T Cell Receptor Revision Does Not Solely Target Recent Thymic Emigrants

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CD4+Vβ5+ T cells enter one of two tolerance pathways after recognizing a peripherally expressed superantigen encoded by an endogenous retrovirus. One pathway leads to deletion, while the other, termed TCR revision, results in cellular rescue upon expression of an alternate TCR that no longer recognizes the tolerogen. TCR revision requires the rearrangement of novel TCR β-chain genes and depends on recombinase-activating gene (RAG) expression in peripheral T cells. In line with recent findings that RAG+ splenic B cells are immature cells that have maintained RAG expression, it has been hypothesized that TCR revision is limited to recent thymic emigrants that have maintained RAG expression and TCR loci in a recombination-permissive configuration. Using mice in which the expression of green fluorescent protein is driven by the RAG2 promoter, we now show that in vitro stimulation can drive reporter expression in noncycling, mature, peripheral CD4+ T cells. In addition, thymectomized Vβ5 transgenic RAG reporter mice are used to demonstrate that TCR revision can target peripheral T cells up to 2 mo after thymectomy. Both sets of experiments strongly suggest that reinduction of RAG genes triggers TCR revision. Approximately 3% of CD4+Vβ5+ T cells in thymectomized Vβ5 transgenic reporter mice have undergone TCR revision within the previous 4–5 days. TCR revision can also occur in Vβ5+ T cells from nontransgenic mice, illustrating the relevance of this novel tolerance mechanism in unmanipulated animals. The Journal of Immunology, 2003, 171: 226–233.

During the course of intrathymic differentiation, maturing T cells engage in a carefully controlled series of recombination events that construct functional TCR α- and β-chain genes from germline-encoded segments (reviewed in Ref. 1). These reconfigured genes encode cell surface receptors for Ag that serve as the basis for the selection of self-tolerant, self-MHC-restricted T cells slated for export to the lymphoid periphery (reviewed in Ref. 2). The sequential, developmentally regulated, and locus-restricted TCR gene rearrangement events are absolutely dependent upon RAG1 and RAG2 activities (3–6), which are tightly controlled at both the transcriptional and post-translational levels (1, 7, 8). After the CD4−CD8− double-negative (DN) thymocyte receives a signal from a functional TCR β-chain, RAG gene transcription ceases, and the cell prepares to divide (9, 10). Entry into the cell cycle triggers the phosphorylation and concomitant cleavage of RAG2 protein, thereby ensuring that the temporal boundary remains intact between the incompatible functions of DNA replication and site-specific recombination (11). After proliferation ceases, RAG expression is reinduced in the CD4+CD8+ double-positive (DP) thymocyte, only to be extinguished again after TCR α-chain gene rearrangement is complete, and positive selection signals that the thymocyte expresses on its surface a functional TCRαβ (9, 12, 13). Thymocytes that have been positively selected enter the CD4+ or CD8+ single-positive (SP) compartment, from which cells are allowed to exit the thymus and seed the peripheral lymphoid organs (2).

Given this tight control of RAG gene expression and sequential accessibility to the recombinase of first the TCRβ and then the TCRα locus, it came as a surprise that RAG gene expression and TCR locus recombination can contribute to the induction of T cell tolerance to peripherally expressed self Ags. CD4+ T cells from Vβ5 transgenic (Tg) mice that encounter a superantigen encoded by the defective endogenous retrovirus mouse mammary tumor virus-8 (Mtv-8) are either rendered anergic and deleted (14–16) or triggered to undergo TCR revision (17, 18). Through this latter tolerance mechanism, Vβ5 expression is lost, and the expression of an alternate, Mtv-8-nonreactive TCR β-chain encoded by a novel rearrangement rescues the cell. While both processes are restricted to CD4+ T cells and are driven by Mtv-8 expression, TCR revision is dependent upon B cells, the expression of CD28, and inducible costimulator molecules, while deletion is not (our unpublished observations and Refs. 17 and 18).

One troubling aspect of TCR revision is that it requires RAG gene expression and TCR locus accessibility in peripheral T cells, both considered undesirable traits in post-thymic T cells. An apparent means of sidestepping this conundrum presented itself by means of a thorough analysis of a population of RAG-expressing splenic B cells (19, 20). By using independent lines of mice in which the expression of green fluorescent protein (GFP) serves as a reporter for RAG expression, three groups separately concluded that RAG+ splenic B cells represent an immature, transitional population of new arrivals from the bone marrow (21–23). Rather than reinitiating RAG expression, these B cells have maintained a low level of RAG expression during this transitional phase and, once this expression is extinguished, appear incapable of its reinduction. Although a similar compartment of transitional T cells has not been described, and intrathymic positive selection signals the abrupt down-regulation of RAG expression in T cells (while a
clear positive selection signal appears to be lacking in B cell maturation, it is tempting to draw parallels between the expression of the recombinase machinery in mature B and T cells.

We now use two lines of RAG reporter mice carrying a V$$\beta$$5 transgene to test the hypothesis that TCR revision is limited to recent thymic emigrants that have maintained RAG expression and TCR loci in a recombination-permissive configuration. We demonstrate that RAG expression and TCR revision can be induced in vitro in mature peripheral T cell populations and are not limited to recent thymic emigrants in vivo. Cells that have been exported from the thymus up to 8 wk previously can reinduce RAG gene expression and enter a tolerance pathway, resulting in TCR revision. Judging from the approximate half-life of GFP and the repression and enter a tolerance pathway, resulting in TCR revision.

To enrich for CD4$$^+$V$$\beta$$5$^+$ T cells in these mice we have undergone TCR revision within the previous 4–5 days. Furthermore, RAG$$^+$CD4$$^+$V$$\beta$$5$^+$ cells are detectable in thymectomized V$$\beta$$5 non-Tg mice, illustrating the relevance of this novel tolerant mechanism in unmanipulated animals.

Materials and Methods

**Mice**

RAG2/GFP knockin mice were obtained from Dr. F. Alt (22), and RAG2p-GFP Tg mice were obtained from Dr. M. Nussenzeneggb (21). The V$$\beta$$5 transgene was crossed onto these lines by a cross/bacross breeding strategy using V$$\beta$$5 Tg C57BL/6 (B6) mice (14). Weanlings were typed for V$$\beta$$5 expression by flow cytometry of PBL. RAG2p-GFP Tg mice were similarly typed for GFP expression, and RAG2/GFP knockin mice were typed by PCR using tail DNA. Both strains express enhanced GFP (24) under the control of the RAG2 promoter. In vitro induction of T cells was performed using one or more of the following: anti-CD3 (0.2 $\mu$g/ml), anti-CD28 (0.5–1.0 $\mu$g/ml), and IL-7 (5 ng/ml). IL-7, a gift from Dr. S. Levin, was obtained from cells transfected with an expression vector encoding murine IL-7 (26). At the beginning of cell culture, the cell cycle inhibitor t-mimosine (Sigma-Aldrich) was added at 150 $\mu$M, a level found by our laboratory (unpublished observations) and others (27) to inhibit cell proliferation, but not cell viability. After 2–4 days in culture, cells were harvested and stained for CD4 and V$$\beta$$5 surface expression and analyzed by flow cytometry as described above. The percentage of GFP$$^+$V$$\beta$$5$^+$ CD4$^+$ cells was determined by setting gates based on the cells in parallel cultures in V$$\beta$$5 Tg B6 mice.

**Detection of RAG gene expression and V(D)J recombination intermediates**

Genomic DNA and total RNA were isolated from sorted cells using an RNA/DNA mini kit according to the manufacturer’s instructions (Qiagen). Total RNA from thymocytes was obtained by Nonidet P-40 lysis of cells, followed by guanidine-buffered extraction as described in detail previously (17). RNA was resuspended in RNase-free water and stored at $-80^\circ$C. Genomic DNA was isolated from liver and thymus by overnight digestion of snap-frozen tissue with 0.1 mg/ml proteinase K in 0.5% SDS, 100 mM NaCl, 10 mM Tris, and 25 mM EDTA. Phenol-extracted, ethanol-purified DNA was resuspended in 10 mM Tris (pH 7.5) and 1 mM EDTA.

DNA-purified RNA was transcribed into cDNA by random hexamers using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). RT-PCR with primers (28) for hypoxanthine-guanine phosphoribosyltransferase (HPRT) was performed on serial 3-fold dilutions of cDNA from sorted cells to determine equivalent amounts of starting material to use in subsequent RT-PCR with the following primer sets: 1) R1-forward primer, corresponding to the 5’–untranslated sequence of HPRT (bp 32–57 bp) (28); and R1-reverse primer, corresponding to bp 569–593 of the coding region of RAG1; and 2) R2-forward primer, corresponding to the 5’ untranslated sequence of RAG2 (bp 91–109 bp) (3); and R2-reverse primer, corresponding to bp 539–562 of the coding region of RAG2. PCR products were electrophoresed in 1% Sea-Kem LE and 0.5% NuSieve (3/1) agarose (Cambrex, East Rutherford, NJ), transferred to GeneScreen Plus (PerkinElmer, Boston, MA), and hybridized according to standard protocols. The membranes were probed with random hexamer-primed 32P-labeled RAG1 and RAG2 nested fragments isolated from plasmids harboring cDNAs (3, 28) to detect 560- and 470-bp products, respectively. An end-labeled internal oligonucleotide probe detected a 350-bp PCR product for HPRT (3, 28) to detect 560- and 470-bp products, respectively. An end-labeled internal oligonucleotide probe detected a 350-bp PCR product for HPRT (3, 28). For a given sequence, the PCR reactions from experimental and control groups were performed at the same time, the products were run on the same gel, and the resulting single blot was then probed and exposed.

Ligation-mediated PCR has been described in detail previously (17, 30). Briefly, to measure T cell-specific intermediates in TCR V to DJ recombination, linkers were ligated to genomic DNA as described above. Control reactions to determine the relative amounts of ligated DNA were performed with primers to CD14 (31). For the detection of signal end breaks, PCR reactions were performed with primers specific for the linker and nested primers specific for TCR DJ2. The primary reactions were performed for 12 cycles (94°C, 1 min; 66°C, 1 min; 72°C, 1 min), followed by one 10-min 72°C step, and the nested reactions were run using 5% of the initial reaction in 30 cycles using the same PCR conditions. PCR products were electrophoresed and blotted as described above, then hybridized with a 32P-end-labeled DJ2-specific nested oligonucleotide probe. Southern blots were probed with RAG1, RAG2, HPRT, and DJ2 signal end breaks were exposed to film for $>$1 wk.

**Results**

**Use of GFP to report RAG expression in T cells**

To more easily analyze the process of TCR revision both in vitro and in vivo, we imported two lines of mice in which GFP serves as a reporter for RAG2 expression. RAG2/GFP knockin mice were engineered to express a fusion protein between RAG2 and GFP, encoded by a gene targeted to the endogenous RAG2 locus (22). This gene is expressed at the level dictated by the RAG2 cis-acting control elements, and the fusion protein undergoes cell cycle-dependent degradation, as does the parent RAG2 protein (11). As such, GFP expression is dim and is lost as thymocytes enter the SP compartment (Fig. 1) (22). In contrast, RAG2p-GFP Tg mice carry a multicopy GFP transgene driven by the RAG2 promoter (21). In

**Abs, cell surface staining and flow cytometric analysis**

Splenocytes and water-lysed PBL were resuspended in HBSS containing 1% BSA for staining. Monoclonal, arachidyl, and intralymph nodules (LN) were harvested, teased, passed through Nitex, and resuspended as described above. To enrich for CD4$^+$ T cells from thymectomized V$$\beta$$5 Tg RAG2p-GFP Tg mice before sorting, pooled splenocytes and LN cells were positively selected using CD4 microbeads and an AutoMACS column, according to the manufacturer’s protocols (Miltenyi Biotech, Auburn, CA). To enrich for CD4$^+$ T cells from V$$\beta$$5 non-Tg RAG2p-GFP Tg mice, negative selection with BioMag beads (Qiagen, Valencia, CA) was performed on cells bearing the following epitopes: CD8, MHC class II, FcR, NK1.1, Ter119, and B220. Enriched CD4$^+$ V$$\beta$$5$^+$ T cells were sorted into GFP$^+$ CD4$^+$ and GFP$^+$ CD4$^+$ populations with a FACS Vantage (BD Biosciences, Mountain View, CA). Flow cytometric analysis of T cells was performed using CellQuest software on either a FACSCalibur or a FACScan flow cytometer (BD Biosciences). The following Abs were used: anti-V$$\beta$$5 TCR (MR9-4), V$$\beta$$8.1 TCR (F23.1), CD3 (145-2C11), CD4 (RM4-5 or RM4-4), CD8 (53-6.7), CD11b (M1/70), CD16/32 (2.4G2), CD28 (37.51), CD45/B220 (RA3-682), Ter-119, NK1.1 (PK136), MHC class II (AF6-120.1), and streptavidin-allophycocyanin. Streptavidin-PE was obtained from Caltag Laboratories (Burlingame, CA). Surface staining was limited to fluorochromes in the second through the fourth fluorescence channels; the first fluorescent channel used for GFP detection.

**In vitro induction of RAG gene expression in CD4$^+$ V$$\beta$$5$^+$ T cells**

Splenocytes from V$$\beta$$5 Tg B6 and V$$\beta$$5 Tg RAG2/GFP knockin mice were seeded in 96-well plates at 5 × 10$^4$ cells/well and incubated at 37° under 7% CO2 in RPMI medium containing 10% FCS, 10 U/ml penicillin-streptomycin, 10 mM HEPES, 5.5 mM 2-ME, and 4 mM l-glutamine. In the indicated cultures T cells were stimulated with one or more of the follow-
these mice GFP expression is much brighter, and the transgene-encoded protein is not subject to post-translational control. SP thymocytes remain GFPbright, and GFP lingers even after cells exit the thymus (Fig. 1). Thus, the knockin mice offer the advantage of accurate, developmentally restricted reporting, while the Tg mice offer the advantage of easy detection of brightly fluorescing cells.

Peripheral CD4+ T cells can be induced to express RAG genes in vitro

As expected, pooled spleen and LN cells from Vβ5 Tg RAG2/GFP knockin mice express no detectable GFP directly ex vivo (Fig. 2A, top panel). CD4+Vβ5+ T cells within this population can be induced to express GFP upon coculture with anti-CD28 and low levels of anti-CD3. GFP expression by these cultured cells can be increased with the addition of IL-7 (Fig. 2A, second panel). Addition of supernatants containing thymic stromal lymphopoietin similarly enhanced GFP expression, while those containing IL-2 did not (our unpublished observations). A distinct population of GFPbright cells can be detected only upon the addition of the cell cycle inhibitor mimosine to the anti-CD3- and anti-CD28-stimulated, IL-7-supplemented cultures. The enhancement of GFP expression in the presence of mimosine is most apparent late in the stimulated, IL-7-supplemented cultures. The enhancement of GFP cell cycle inhibitor mimosine to the anti-CD3- and anti-CD28-

FIGURE 1. Thymocytes and peripheral T cells from two lines of mice in which GFP serves as a reporter for RAG expression differ in GFP intensity and in developmental stage-specific restriction of GFP expression. Thymocytes and splenocytes from 7- to 8-wk-old RAG2/GFP knockin (left panels) and RAG2p-GFP Tg mice (right panels) were stained with anti-CD4 and anti-CD8, and gated DN (CD4+CD8-), DP (CD4+CD8-), and SP (CD4-CD8+) thymocytes and peripheral T cells were analyzed for green fluorescence. DN thymocytes from a B6 mouse (bold line, top panels) are shown for a GFP- control. The following mean fluorescence intensities were calculated for the GFP+ cells from RAG2p-GFP Tg mice: GFP+ DP thymocytes, 1030 (99% GFP+); CD4+ SP thymocytes, 480 (88% GFP+); and CD4+ splenocytes, 67 (37% GFP+).
after RAG expression is extinguished intrathymically by positive selection. Although we determined by Southern analysis that RAG2p-GFP Tg mice carry the endogenous retrovirus that encodes Mtv-8 (our unpublished observations), it was essential to verify whether CD4⁺ Vβ5⁺ T cells from Vβ5 Tg RAG2p-GFP Tg mice are subject to tolerance induction by the two pathways, deletion and TCR revision, previously defined in Vβ5 Tg B6 mice (14–18). Deletion of CD4⁺ peripheral T cells, measured by an inversion of the CD4/CD8 ratio, is readily detected in thymectomized Vβ5 Tg RAG2p-GFP Tg mice (Fig. 3, top panel), as is the gradual loss of transgene expression that is the end result of TCR revision (Fig. 3, middle panel). Both the deletion of CD4⁺ T cells and the loss of transgene expression among the surviving CD4⁺ cells occur with kinetics similar to those seen in thymectomized Vβ5 Tg B6 mice (14).

**TCR revision occurs with measurable frequency in thymectomized Vβ5 Tg mice**

If thymectomized Vβ5 Tg RAG2p-GFP Tg mice are to serve as an in vivo model system for TCR revision, it is essential that the CD4⁺ population includes a measurable frequency of GFP⁺ cells, and that this frequency exceeds that found in thymectomized Vβ5 non-Tg RAG2p-GFP Tg mice. Such is the case, as illustrated in Fig. 3, bottom panel. Approximately 60% of CD4⁺ T cells are GFP⁺ in 5-wk-old animals just before thymectomy, and this proportion is the same regardless of whether the animals carry the Vβ5 transgene. The representation of GFP⁺ cells in the CD4⁺ population drops precipitously in both sets of animals in the early weeks after thymectomy, reflecting the loss of recent thymic emigrants. However, the percentage of CD4⁺ T cells that are GFP⁺ in Vβ5 Tg RAG2p-GFP Tg mice levels out at 3–7% by wk 3 post-thymectomy, while that in Vβ5 non-Tg RAG2p-GFP Tg mice is lower, at an average of 1.7%. RAG-expressing immature B cells are excluded by the CD4 gate imposed on cells from both sets of mice, and further analyses reveal that the GFP⁺CD4⁺ cells are CD19⁺, B220⁺, and pan-TCRβ⁺ (our unpublished observations). Thus, GFP⁺CD4⁺ T cells appear with measurable frequency in thymectomized Vβ5 Tg RAG2p-GFP Tg mice, and this frequency slightly, but reproducibly, exceeds that found in thymectomized Vβ5 non-Tg littermates.

**GFP⁺CD4⁺ T cells express RAG1 and RAG2 and contain TCRβ recombination intermediates**

To determine directly whether the GFP⁺ CD4⁺ peripheral T cells in thymectomized Vβ5 Tg RAG2p-GFP Tg mice have begun the process of TCR revision, we analyzed RAG expression in these cells relative to their GFP⁻ counterparts. RT-PCR analysis revealed that both RAG1 and RAG2 are expressed in GFP⁺ CD4⁺ cells from these animals (Fig. 4A). The GFP⁻ CD4⁺ T cell population from some animals was found to express low levels of RAG1, but RAG2 expression was restricted to the GFP⁺ compartment despite equal loading of cDNA from each source (Fig. 4A, HPRT lanes). To determine whether the RAG1 and RAG2 gene products are functional and whether the TCR loci are accessible to the recombination machinery in these cells, ligation-mediated PCR was used to detect the RAG-mediated, double-strand DNA breaks that initiate the recombination of TCR Vβ segments to previously rearranged D-Jβ gene elements. The presence of T cell-restricted recombination intermediates in the GFP⁺, but not GFP⁻, CD4⁺ peripheral T cell populations from thymectomized Vβ5 Tg RAG2p-GFP Tg mice defines the former cells as intermediates in the process of TCR revision (Fig. 4B). The RAG dependency of these double-strand breaks in the TCRβ locus is demonstrated by their absence in RAG-null thymocytes. Because the experimental

**FIGURE 3.** Thymectomized Vβ5 Tg RAG2p-GFP Tg mice serve as an in vivo model system for Mtv-8-driven deletion and TCR revision in the CD4⁺ peripheral T cell population. Vβ5 Tg and non-Tg RAG2p-GFP Tg mice were thymectomized at 5 wk of age, and at the indicated time points PBL were stained to detect surface expression of Vβ5, CD4, and CD8. Symbols represent individual Vβ5 Tg (●) or Vβ5 non-Tg mice (○). Top panel, The CD4/CD8 ratio is plotted against weeks post-thymectomy for Vβ5 Tg RAG2p-GFP Tg mice. Middle panel, The percentage of gated CD4⁺ T cells that are Vβ5⁺ or Vβ5⁻ is plotted against weeks post-thymectomy for Vβ5 Tg RAG2p-GFP Tg mice. Bottom panel, The percentage of gated CD4⁺ T cells that are GFP⁺ is plotted against weeks post-thymectomy for both Vβ5 Tg and non-Tg RAG2p-GFP Tg mice. ■ and □, average percentage at each time point. Data from mice just before thymectomy are illustrated in a separate bar graph.
mice had been thymectomized 5 wk or more previously, these results also indicate conclusively that TCR revision does not solely target recent thymic emigrants.

RAG2 expression is extinguished in cells that have recently emigrated from the thymus

To assess whether GFP+ recent thymic emigrants maintain RAG expression at detectable levels, RAG2 expression was analyzed in sorted CD4+ spleen and LN cells from euthymic Vb5 non-Tg RAG2p-GFP Tg mice. No RAG2-specific message was detectable even in sorted CD4+GFP+ cells (Fig. 5). The absence of detectable RAG2 expression by recent thymic emigrants strongly suggests that reinduction of RAG expression triggers TCR revision in Vb5 Tg mice.

FIGURE 4. GFP+CD4+ peripheral T cells from thymectomized Vb5 Tg RAG2p-GFP Tg mice express RAG1 and RAG2 and contain recombination intermediates at the TCRβ locus. Splenic and LN CD4+ T cells from a pool of three Vb5 Tg RAG2p-GFP Tg mice thymectomized 5 wk previously were sorted into GFP+ and GFP− populations. Postsort analysis indicated a purity of 98% for GFP+ CD4+ cells and 86% (99% CD4+) for GFP−CD4+. This latter population was 99% CD4+pan-TCRβ+, and the remaining 14% fell just below the GFP cutoff, probably as the result of inevitable photobleaching during the sorting process itself. RNA and genomic DNA were isolated from both sorted populations. A, GFP+CD4+ T cells re-express RAG1 and RAG2 mRNA. RT-PCR on serial 3-fold dilutions of cDNA were performed for each sample. Southern blots were hybridized with the probes indicated at the left of each blot. Control reactions for RAG1 and RAG2 were B6 thymus, starting at 1/80th the relative amount of cDNA compared with the sorted samples. HPRT was used to normalize the total cDNA levels of the sorted samples to each other; the B6 thymus samples were below the range of detection with this probe. Results are representative of four independent experiments using mice thymectomized 4–8 wk previously. B, GFP+CD4+ T cell populations contain double-strand signal end breaks within the TCRβ locus. A blunt-ended linker was ligated onto genomic DNA samples from sorted populations and control tissues as indicated. Nested PCR was performed using primers specific for the linker and Dβ2 to detect broken signal ends associated with Dβ2. The PCR products were visualized with a nested Dβ2-specific probe. A second PCR reaction for CD14 was used to normalize the relative amount of linker-ligated DNA. Results are representative of two independent experiments using mice thymectomized 5–6 wk previously.

FIGURE 5. RAG2 expression is extinguished in cells that have recently emigrated from the thymus. Spleen and LN cells from 5- to 6-wk-old euthymic Vb5 non-Tg RAG2p-GFP Tg mice were enriched for CD4+ T cells and sorted on the basis of GFP expression, with a resulting purity of 96% for both sorted populations. RNA isolation, RT-PCR, and Southern analysis were performed as described in Fig. 4.

TCR revision occurs in non-Tg mice

Vb5 Tg RAG reporter mice have greatly facilitated the study of Mtv-8-driven TCR revision among CD4+Vb5+ peripheral T cells. However, in these Tg animals, recombination at the endogenous TCRβ locus cannot excise the transgenes that encode the Vb5+ TCR. The question therefore arises whether Ag receptor revision in this system can occur when the autoreactive Vb5+ TCR is encoded within the normal endogenous locus. An analysis of the percentage of GFP+ cells in individual thymectomized Vb5 non-Tg RAG2p-GFP Tg mice reveals that RAG-expressing cells are concentrated in the peripheral CD4+Vb5+ population, relative to CD8+Vb5+ cells or CD4+ cells that are either Vb5− or Vb8+ (Fig. 6). This trend is clear despite the fact that the CD4+Vb5+ population probably includes cells that are undergoing revision

FIGURE 6. TCR revision occurs in CD4+Vb5+ cells from Vb5 non-Tg mice. Spleen cells from four individual thymectomized Vb5 non-Tg RAG2p-GFP Tg mice were stained for CD4, CD8, and Vb5 or Vb8, and the percentage of GFP+ cells was determined for gated CD4+Vb5+ (■), CD4+Vb5− (▲), CD4+Vb8+ (○), and CD8+Vb5+ cells (□). Donor 1 was 5 wk, donors 2 and 3 were 6 wk, and donor 4 was 11 wk post-thymectomy. Results are representative of data from six mice.
and have down-regulated Vβ5 surface expression. Thus, CD4+Vβ5+ peripheral T cells in Vβ5 non-Tg mice can undergo Ag receptor revision.

Discussion

The induction of tolerance among mature peripheral T cells operates through multiple pathways, including clonal exhaustion (32), nutrient deprivation (33), active suppression (34, 35), and anergy, either with or without alteration in Ag receptor or coreceptor expression (36). Two distinct mechanisms, deletion and TCR revision, operate in Vβ5 Tg B6 mice to tolerate CD4+Vβ5+ peripheral T cells to the endogenous superantigen encoded by Mtv-8. These two tolerance pathways operate by distinct rules; Mtv-8-driven TCR revision requires B cells and the expression of CD28 and inducible costimulator molecules, while Mtv-8-dependent deletion does not (Refs. 17 and 18 and our unpublished observations). Recent work from the Kanagawa laboratory (37) has also suggested that TCR revision is induced by encounter only with superantigens, not conventional Ags.

By crossing a transgene encoding an Mtv-8-reactive Vβ5+ TCR onto lines of mice in which green fluorescence can be used to signal RAG expression, we now demonstrate that the expression of a RAG2-GFP fusion protein encoded within the endogenous RAG2 locus can be induced upon in vitro stimulation of GFP+ mature peripheral T cells (Fig. 2). Stimulation with a low dose of anti-CD3 in conjunction with anti-CD28 signals GFP expression, the level of which is up-regulated in the presence of IL-7. The requirement for CD28 cross-linking may explain the dependence of TCR revision on CD28 molecules (18). The influence of IL-7 on the induction of RAG expression in CD4+Vβ5+ T cells is compatible with its role in facilitating Ag receptor recombination (38, 39) and RAG expression by LPS-stimulated splenic B cells (19, 40). Addition of mimosine to block cell cycle progression in late G1 results in the accumulation of a distinct population of GFP+CD4+ T cells (Fig. 2), probably due to the loss of RAG2 degradation that normally occurs upon entry into the S phase (41). Cell cycle blockade also rules out the possibility that the GFP+ cells detected in stimulated cultures are derived from the selective survival and proliferation of GFP+ cells present in undetectable numbers at the initiation of the culture. Thus, these in vitro results offer strong evidence for the reinduction of RAG gene expression in peripheral T cell populations.

It is of interest that only CD4+ T cells from Vβ5 Tg RAG2/GFP knockin mice are triggered by TCR stimulation to express GFP in vitro. The fact that cocultured CD8+ T cells remain GFP− may imply that TCR revision is limited to CD4+ T cells, a hypothesis compatible with some experimental evidence (17, 18, 37). Alternatively, GFP expression in vitro in our system may be restricted to CD4+ cells that have received some required signal in vivo. If so, recent evidence that TCR revision can target CD8+ T cells suggests that such a hypothetical signal for TCR revision may help determine whether TCR revision targets CD4+ or CD8+ T cells (42).

We next turned to a wholly in vivo system, thymectomized Vβ5 Tg RAG2p-GFP Tg mice. As expected, CD4+ T cells are deleted, and CD4+Vβ5+ cells accumulate in these mice with kinetics similar to those seen previously in thymectomized Vβ5 Tg B6 mice (Fig. 3) (14). Of more significance is the observation that the fraction of CD4+ T cells that are GFP+ in thymectomized Vβ5 Tg RAG2p-GFP Tg mice is invariably greater by ~3% than in their Vβ5 non-Tg counterparts. We suggest that it is these cells that are undergoing Mtv-8-driven TCR revision, a hypothesis substantiated by the expression of RAG1 and RAG2 only in these GFP+ cells, a population also found to contain T cell-restricted intermediates in recombination at the TCRβ locus (Fig. 4).

Vβ5 Tg RAG2-GFP Tg mice have permitted the first estimation of the frequency of cells undergoing TCR revision. Using cells carrying a gene encoding an IL-4/EGFP fusion protein targeted to the IL-4 locus (43), the half-life of GFP has been estimated to be 16–20 h in murine T cells cultured in the presence of translation inhibitors (M. Mohrs and R. Locksley, unpublished observation). If peripheral T cells undergoing TCR revision express RAG2 at a level comparable to DP thymocytes, these cells should register as GFP+ for approximately five GFP half-lives, or 4–5 days (Fig. 1). If the cells divide or express less RAG2 than thymocytes (see below), they will fluoresce for fewer days. By this logic, an estimated maximum of 3% of peripheral CD4+ T cells in thymectomized Vβ5 Tg RAG2p-GFP Tg mice have undergone TCR revision in the previous 4–5 days. That TCR revision is not a rare event has been suggested previously by the diverse repertoire of endogenous TCR β-chains in Vβ5+ CD4+ T cells from individual Vβ5 Tg B6 mice (18). The frequency of cells undergoing TCR revision in vivo is much lower than that achieved in vitro by stimulation with anti-CD3 and anti-CD28 in the presence of IL-7 and mimosine (Fig. 2), a system that visualizes cells capable of undergoing TCR revision upon synchronous stimulation under conditions in which accumulation of GFP is optimized and progression along the pathway of TCR revision is blocked.

By semiquantitative RT-PCR analysis of multiple samples of wild-type thymus and GFP+CD4+ cells from thymectomized Vβ5 Tg RAG2p-GFP Tg mice, we estimate that the RAG1 signal is 80–100 times stronger in thymus (predominantly DP thymocytes) than in the GFP+CD4+ cells, an estimation borne out by the intensity of the GFP signal in these two populations (Fig. 1 and data not shown). RAG1 expression not associated with recombinase activity was occasionally detectable in GFP+CD4+ T cells from thymectomized Vβ5 Tg RAG2p-GFP Tg mice (Fig. 4) and CD4+ Vβ5+ cells sorted from Vβ5 Tg B6 mice (our unpublished observations), and RAG1 expression in the absence of RAG2 has been documented previously in the CNS (44). In the case of cells undergoing TCR revision, RAG2-independent expression of RAG1 may reflect either a threshold effect (RAG1 message is more abundant than RAG2 message) or a temporal progression (RAG1 expression may be initiated slightly before RAG2 expression or extinguished slightly later).

The analyses of GFP+CD4+ cells from thymectomized Vβ5 Tg RAG2p-GFP Tg mice clearly indicate that T cells migrating from the thymus at least 5 wk previously can be triggered to undergo TCR revision (Figs. 3 and 4). Thus, this process does not solely target recent thymic emigrants, a finding entirely in keeping with previous observations. The absence of Vβ5+CD4 SP thymocytes, even in mice in which most peripheral CD4+ T cells are Vβ5−, the nontemplated nucleotide sequences in the revised TCR β-chain genes that are atypical of those generated in the adult thymus (18), and the kinetics of accumulation of Vβ5+CD4+ peripheral T cells that remain unchanged after thymectomy (14), all point to the peripheral origin of cells undergoing TCR revision. Although extra-thymic RAG expression has been reported previously in specialized tissue such as intestinal cryptopatches, it is not associated with TCRβ+CD4+ T cells located in other secondary lymphoid tissues (45, 46). The in vitro induction of GFP expression in peripheral GFP+CD4+ T cells from Vβ5 Tg RAG2/GFP knockin mice (Fig. 2) offers evidence that TCR revision requires the reinduction of RAG gene expression, rather than its continued maintenance. This evidence is strengthened by our inability to detect RAG2 transcription in CD4+ recent thymic emigrants (Fig. 5), defined as GFP+ cells in euthymic Vβ5 non-Tg RAG reporter mice (Fig. 3).
It is also unlikely in the case of RAG2p-GFP Tg mice that the GFP we detect is a result of protein lingering after RAG2 expression has been terminated. The 16–20 h half-life of GFP in murine T cells and the fact that RAG2 is expressed and functional in these cells (Fig. 4) both speak strongly against this possibility. Thus, unlike transitional RAG+ splenic B cells that appear to maintain RAG expression before Ag contact, T cells undergoing TCR revision enter this tolerance pathway and appear to reactivate RAG gene expression. This finding is compatible with what is known to distinguish B and T cell differentiation. B cells do not receive a definitive signal to extinguish RAG expression, while intrathymic positive selection signals the abrupt cessation of RAG expression in T cells before their exit from the thymus (Fig. 5) (9, 13).

Our data also demonstrate, for the first time, that TCR revision in CD4+ T cells can occur in mice carrying unmanipulated TCR loci (Fig. 6), while previous studies have been limited to TCR Tg or knockin mice (17, 37). TCR revision provides a mechanism for the previously documented age-dependent loss of Vβ5 CD4+ T cells in non-Tg mice (15). Furthermore, the appearance of CD4+ TCRαβ RAG peripheral T cells in normal humans and in increased numbers in patients with defective responses to DNA damage (47, 48) suggest that TCR revision occurs in humans. These findings are made all the more surprising, given the risks inherent in RAG gene expression in the lymphoid periphery. Risks include an increased potential for transformation caused by the aberrant juxtaposition of lymphocyte-specific promoters and cellular oncogenes (49, 50). As previously discussed (37), the loss of superantigen recognition could also influence the outcome of infections with superantigen-expressing bacterial or viral pathogens such as Yersinia pseudotuberculosis, Treponasoma cruzi, murine γ herpes virus, and the bacteria associated with Crohn’s disease (51–54). Our results clearly demonstrate that TCR revision can contribute to the induction of tolerance to peripheral expressed self Ags and to the enhancement of TCR diversity in aging mice. Thus, the benefits of generating a broad TCR repertoire expressed by self-tolerant, functional T cells in individuals confronting diminished CD4+ T cell numbers outweigh the risks associated with peripheral expression of RAG genes.

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References


