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Self-Tolerance Checkpoints in CD4 T Cells Specific for a Peptide Derived from the B Cell Antigen Receptor

Thiago Detanico,*† Ryan A. Heiser,* Katja Aviszus,* Cristina Bonorino,† and Lawrence J. Wysocki*

Linked recognition of Ag by B and T lymphocytes is ensured in part by a state of tolerance acquired by CD4 T cells to germline-encoded sequences within the B cell Ag receptor (BCR). We sought to determine how such tolerance is attained when a peptide from the BCR variable (V) region is expressed by small numbers of B cells as it is in the physiological state. Mixed bone marrow (BM) chimeras were generated using donor BM from mice with B cells that expressed a transgene (Tg)-encoded κ L chain and BM from TCR Tg mice in which the CD4 T cells (CA30) were specific for a Vκ peptide encoded by the κTg. In chimeras where few B cells express the κTg, many CA30 cells were deleted in the thymus. However, a substantial fraction survived to the CD4 single-positive stage. Among single-positive CA30 thymocytes, few reached maturity and migrated to the periphery. Maturation was strongly associated with, and likely promoted by, expression of an endogenous TCR α-chain. CD4+ CA30 cells that reached peripheral lymphoid tissues were Ag-experienced and anergic, and some developed into regulatory cells. These findings reveal several checkpoints and mechanisms that enforce a state of self-tolerance in developing T cells specific for BCR V region sequences, thus ensuring that T cell help to B cells occurs through linked recognition of foreign Ag. The Journal of Immunology, 2011, 187: 82–91.

The generation of high-avidity Ab responses requires linked recognition of Ag by specific B cells and CD4 T follicular helper cells in the context of a germinal center (GC) reaction. Within the GC, B cells mutate genes encoding the B cell Ag receptor (BCR) variable (V) region in a process that ultimately results in the maturation of Ab affinity and fine specificity (1–4). A requirement for Ag-specific T cell help to B cells during the GC reaction is thought to be an important regulatory checkpoint, ensuring that only B cells with high-avidity BCR for foreign Ags receive appropriate signals from T follicular helper cells that promote B cell growth and differentiation.

A potential caveat in this scenario is that, along with foreign Ag, peptides from the BCR are also processed and presented on the B cell surface in MHC II (5–12). CD4 T cells with specificity for V region peptides derived from the BCR could potentially provide an avenue of help to the B cell, in violation of the principle of linked Ag recognition (13). Use of this pathway is plausible because of the enormous sequence diversity within the repertoire of V regions expressed by B cells. Some of this diversity is germline-encoded, and some is generated by somatic recombination during lymphopoiesis in the bone marrow (BM) and by somatic hypermutation in the periphery. Ag-unlinked help to the B cell, directed by BCR peptides, is potentially dangerous, as underscored in transgene (Tg) models where such help results in autoimmune development and manifestations of systemic autoimmune disease (14, 15).

Prior studies have demonstrated that CD4 T cells attain a state of tolerance to germline-encoded Ab diversity. This was shown by immunizing mice with unmutated mAbs and sampling T cell hybridomas for responses to the mAb V region peptides in the context of MHC II (16, 17). Additional studies using Tg models revealed that this special case of self-tolerance among CD4 T cells takes place by central deletion within the thymus. However, these studies were performed in mice with nearly monoclonal populations of B and T cells and with high concentrations of serum mAb bearing antigenic V region peptides (14, 18, 19). In these “monoclonal” models, even maternally transmitted mAb resulted in thymic deletion of CD4 T cells specific for peptides from the mAb (14, 20). Complementary experiments demonstrated that large quantities of injected IgG could similarly induce thymic deletion in CD4 T cells reactive to a V region peptide (18).

While it is clear that CD4 T cells in wild-type (WT), nontransgenic mice are rendered tolerant to germline-encoded peptides derived from Ig V regions and that T cells specific for such peptides are deleted in the thymus of Ig transgenic mice, the mechanism(s) of tolerance to BCR and Ig V regions present at physiological levels are unknown. To gain insight into this problem, we generated mixed BM chimeras in which Vκ peptide-specific T cells developed in the presence of physiological numbers of B cells expressing the cognate κV region. Our experiments reveal multiple checkpoints in tolerance culminating in the development of rare Vκ-specific regulatory T cells (Treg) in the periphery.

Materials and Methods

Mice

A complementary pair of mice expressing either a complete Igκ Tg containing a Vκ36-71 exon (κTg mouse) or a Tg encoding an αβ TCR (Val1/
V38) specific for a peptide from Vx36-71 in the context of I-A\(\delta\) (CA30 mouse) has been described (14). These Tgs are carried by mice with an A/J genetic background through more than 25 backcross generations. Large populations of lymphocytes expressing the respective Tgs are present in a resting state, as assessed in the CA30 mouse by low frequencies of T cells expressing activation markers. In the kTg mouse, this resting state is evidenced by large numbers of high-density B cells (\(>1.079\times10^{9}\)) (5). B6, PL-Thy1\(^{-}\)a\(^{-}\)/CyJ were purchased from The Jackson Laboratory (Bar Harbor, ME). BM from (A/J.\(\times\)C57BL/6)F\(_1\) and (A/J.CA30 \(\times\) B6, PL-Thy1\(^{-}\)/CyJ\(\times\)F1) mice were used to create mixed BM chimeras in this study. In some experiments, transgenic mice expressing the F508 \(\alpha\)BCTCR on a B6.PL-Thy1\(^{-}\)/CyJ background were crossed with the A/J. CA30 mice to produce an F1 mouse expressing two TCRs. F508 T cells Harbor, ME). BM from (A/J.\(\times\)C57BL/6)F\(_1\) and (A/J.CA30 \(\times\) B6, PL-Thy1\(^{-}\)/CyJ\(\times\)F1) mice were used to create mixed BM chimeras in this study.

**Mixed BM chimeras**

C57BL6 \(\times\) A/J F1 (B6AF1) recipient mice (The Jackson Laboratory) were lethally irradiated [130 rad in two doses (800\(\times\)500), 3 h apart] and reconstituted i.v. with a total of \(2\times10^{6}\) BM cells, previously depleted of mature leukocytes with the EasySep BM progenitor enrichment kit (Stemcell Technologies, Vancouver, BC, Canada). Various ratios of BM cells from CA30 F1 and kTg F1 or control B6AF1 mice were used as described in the figures and legends of this article. Chimeras were maintained under specific pathogen-free conditions in microisolator cages on a food source fortified with antibiotics (SEPTRA, Harlan Laboratories, Denver, CO) until sacrificed at 12–16 wk for various analyses of tolerance.

**Flow cytometry**

Lymph nodes and spleen cells were pooled, and cells were incubated for 30 min on ice with a mixture of labeled Abs in staining buffer (PBS plus 2% FCS plus 0.01% NaN\(_3\)). The following labeled mAbs were purchased from BioLegend (San Diego, CA): anti-B220, anti-CD44, anti-CD69, anti-CD19, anti-CD8, anti-CD3 (clone R4-3A1), anti-CD25, and anti-CD62L (clone M70-188). The following Abs were purchased from eBioscience (San Diego, CA): anti-CD45, anti-CD69, anti-CD4, anti-CD3, and anti-CD19 (clone B33-1, A52, 30F11, and 1D3). Blocking Abs were purchased from eBioscience (San Diego, CA) and used at 10\(\mu\)g/ml.

**Quantifying serum Ig**

To quantify serum IgG and IgM, 96-well trays were coated overnight at 4\(\degree\)C with 1 \(\mu\)g/ml of H chain-specific goat anti-mouse IgG or IgM (Sigma, St. Louis, MO). Trays were blocked as above and incubated with blocking buffer for 1 h at 37\(\degree\)C. Serial dilutions of mouse sera (starting at 1:1000) were incubated for 1 h at 37\(\degree\)C. Plate-bound Ig was detected with biotinylated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) followed by europium-labeled streptavidin (Wallac, Turku, Finland). Europium fluorescence at 615 nm was measured on a Wallac Victor\(^{\text{TM}}\) 1420 multilabel counter using an excitation wavelength of 340 nm as described (22). Standard curves for quantification were generated with known quantities of IgG and IgM mAbs.

A competition immunoassay was used to quantify serum Ig containing the transgenic Vx36-71 L chain. The 96-well trays were coated overnight at 4\(\degree\)C with a mixture of mAb17-63 (1.66 \(\mu\)g/ml) and normal bovine \(\gamma\)-globulin (3.33 \(\mu\)g/ml, Sigma). Trays were blocked as above and incubated with serial dilutions of sera (starting at 1:25) mixed with biotinylated mAb36-71 FR1-I-A\(^k\) (25 ng/well). Biotinylated Abs were detected by europium-labeled streptavidin. A standard curve for quantification was performed with a complete Igk Tg with a Vx-Jk exon derived from the 36-71 hybridoma (24). The first 13 aa of framework 1 in this Vx compose an immunogenic MHC II epitope. B cells of this line exhibit good isotypic exclusion, reside in the context of normal lymphoid architecture, and contain a substantial fraction that are in a resting state, as assessed by a variety of criteria (5). CA30 Tg mouse carry \(\alpha\)BCTCR Tgs that encode a TCR reactive to the Vx epitope in the context of I-\(\alpha\). CD4 T cells in this mouse are present in normal numbers and largely Ag inexperienced (14). Both lines were on an A/J genetic background. To produce F1 donors for the chimeras studies, A/J kTg mice were crossed with B6 mice, and A/J CA30 mice were crossed with B6 Thy1.1 mice to permit tracking of CA30 T cells. For tolerance studies, B6AF1 recipients were reconstituted with donor BM at low CA30/kTg ratios to generate chimeras in which frequencies of kTg B cells were approximately those expected for B cells expressing any given V region in a WT mouse (Fig. 1).

**Chimeras with low frequencies of tolerogenic kTg B cells**

In initial experiments, we reconstituted mice with donor BM enriched for hematopoietic precursors at two CA30/kTg cell ratios. At 12 wk, the degree of chimerism was assessed in blood, spleen, lymph nodes, and thymus using an mAb (mAb17-63) directed against the kTg B cells and corresponding serum Igk.**results**

Analyzing CD4 T cell tolerance to germline-encoded BCR V regions in mixed BM chimeras

To analyze mechanisms of CD4 T cell tolerance to germline-encoded BCR V regions, we developed a mixed BM chimera model using a complementary pair of donor Tg mice. The kTg animal expresses a complete Igk Tg with a Vx-Jk exon derived from the 36-71 hybridoma (24). The first 13 aa of framework 1 in this Vx compose an immunogenic MHC II epitope. B cells of this line exhibit good isotypic exclusion, reside in the context of normal lymphoid architecture, and contain a substantial fraction that are in a resting state, as assessed by a variety of criteria (5). CA30 Tg mice carry \(\alpha\)BCTCR Tgs that encode a TCR reactive to the Vx epitope in the context of I-\(\alpha\). CD4 T cells in this mouse are present in normal numbers and largely Ag inexperienced (14). Both lines were on an A/J genetic background. To produce F1 donors for the chimeras studies, A/J kTg mice were crossed with B6 mice, and A/J CA30 mice were crossed with B6 Thy1.1 mice to permit tracking of CA30 T cells. For tolerance studies, B6AF1 recipients were reconstituted with donor BM at low CA30/kTg ratios to generate chimeras in which frequencies of kTg B cells were approximately those expected for B cells expressing any given V region in a WT mouse (Fig. 1).

**Chimeras with low frequencies of tolerogenic kTg B cells**

In initial experiments, we reconstituted mice with donor BM enriched for hematopoietic precursors at two CA30/kTg cell ratios. At 12 wk, the degree of chimerism was assessed in blood, spleen, lymph nodes, and thymus using an mAb (mAb17-63) directed against the kTg B region. Flow cytometric analyses indicated that chimeras containing low frequencies of kTg B cells had been generated in both cases (Fig. 2A, 2B). Moreover, the frequencies of kTg B cells and the amount of kTg-derived serum Ab were roughly proportional to the ratios of reconstituting kTg and CA30 BM (Fig. 2C). In mice that received the lowest proportion (6%) of kTg BM (k\(\times\)m)\(\times\)CyJ, kTg B cells comprised from 1% to 2% of all B220\(^+\) cells, and kTg Ab was present in sera at \(\approx 5\) \(\mu\)g/ml. These approximate the physiological frequency of B cells and quantity of Ab expected for any given Vk gene in a WT mouse. In mice reconstituted with twice as many kTg BM cells (k\(\times\)h\(\times\)m\(\times\)CyJ), these numbers were approximately twice as high (Fig. 2B, 2C).

**FIGURE 1.** Generation of mixed BM chimeras. Lethally irradiated B6AF1 recipient mice were reconstituted i.v. with \(2\times10^{6}\) BM cells depleted of mature leukocytes. CA30 and kTg BM cells were mixed at the indicated frequencies. Recipient mice were sacrificed for analysis at 12–16 wk.
Moreover, as shown in Fig. 2D, the low levels of κTg serum Ig in mixed chimeras were not a consequence of a reduced quantity of total Ig, as mg/ml quantities of serum Ig were present in chimeric mice.

Incomplete negative selection of BCR peptide-specific thymocytes in chimeric mice

Prior studies using double-Tg mice carrying 36-71 κ and CA30 TCR Tgs demonstrated that CA30 T cells were deleted during thymocyte development. To determine whether this was also true in chimeras with small numbers of κTg B cells, we stained thymocytes for CA30 T cells using an anti-Thy1.1 Ab. In CA30/κTglow mice, <10% of CD4 single-positive (SP) thymocytes were Thy1.1+, and in CA30/κTgint mice they were <5% (Fig. 3A), suggesting that CA30 thymocytes were deleted during development in the thymus. Thymic deletion was also suggested by the reduced frequencies of CD4 SP cells among total Thy1.1+ thymocytes in mixed BM chimeras (Fig. 3B). In thymi of control mice that were reconstituted only with CA30 BM, most of the cells (~68%) were CD4 SP, as expected for an MHC II-restricted αβTCR. In contrast, this number dropped to ~25% and ~10% in CA30/κTglow and CA30/κTgint chimeras, respectively (Fig. 3B). Although negative selection in the thymus was evident, it was nevertheless incomplete. This distinguishes tolerance in the chimeric model from that observed in prior studies of double-transgenic CA30 × κTg mice and in a related double-Tg model where CD4 SP cells were undetectable (14, 18). Incomplete tolerance indicated that either some CA30 T cells were able to escape tolerance in the chimeras or that there was an additional tolerance checkpoint.

Deletion of BCR peptide-specific thymocytes in mixed BM chimeras

Less than 10% of the all CD4 SP thymocytes were CA30 (Thy1.1+) in CA30/κTg chimeras. We interpret this to indicate that thymic deletion by cognate self-antigen rather than competition between CA30 and WT thymocytes (from the κTg donor) was responsible for this low frequency because in both sets of chimeras, a large proportion (94 and 88%) of reconstituting BM was from CA30 donors. Also, the 2-fold drop in the proportion of CD4 SP CA30 thymocytes between the CA30/κTglow and CA30/κTgint chimeras did not reflect the small change in the proportion of reconstituting CA30 BM (Fig. 3A). To determine if competition by non-Tg thymocytes could account for the low numbers of CA30 Tg thymocytes in mixed BM chimeras, we reconstituted B6AF1 mice with equal numbers of BM cells from B6AF1 and CA30 mice. In these chimeras, WT CD4 SP thymocytes demonstrated an advantage over CA30 CD4 SP thymocytes (~29% of the CD4 SP thymocytes were Thy1.1+). However, this appeared insufficient to account for the low frequencies of CD4 SP CA30 thymocytes observed in the mixed BM chimeras (Supplemental Fig. 1).

To provide a definitive test of clonal deletion mediated by κTg B cells, we generated mixed BM chimeras in which the proportion of κTg BM in the experimental group (2%) was matched in the control group by WT B6AF1 BM. As shown in Fig. 3C and 3D, CA30 Tg thymocytes decreased in number as they progressed to the CD4 SP stage in CA30/κTg chimeras but not in the control CA30/B6AF1 chimeras. The absolute numbers of Thy1.1+ CD4 SP thymocytes were also severely reduced in the experimental group relative to those in the control group (p < 0.0001). Collectively, these results indicate that CA30 T cells were subject to thymic deletion in chimeric mice harboring low frequencies of κTg B cells but that deletion was incomplete.

Escape of BCR peptide-specific T cells to the periphery

To determine if any CA30 T cells from mixed BM chimeras attained maturity and seeded the periphery, we stained lymphocytes from spleen and lymph node with anti-Thy1.1. Fig. 4A and 4B show that Thy1.1+ CD4 T cells were present in low numbers in peripheral lymphoid organs. In CA30/κTglow chimeras, ~1% of spleen and lymph node CD4+ T cells stained with anti-Thy1.1, whereas in the CA30/κTgint group, this number was <0.25%. These low frequencies indicated that there were 10- to 20-fold reductions in the proportion of peripheral CD4 cells that were Thy1.1+ (CA30) relative to the proportion of Thy1.1+ cells among CD4+ thymocytes.

CD4 SP thymocytes specific for a BCR peptide are mostly immature

The large difference in frequency of Thy1.1+ CD4+ T cells between the thymus and the periphery could be due to deletion of immature thymocytes at the CD4 SP stage. To test for this, we assessed thymocytes for surface expression of CD24, which distinguishes immature cells poised for apoptosis upon TCR signaling (CD24high) from mature, apoptosis-resistant cells (CD24low) (25). In experimental BM chimeras, an average of only 25% of Thy1.1+...
CD4 SP thymocytes were mature. In contrast, ∼67% were mature in the CA30-only controls (Fig. 4C, 4D). This difference was even larger when experimental and control chimeras analyzed on the same day were individually compared and fold-differences averaged (Fig. 4E). This indicated that in addition to deletion at or before the CD4+CD8+ double-positive (DP) stage, late-stage deletion of CD4 SP cells also occurred. Moreover, in these chimeras, the proportion of all CD4 SP thymocytes that were mature was close to the proportion of peripheral CD4+ cells that were Thy1.1+, indicating that the reduction of CD4+ CA30 cells occurring between the thymus and the periphery could be largely accounted for by late-stage deletion of immature CD4 SP thymocytes. Notably,

**FIGURE 3.** Partial deletion of CA30 thymocytes in chimeras with kTg B cells. A, Mean percentage of total CD4+ SP thymocytes that were Thy1.1+ in CA30/kTg mixed BM chimeras. B, Distribution of CA30 (Thy1.1+) thymocyte subpopulations in representative CA30/ kTg chimeras. Thymocytes were segregated on the basis of the Thy1.1 marker (CA30 cells) and analyzed for the expression of CD4 and CD8. Error bars indicate SEM (n = 5). C, Donor BM ratios used to reconstitute lethally irradiated B6AF1 mice. D, Numbers of thymocytes in mixed chimeras generated as indicated in C (RBC lysed). Statistics were calculated using a one-tailed paired t test. Data represent the composite of two independent experiments. DN (CD4−CD8− double negative), DP (CD4+CD8+ double positive), and CD4 SP (single positive). **p = 0.0023, ***p < 0.0001.

**FIGURE 4.** A majority of CD4 SP CA30 thymocytes in mixed BM chimeras have an immature phenotype. A, Percentage of CD4+ splenocytes that were Thy1.1+ Vβ8+. “NonTg” denotes splenocytes of negative control Thy1.2+/+ mouse. B, Summary of A. Error bars indicate SEM (n = 5). C, Representative frequencies of mature CA30 CD4 SP thymocytes defined on the basis of CD24 expression levels. D, Percentage of mature CA30 thymocytes among total CD4 SP CA30 thymocytes. Matching symbols denote experimental and control mice analyzed on same day. Horizontal bars depict mean value for all animals in each group. E, Fold-difference in percentage of mature CA30 thymocytes between control and experimental chimeras. Graph depicts mean fold-difference calculated using individual fold-differences for paired experimental and control chimeras. F, Low frequencies of kTg B cells among B220+ CD19+ splenocytes in mixed BM chimeras generated with a CA30/kTg or CA30/B6AF1 (control) donor BM ratio of 98:2 for experiments in this figure. Error bars indicate SEM (n = 8, combined results of three experiments).
in this cohort of chimeras generated using donor BM at a CA30/κTg reconstitution ratio of 98:2, only 0.5% of peripheral B cells were κTg+ (Fig. 4F).

Poor allelic exclusion in BCR peptide-specific T cells in secondary lymphoid tissue

To analyze TCR expression by peripheral CD4+ Thy1.1+ T cells of CA30/κTg chimeric mice, we stained splenocytes with a Vx36-71 FR1-I-Aκ tetrameric reagent. As shown in Fig. 5A, Thy1.1+ CD4+ T cells expressed the CA30 TCR, albeit at reduced levels compared with cells in the control mice reconstituted with CA30 and B6AF1 BM. This suggested that peripheral CA30 T cells in the mixed chimeras had down-modulated their TCR. When stained with an Ab directed to the Vβ8.2 component of the CA30 TCR, however, Thy1.1+ CD4+ T cells in the chimeras and controls were nearly indistinguishable, indicating that the low level of staining with the MHC–peptide tetramer was due instead to expression of a second TCR (Fig. 5A). To confirm this interpretation, we stained lymphocytes from the chimeras with an Ab to Vα2, which is distinct from the Vα component (Vα1) of the CA30 TCR. As shown in Fig. 5B, peripheral CA30 T cells (Thy1.1+ CD4+ Vβ8+ in chimeras stained at a frequency that was ~30 times greater than that of CA30 T cells from control animals, indicating frequent dual TCRα-chain expression in CA30 T cells of chimeric mice (Fig. 5B, top panels).

To determine whether this elevated frequency of peripheral CA30 T cells expressing an endogenous TCRα-chain was due to high-frequency expression in mature thymocytes, we analyzed mature Thy1.1+ thymocytes (CD4 SP Vβ8− CD24low) from CA30/κTg chimeras by flow cytometry and found that they indeed expressed Vα2 at a high frequency relative to controls and at a similar frequency to that seen in the periphery (Fig. 5B, lower panels). Mature Thy1.1+ thymocytes from CA30/κTg mice also stained more weakly with the Vx36-71 FR1-I-Aκ tetramer than did controls from CA30 control mice (Fig. 5A). Collectively, these results suggested that expression of an endogenous Vα due to receptor editing or failed allelic exclusion promoted survival and maturation of CA30 thymocytes in BM chimeras containing κTg B cells.

BCR peptide-specific thymocytes protected from late thymic deletion by a second TCR

To determine if expression of a second TCR by autoreactive CA30 thymocytes would protect them from thymic deletion, we bred a second TCR Tg from the F508 TCR Tg mouse into CA30 donors to produce dual TCR (2TCR) mice. The F508 TCR is encoded by Vα2/Vβ14 Tgs and binds an immunogenic peptide called 3K in the context of I-Aκ (21). Somewhat unexpectedly, 2TCR T cells expressed surface Vβ8 and Vα2 at levels that were comparable with those of pure single receptor CA30 or F508 T cells, respectively. This was accompanied by some, albeit poor, expression of the Vβ14 gene in 2TCR cells, which can be seen in comparative stains of F508 and 2TCR T cells (Fig. 6A). This suggests that 2TCR T cells expressed slightly more total TCR than did CA30 T cells. Similar results were observed with 2TCR thymocytes (data not shown). Importantly, 2TCR T cells stained poorly with the Vx36-71 FR1-I-Aκ tetramer, indicating reduced expression of the CA30 TCR (Supplemental Fig. 2). We can infer that most of this reduction was likely due to competition by the F508 Vα2 for chain pairing with the CA30 Vβ8.2 because Vα2 was expressed at high levels on 2TCR T cells (Fig. 6A, third panel).

As a measure of TCR signaling potential and function, we compared the ability of 2TCR and CA30 T cells to respond to the Vx36-71 FR1 peptide. As expected, there was a reduced in vitro proliferative response to the Vx36-71 FR1 peptide by naive 2TCR T cells relative to CA30 T cells (Supplemental Fig. 3A). This was particularly evident at the lowest concentrations of peptide, indicating a higher threshold concentration of peptide required for proliferation of 2TCR T cells. Results of in vivo tests were similar. Nine days after adoptive transfer of 2TCR or CA30 T cells (Thy1.1+) and immunization with the Vx36-71 FR1 peptide, CA30 T cells proliferated more extensively than 2TCR T cells (Fig. 6B). At the same time, there was no difference in the percentage of IL-2–producing cells among recovered Thy1.1 CD4+ T cells.

**FIGURE 5.** Poor TCRα allelic exclusion in CA30 T cells of mixed BM chimeras. A. Tetramer and Vβ8 stains of CD4+ Thy1.1+ splenocytes and mature thymocytes from CA30/κTg chimeras (thick line) or control CA30/B6AF1 chimeras (thin line). “NonTg” denotes CD4+ Thy1.1− cells from CA30/κTg chimera (dotted line). Representative data for one of three experiments (eight mice total). B. Poor TCRα allelic exclusion in CA30 splenocytes (left panel) and thymocytes (right panel) in CA30/κTg chimeras. Representative data for one of three experiments (eight mice total).
T cells from the two groups, suggesting that the 2TCR T cells were functionally normal (Supplemental Fig. 3B). In addition, no phenotypic differences were observed between naive 2TCR and CA30 T cells with respect to expression of CD44, CD62L, and CD25 (Supplemental Fig. 3C,3D), indicating that like CA30 T cells, 2TCR T cells were in a resting state. These results were consistent with the interpretation that expression of a second TCR diminished functional responses to Ag due to diminished signaling mediated by the CA30 TCR, without obvious effects on T cell physiology.

To determine if a “diluted” CA30 TCR on 2TCR T cells would promote thymocyte maturation and migration to the periphery, we generated mixed chimeras with donor 2TCR or CA30 and kTg BM (95:5 ratio). Fig. 6C illustrates 2TCR thymocyte progression in mixed BM chimeras with (lower panel) or without (upper panel) kTg B cells. Somewhat unexpectedly, expression of a second TCR did not rescue Thy1.1+ T cells at the DP stage. However, at the CD4 SP stage, a substantial proportion of Thy1.1+ thymocytes (~50%) advanced to maturity as assessed with CD24. This frequency was similar to that (~60%) of control mice reconstituted with 2TCR and B6AF1 BM (95:5 ratio) (Fig. 6D).

In the periphery of 2TCR/kTg mice, Thy1.1+ 2TCR cells constituted ~0.8% of all CD4 T cells, which is modestly higher than the frequency observed for the CA30/kTg chimeras (~0.4%), in which a similar degree of reconstitution by kTg B cells was attained (6–7%) (Fig. 6E). However, 2TCR T cells were far less competitive than CA30 T cells, as assessed by their low percentages in the thymus and periphery of control mice in which no kTg B cells were present (Fig. 6F and data not shown). CD4 SP CA30 (Thy1.1+) thymocytes constituted ~8% of all CD4 SP thymocytes in CA30/kTg chimeras, whereas this value was ~2% for the 2TCR/kTg chimeras (Fig. 6E). Therefore, in going from the thymic CD4 SP stage to the periphery, there was an ~20-fold reduction in the fraction of CA30 T cells in CA30/kTg chimeras (8–0.4%), whereas the reduction was only ~2.5-fold in the case of the 2TCR/kTg chimeras (Fig. 6G). Collectively, these results indicate that expression of a second TCR by CA30 T cells enhanced their maturation in the thymus at the CD4 SP stage, which was reflected by increased frequencies of CA30 T cells in the periphery. This, together with preceding results indicating poor Vα allelic exclusion in mature CA30 CD24low thymocytes and peripheral CA30 T cells of CA30/kTg chimeras, support the
conclusion that autoreactive CA30 T cells escaped thymic deletion in part due to expression of a second TCRα-chain. Although we cannot formally rule out the possibility that mature 2TCR thymocytes and T cells occupied larger niches due to selection mediated by a second receptor, this possibility seems inconsistent with the observation that in hosts lacking the κTg self-antigen, 2TCR thymocytes competed more poorly with WT thymocytes than did CA30 thymocytes, and this was reflected by reduced frequencies of 2TCR T cells in the periphery.

Unresponsive state of BCR peptide-specific T cells in the periphery

Peripheral CA30 T cells (CD3+ Thy1.1+ Vβ8+) in CA30/κTg chimeras segregated on the basis of CD4 expression (Fig. 7A). Compared with those in the control CA30/B6AF1 chimeras, the peripheral CD4+ CA30 T cells in CA30/κTg chimeras expressed high levels of CD44 and PD-1 and had modestly increased levels of CD5 (Fig. 7B). These surface molecules are considered to be indicators of Ag experience. However, when CD4+ CA30 T cells were stimulated in vitro with the Vκ36-71 FR1 peptide, they failed to proliferate, as assessed by CFSE dilution (Fig. 7B) or by incorporation of [3H]thymidine (data not shown). The unresponsive state was overcome by addition of rIL-2 at a high dose (50 U/ml), indicating that the cells were anergic (Fig. 7C). CD4 SP thymocytes from CA30/κTg mice behaved similarly, indicating that the anergic state was induced during T cell development in the thymus (Fig. 7C). Mature Thy 1.1+ thymocytes and peripheral Thy 1.1+ CD4 T cells from 2TCR cells in the 2TCR/κTg chimeras displayed a similar Ag-experienced/anergic phenotype (Supplemental Fig. 4).

Anergic CA30 T cells have a regulatory phenotype

Prior studies have demonstrated that autoreactive thymocytes sometimes develop into natural Treg that seed the periphery (26–29). There are also reports that Treg do not proliferate in response to cognate peptide–MHC II unless IL-2 is provided at a high concentration (30). These similarities with our model prompted us to determine whether anergic peripheral CA30 T cells expressed hallmarks of Treg. As seen in Fig. 8, ∼25% of peripheral CD4+ CA30 T cells derived from CA30/κTg chimeras expressed CD25 and Foxp3, which are characteristic of Treg. This stands in contrast to CD4+ CA30 T cells from control CA30/κTg chimeras, which were negative for both molecules (∼0.7%). The presence of such cells in CA30/κTg mice suggested that they might be capable of enforcing self-tolerance to Ig and BCR-derived peptides in the periphery.

To test for potential regulatory function, we cultured purified CFSE-labeled CD4 CA30 T cells with or without Thy 1.1+ CD4 T cells from CA30/κTg chimeras at various ratios and evaluated proliferation in response to the Vκ36-71 FR1 peptide. Results of Fig. 8c show that proliferation was strongly suppressed in cultures containing peripheral CA30 T cells derived from CA30/κTg BM chimeras.

Discussion

The repertoire of CD4 T cells attains a state of self-tolerance to BCR V regions as part of a larger tolerance program ensuring that T cell help to B cells is restricted through linked recognition of Ag. Prior studies with intact BCR and TCR double-transgenic mice have shown that such tolerance occurs in the thymus by deletion at/or preceding the CD4 SP stage of development (14, 18, 19). In contrast, using mixed BM chimeras to achieve physiological numbers of B cells expressing a tolerogenic BCR, we observed at least three checkpoints at which reactive CD4 T cells were rendered tolerant. We found that the extent of thymocyte deletion preceding the CD4 SP stage was dependent on the proportion of B cells in the repertoire that expressed the Vκ epitope seen by the T cells. This is consistent with the massive thymocyte deletion observed in prior studies involving “monoclonal” mice, where virtually all B cells expressed a single tolerogenic BCR (14, 18). In chimeric mice containing physiological numbers of relevant B cells, early-stage thymocyte deletion was followed by a second deletional checkpoint at the CD4+ SP stage, where few cells reached maturity. A third censorship point was revealed by the anergic state of those thymocytes that expressed a second receptor, reached maturity, and migrated to the periphery. At least some of these cells had developed regulatory capabilities.

Although our work reveals multiple checkpoints to eliminate or contain CD4 T cells that might otherwise provide help to B cells via BCR-derived peptides, it is unclear how large of a role the
nondeleterial tolerance checkpoints play in WT animals. Our chimeras contained large numbers of CA30 precursor cells, which conceivably may compete for tolerogenic self-antigen in ways that may not be true of WT mice. And even with large precursor numbers, tolerance in the thymus was impressively efficient, suggesting that in WT animals few if any BCR peptide-specific T cells ever reach the periphery. However, the efficiency of thymic deletion may be variable among clones and likely depends upon a number of factors, such as the affinity of the TCR for BCR peptide–MHC, the affinity of the BCR peptide for MHC, and the abundance of B cells and perhaps other APC that present the cognate BCR peptide. In this regard, the CA30 clone is strongly reactive to the Vk36-71 FR1 peptide and consistently predominates in CD4 T cell responses in mice immunized with whole mAb36-71, which may explain why this clone is prone to efficient thymic deletion in chimeras. In addition, the CA30 TCR is expressed at an unusually early stage of development (double-negative [DN]) because the TCR Tgs are driven by a CD2 promoter. DN cells in chimeras with large numbers of CA30 precursors may have an opportunity to encounter the Vk36-71 FR1 peptide at an earlier stage of development than normal.

It is conceivable that there is an additional late tolerance mechanism, as represented by the DN CA30 T cells observed in the periphery. Down-modulation of CD4 and CD8 coreceptors has been observed in other Tg models of αβ T cell tolerance and has been proposed as a potential censorship mechanism for self-reactive cells (31, 32). Alternatively, peripheral DN T cells could be artifacts of premature expression of the transgenic αβTCR at the DN stage of thymocyte development as mentioned above (33). Some authors have argued that premature αβTCR expression may preemp coreceptor expression and MHC selection in the thymus and allow peripheral migration of αβ analogues of γδ T cells (34, 35). Because we cannot conclusively distinguish between these alternatives, the DN peripheral CA30 T cells were not incorporated into our tolerance scheme.

We found that the CA30 clone was severely underrepresented among peripheral CD4 T cells relative to its representation among CD4 SP thymocytes. In most chimeras, this proportional reduction from the thymus to the periphery was greater than 10-fold and could be largely accounted for by deletion at the mature CD4αβ SP thymocyte stage: among WT CD4αβ thymocytes, ~67% were mature (CD24low), whereas this value was ~25% in the mixed BM chimeras. Those CA30 T cells that escaped to the periphery apparently did so due to expression of a second TCRα-chain, as inferred from the relatively high frequency (7%) of peripheral CA30 T cells expressing a non-transgenic Vγ2 for which a diagnost Ab was available. This was not seen in peripheral CA30 T cells from control chimeras lacking κTg B cells. This is reminiscent of observations made in other TCR Tg models of tolerance where expression of a second TCRα-chain was associated with a state of peripheral tolerance (36, 37). For example, using moth cytochrome c-specific CD4 T cells crossed with Ag Tg mice, Girgis et al. (36) found that cytochrome c-specific T cells were rendered anergic to cognate peptide. Anergy was the consequence of suboptimal TCR signaling on peripheral T cells due to dilution of the specific TCR by an endogenous TCRα-chain (36). Because of the A/J genetic background of our CA30 and κTg lines, this interpretation could not readily be tested using RAG-deficient animals. However, other groups have reported increased frequencies of anergic/regulatory T cells in TCR/Ag double-transgenic RAGα/β mice relative to those of RAG−/− mice.

Rather than using RAG−/− mice, we found support for the Vα-rescue interpretation by using double-TCR transgenic (CA30× F508/F1 mice [2TCR]) as a source of donor BM in mixed 2TCR/κTg chimeras (38). In these chimeras, the frequencies of mature CA30 CD4αβ thymocytes were increased relative to those of mixed CA30/κTg BM chimeras. This is noteworthy that expression of a second TCR did not diminish the degree of thymic deletion at the first checkpoint preceding the CD4 SP stage, as the frequencies of CD4 SP thymocytes were comparable in 2TCR/κTg and CA30/κTg mixed chimeras. Instead, the second TCR increased the proportion CA30 CD4 SP thymocytes that survived to maturity (CD24low). The most straightforward interpretation of this observation is that the signaling threshold for deletion at this second checkpoint is higher than that for deletion at the first because expression of a second TCR promoted survival of thymocytes only at the second checkpoint. This is consistent with results of prior studies using model peptides and Ags, where the Ag concentration threshold for negative selection was found to be lower for immature thymocytes than for mature thymocytes (39–41).

Results of studies with the 2TCR mice must be interpreted with caution, however, because 2TCR cells express a second Vβ, and because there is a lack of diversity in the second TCRα-chain, neither of which is physiological. The CA30 TCR in 2TCR T cells was probably more effectively “diluted” than that of CA30 T cells expressing only an additional endogenous TCRα-chain. Nevertheless, CA30 T cells and 2TCR T cells of tolerogenic hosts had similar phenotypes and were functionally unresponsive to peptide stimulation in vitro, unless provided with high doses of rIL-2 (Supplemental Fig. 4). And the results obtained with 2TCR mice were predicted based on poor allelic exclusion by mature CA30 thymocytes and peripheral CA30 T cells in CA30/κTg chimeras, as inferred by high-frequency expression of an endogenous Vα and poor tetramer staining.
Cells with a Treg phenotype (CD25+, Foxp3+) were enriched among peripheral CD4+ CA30 T cells in mixed chimeras, suggesting that they were positively selected to enter the peripheral pool. They comprised ~15% of CD4 CA30 T cells, which was ~15 times higher than the percentage observed in control chimeras lacking αTg B cells. Still, a majority of CD4 CA30 T cells did not have the Treg phenotype. This heterogeneity among CD4 CA30 T cells could be due to several causes discussed in related prior studies that include homeostatic constraints on the size of the Treg pool, differences in signal strength among CA30 T cells expressing diverse endogenous TCR α-chains, or constraints on the APC niche required for Treg development (27, 42–47). In addition, it is possible that the CD25+ Foxp3+ population of CA30 T cells possess regulatory activity, as CD25+ Treg have been seen in another Tg model of tolerance (28). In this vein, several reports have shown that the most biologically potent Treg expressed in another Tg model of tolerance (28). In this vein, several reports have shown that the most biologically potent Treg expressed in another Tg model of tolerance (28). In this vein, several reports have shown that the most biologically potent Treg expressed in another Tg model of tolerance (28).


