mice of the indicated backgrounds and ages were stained and analyzed as in Fig. 1 to determine the CD4:CD8 ratio. Each symbol represents data from an individual mouse. B, Both Mtv-8 and Mtv-9 can drive the loss of transgene expression in CD4+ peripheral T cells in Vβ5 Tg mice. The percent Vβ5+ T cells among CD4-gated cells was determined by flow cytometric analysis of PBL prepared as above; each symbol represents data from an individual mouse.
non-Tg mice (Fig. 5) results in an apparent over-representation of Vα7 expression among CD8+ T cells from old Tg mice (Table III). Overall, one of the most dramatic differences is the over-representation of Vα4 among CD4+ and CD8+ T cells in old Tg animals (Table III).

To elucidate further the effect of chronic tolerance induction on the Vα repertoire, we compared the Vα repertoire of CD8low and CD8high cells isolated from 15- to 18-wk-old Vβ5 B6 × BXD15 animals at the peak of CD8low formation (Table IV). Expression of Vα1, Vα3, Vα4, Vα7, Vα8, and Vα11 do not significantly differ between CD8low and CD8high cells (Table IV). As predicted by the data in Fig. 4, CD8low cells express almost fivefold less Vα2 mRNA than do CD8high cells (Table IV). Vα13 and Vα18 expression levels are also low in CD8low cells (Table IV). Compared with CD8high cells, CD8low cells express more Vα4.4, Vα5, and especially Vα9/10 (Table IV). Thus, tolerance induction among both CD4+Vβ5+ and CD8+Vβ5+ T cells is associated with reproducible alterations in the Vα repertoire.

CD4+CD8− (DN) Vβ5+ T cells develop in aging Vβ5 Tg and non-Tg mice and demonstrate selection for expression of a particular Vα-chain

A third compartment that alters with age in Vβ5 Tg mice is the peripheral CD4−CD8− DN compartment. The percentage of DN peripheral T cells dramatically increases in an age-dependent manner among Vβ5+ but not among Vβ5− T cells in Vβ5 Tg mice (Fig. 6). In non-Tg B6 mice, the representation of DN cells is much greater among Vβ5+ T cells than among Vβ5− T cells, and this over-representation is relatively stable in non-Tg mice of various ages (Fig. 6). To elucidate the origin and TCR repertoire of these coreceptor-null T cells, we compared the Vα repertoire of DN T cells to age-matched CD4+ and CD8+ T cells from Tg mice. DN T cells in middle-aged mice demonstrate increased expression of many Vα families relative to either CD4+ or CD8+ T cells (Table V). Poor expression of Vα1 among CD8+ T cells (Fig. 5) leads to an exaggerated expression of Vα1 among DN T cells relative to CD8+ T cells from old Tg mice. However, DN T cells in old mice demonstrate consistent over-representation of Vαe4.4, and low expression of nearly all other Vα families (Table V). Therefore, the induction of peripheral tolerance in Vβ5 Tg mice leads to the predominance of a subpopulation of DN T cells in old mice, many of which express a Vα4.4+Vβ5+ TCR.

Discussion

Using Vβ5 Tg mice as a model system, we have explored how the induction of tolerance shapes different cellular compartments and their repertoire of expressed TCRs. In B6 Vβ5 Tg mice, tolerance to endogenous Ags drives the deletion of CD4+ T cells, the formation of CD8low cells, and the development of large numbers of peripheral DN T cells. Together these processes shape the peripheral immune repertoire of Vβ5 Tg mice. While reactivity of Vβ5+ T cells to vSAG6, vSAG8, vSAG9, and vSAG11 has been previously demonstrated (25, 38, 39), here we have shown that MHC class II I-A presentation of vSAG8 or vSAG9 drives the deletion of CD4+ Vβ5+ T cells in the periphery of H-2b, I-E− mice. Surprisingly, this deletion is no more rapid or complete in mice carrying both Mtv-8 and Mtv-9 than in mice carrying only one Mtv (Fig. 2), which explains our earlier finding that Mtv-9 did not drive Vβ5+CD4+ T cell deletion in mice that carried Mtv-8 (20). The inversion of the CD4:CD8 ratio among Vβ5+ T cells that results from this deletion is more rapid and extensive than the age-dependent decline in the CD4:CD8 ratio among total T cells in B6 mice (Figs. 1 and 2A) or in T cells from other murine or human studies (40–42), highlighting how dramatically tolerance induction can alter the balance in peripheral lymphocyte subsets.

The CD8+ T cell compartment of Vβ5 Tg mice is shaped by different factors than is the CD4+ T cell compartment in Vβ5 Tg mice. MHC class II presentation of vSAGs does not drive the formation of CD8low deletional intermediates, and the absence of a tight correlation between Vβ5 expression and CD8low formation makes it unclear whether a SAg (not one encoded by an Mtv) or a conventional Ag with a Vβ preference drives CD8low formation. While SAgs may be presented to T cells in the absence of MHC class II molecules and activate T cells bearing a specific β-chain (43), the CD8+ T cell responses of B6 mice to Moloney murine leukemia virus and OVA (44, 45) and the CD4+ T cell response of B10.A mice to pigeon cytochrome C (46) demonstrate a Vβ preference. Therefore, either class of tolerogen could contribute to...
T cells among VαT cell to escape deletion (14, 15). The increased frequency of DN action between a T cell and its tolerogen sufficiently to allow the cells. This down-regulation may decrease the avidity of the inter-

1

CD8lowVβ5low formation and the modulation of the mature CD8+ T cell pool in Vβ5 Tg mice. An alternative tolerance pathway to CD4+ T cell deletion or CD8low formation is coreceptor down-regulation to form DN T cells. This down-regulation may decrease the avidity of the interaction between a T cell and its tolerogen sufficiently to allow the T cell to escape deletion (14, 15). The increased frequency of DN T cells among Vβ5+ cells in non-Tg B6 mice (Fig. 6) in comparison to their normally minor representation among total T cells (Fig. 6 and Ref. 47–50) suggests that this “escape” pathway is common among Vβ5+ T cells in B6 mice. The low frequency of DN T cells in Mtv− mice (data not shown) suggests that DN T cells derive from CD4+Vβ5+ T cells, not from peripheral CD8+ T cells or from distinct thymic precursor cells as has been suggested for the subpopulation of DN T cells characterized previously (for review see Ref. 51). In addition, the DN T cells that were described previously express predominantly Vα4.4+Vβ5+ TCRs rather than the Vα4.4+Vβ5+ TCRs we observe among DN T cells in old B6 mice, further suggesting that Vβ5+ DN T cells in B6 mice are distinct from previously described αβTCR+ DN T cells (51).

Our ability to study Vβ5+ T cells in this mouse model system has allowed us to identify three distinct pathways of tolerance induction in B6 mice and their role in shaping the CD4+, CD8+, and DN T cell compartments of mature animals. It is likely that similar pathways occur among other T cell subsets in response to a variety of Ags; however, the resulting changes in the peripheral T cell pool may be difficult to distinguish within a diverse T cell population. To extend these studies further, we examined the TCR Vα repertoire of the various subsets. Both the documentation of TCRα-chain involvement in SAg recognition in other systems (52–58) and the inefficient formation of CD8low cells among Vα2 Vβ5+ T cells from B6.OT-1 Tg mice (Fig. 4) hint that the TCR Vα-chains expressed by Vβ5 Tg mice influence tolerance induction. Age-related changes in the Vα repertoire could explain the increase in both the CD4:CD8 ratio and the percentage of peripheral CD4+ T cells expressing Vβ5 in Tg mice aged 50–60 wk (Fig. 4). For instance, CD4+ T cells bearing Vα-chains that contribute to poor SAg interaction when paired with Vβ5 (52–58) could selectively survive or expand during old age, while the more SAG-reactive CD4+ T cells would be deleted, accounting for the upswing in the CD4:CD8 ratio and the percentage of CD4+ T cells expressing Vβ5.

In many cases, the TCR Vα repertoire follows the anticipated pattern. Young mice (8–10 wk) are beginning to delete their CD4+ T cells (Figs. 1 and 2), and the increased expression of Vα3, Vα9/10, and Vα13 in Tg relative to non-Tg mice (Table III) may reflect SAg-mediated expansion of cells bearing these Vα families. These same Vα families are under-represented among CD4+ T cells from middle-aged and old Tg mice relative to non-Tg mice, perhaps because most of the CD4+ T cells bearing these SAG-responsive Vα elements are efficiently deleted with age. The over-representation of Vα4.4 and Vα5 among CD4+ T cells in old Tg animals may reflect poor interaction with vSAG8 and vSAG9, which allows T cells bearing these α-chains to survive in old animals (Table III).

CD8lowVβ5low formation and the modulation of the mature CD8+ T cell pool in B6.OT-1 Tg mice. The frequency of CD8low/CD8high T cells among PBL from B6.OT-1 Tg mice of the indicated ages was stained with FITC-anti-Vβ, PE-anti-CD8α, and biotin-anti-Vα2 followed by TC-streptavidin. The percentage of Vα2+ cells among CD8low and CD8high was determined by gating on CD8low or CD8high cells and analyzing the percent expressing Vα2. Results are from 3–7 mice per age group, and error bars represent SEs of the means.

### FIGURE 4. The formation of CD8low cells in Vβ5 Tg mice is CD8-independent but TCR-dependent. A, CD8low cells develop in Mtv− and Mtv-8’9’ Vβ5 Tg mice, but not in B6 OT-1 Tg mice. The percent CD8low/total CD8 T cells among PBL from Mtv− non-Tg, Mtv-8’9’ Tg, Mtv− Tg, and B6 OT-1 mice (3–5 mice per age group) was determined by analyzing CD8- and Vβ-stained cells by flow cytometry. B, The CD8low compartment in B6.OT-1 mice is enriched for cells that do not express the transgenic TCR α. PBL from B6.OT-1 TCR Tg mice of the indicated ages were stained with FITC-anti-Vβ, PE-anti-CD8α, and biotin-anti-Vα2 followed by TC-streptavidin. The percentage of Vα2+ cells among CD8low and CD8high was determined by gating on CD8low or CD8high cells and analyzing the percent expressing Vα2. Results are from 3–7 mice per age group, and error bars represent SEs of the means.
The Vα repertoire of CD8+ T cells in Vß5 Tg and non-Tg B6 mice shows many of the same trends as does the Vα repertoire of CD4+ T cells. One similarity is the poor expression of all Vα family members in middle aged Tg but not non-Tg animals (Table III), which could result from increased apoptosis and RNA degradation among the largely anergic population of Vß5+ T cells in middle-aged mice. Expression of the Vα7 family and the Vα4.4 gene are increased in CD8+ T cells from old Tg relative to old non-Tg animals, implying that T cells bearing these Vα-chains are resistant to becoming CD8 low cells and being deleted. To gain further insight into the induction of tolerance among CD8+ T cells, we also compared the Vα repertoire between CD8 low and CD8 high cells sorted from Vß5 Tg mice aged 15–18 wk.

Table III. Ratio of HPRT-normalized Vα expression levels in Tg:non-Tg mice

<table>
<thead>
<tr>
<th>Vα Family</th>
<th>Young</th>
<th>Middle-Aged</th>
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<tr>
<td></td>
<td>CD4+</td>
<td>CD8+</td>
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<tr>
<td>1</td>
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<td>1:4:1</td>
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<td>1:4:1</td>
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<td>1:4:1</td>
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</tr>
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<td>4.4</td>
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<td>1:2:1</td>
</tr>
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</tr>
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Table IV. Ratio of HPRT-normalized Vα expression levels in CD8 low: CD8 high cells

<table>
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<th>Vα Family</th>
<th>CD8 low:CD8 high</th>
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<td>8</td>
<td>1:1:1</td>
</tr>
<tr>
<td>9/10</td>
<td>6:4:1</td>
</tr>
</tbody>
</table>

The HPRT-normalized Vα expression levels (Fig. 5) were compared between CD4+ and CD8+ T cells from Tg and non-Tg animals of the indicated ages. Differences greater than twofold are either set in boldface type or underlined. Boldface type identifies differences in which the expression in Tg mice exceeds that in non-Tg mice. Underlining identifies differences in which the expression in non-Tg mice exceeds that in Tg mice. Differences less than twofold are considered insignificant and are not set bold or underlined.

*Expression levels of each Vα family, normalized to HPRT expression levels, were compared between CD8 low and CD8 high cells sorted from splenocytes of five Vß5 Tg mice aged 15–18 wk. Differences greater than twofold are either set in boldface type or underlined. Boldface type identifies differences in which expression in CD8 low cells exceeds that in CD8 high cells. Underlining identifies differences in which expression in CD8 high cells exceeds that in CD8 low cells. Differences less than twofold are considered insignificant and are not set bold or underlined.
cells was necessary because these deletional intermediates do not survive the in vitro culture period (17). Furthermore, their characterization allows us to study cells that are undergoing tolerance induction rather than those cells that are left behind. As expected from the data in Fig. 4, Vα1 expression was almost fivefold lower in CD8\(^\text{low}\) than in CD8\(^\text{high}\) cells, implying that Vα1 expression in CD4\(^+\) T cells exceeds that in CD4\(^-\) T cells. In Vβ5 Tg mice, the percent Vα5 cells increases with age, due in part to rearrangement of endogenous TCR genes (63). The percent DN of Vβ5\(^+\) T cells was calculated by adding the percent CD4\(^+\) Vβ5\(^+\) and the percent CD8\(^-\) Vβ5\(^+\) and subtracting this value from the percent Vβ5\(^+\). This value was then divided by the percent Vβ5\(^+\) to eliminate fluctuations due to age-related variation in the number of T cells. Error bars represent SEs of data averaged from 3–10 mice per age group. In Vβ5 Tg mice, the percent DN T cells differed significantly (p < 0.05 by a paired Student’s t test) between Vβ5\(^+\) T cells and Vβ5\(^-\) T cells in all age groups except 8–14 wk (when there are few Vβ5\(^+\) cells) and 43–60 wk. In non-Tg mice, the percent DN T cells differed significantly (p < 0.05 by a paired Student’s t test) between Vβ5\(^+\) T cells and Vβ5\(^-\) T cells in each age range.

Finally, we analyzed the Vα repertoire of DN T cells and discovered a dramatic over-representation of Vα4.4 among DN T cells in old Tg mice (Table V). A similar over-representation of Vα4.4 \(^+\) T cells was seen among CD4\(^+\) and CD8\(^-\) T cells in old Tg mice (Table III), which, along with the suggestion that DN T cells arise from CD4\(^+\) T cells, implies that Vα4.4 among DN T cells may survive in old mice because their TCR interacts poorly with vSAG8 and vSAG9 (52–58). Therefore, multiple interactions of Vα4.4 with Vβ5\(^+\) CD4\(^+\) T cells with vSAG8 or vSAG9 may lead to coreceptor down-regulation and cell survival as DN T cells.

Overall, our analyses indicate that the Vα repertoires of both non-Tg and Vβ5 Tg mice undergo significant age-related variations as a result of the chronic induction of peripheral tolerance (Fig. 5 and Table III–V), suggesting that the T cell repertoire is continually modulated in vivo. While our data provide the first characterization of the TCR Vα repertoire in B6 mice, our finding that Vα6, Vα7, and Vα12 expression levels are low (data not shown) has been independently confirmed in B10, B10.BR, B10.Q, and C57L mice (59, 60). Previously, modifications of the TCR repertoire have been difficult to detect in the context of heterogeneous peripheral T lymphocyte populations. With the exception of expansion of oligoclonal populations of CD8\(^-\) T cells in old mice (40) and a restriction in the diversity of the Vβ-chain in TCR Vα Tg mice (61), alterations in TCR Vβ expression as a result of clonal expansion or deletion have not been noted. Previous studies of the Vα repertoire in mice (59, 60) and humans (62) quantitated the percentage of cells expressing a particular α-chain at one age, while our comparison of Vα expression between T cell subsets highlights age-related changes in expression during tolerance induction.

In Vβ5 Tg mice, the induction of tolerance affects three different cell populations in distinct ways. Most CD4\(^-\) Vβ5\(^+\) T cells are deleted by vSAG8 and vSAG9, but a Vα4.4\(^+\) subset may survive and give rise to a population of DN T cells. Others survive by...
down-regulation of Vβ5, reexpression of recombination machinery, and rearrangement and expression of endogenous Vβ genes (63). CD8+ Vβ5 T cells are driven through a CD8αβ deletion intermediate by their encounter with a tolerogen not encoded by an endogenous Mtv. Each tolerance pathway results in significant modifications in the TCR repertoire expressed by that compartment. Although the thymus plays an important role in shaping the developing immune repertoire, age-related changes in expression of Vα families in both Vβ5 Tg and non-Tg B6 mice indicate that the balance between self-tolerance and a diverse TCR repertoire is subject to consistent and dramatic adjustments in the lymphoid periphery.

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