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Thymic Expression of a Gastritogenic Epitope Results in Positive Selection of Self-Reactive Pathogenic T Cells

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Intrathymic expression of tissue-specific self-Ags can mediate tolerance of self-reactive T cells. However, in this study we define circumstances by which thymic expression of a tissue-specific autoepitope enhances positive selection of disease-causing, self-reactive T cells. An immunodominant gastritogenic epitope, namely the gastric H/K ATPase β subunit (H/Kβ253–277), was attached to the C terminus of the invariant chain (II) and the hybrid II (II-H/Kβ253–277) expressed in mice under control of the II promoter. The II-H/Kβ253–277 fusion protein was localized to MHC class II-expressing cells in the thymus and periphery of II-H/Kβ253–277 transgenic mice. In one transgenic line the level of presentation in the periphery (spleen) was insufficient to activate naive, low affinity II-H/Kβ253–277-specific transgenic T cells (1E4-TCR), whereas thymic presentation of H/Kβ253–277 enhanced positive selection of 1E4-TCR cells in II-H/Kβ253–277/1E4-TCR double-transgenic mice. Furthermore, II-H/Kβ253–277/1E4-TCR double-transgenic mice had an increased incidence of autoimmune gastritis compared with 1E4-TCR single-transgenic mice, demonstrating that the 1E4 T cells that seeded the periphery of II-H/Kβ253–277 mice were pathogenic. Therefore, low levels of tissue-specific Ags in the thymus can result in positive selection of low avidity, self-reactive T cells. These findings also suggest that the precise level of tissue-specific Ags in the thymus may be an important consideration in protection against autoimmune disease and that perturbation of the levels of self-Ags may be detrimental. The Journal of Immunology, 2004, 172: 5994–6002.

Multiple mechanisms of T cell tolerance to tissue-specific Ags have been identified that deplete the repertoire of self-reactive T cells. Nonetheless these active tolerance mechanisms are incomplete, and a residual population of self-reactive T cells, characterized by a low avidity phenotype for self Ags, remains in the mature repertoire of normal individuals (1–6). For example, myelin basic protein-specific T cells in the periphery of healthy mice represent the residual low avidity T cell repertoire after tolerance of the high avidity myelin basic protein-specific T cells (7). Additional mechanisms of peripheral tolerance, mediated by dendritic cells and regulatory T cells, are likely to play important roles in influencing the activation of self-reactive T cells that remain after clonal deletion (8–11). Nonetheless, abrogation of these control mechanisms, for example, by immunization with strong inflammatory stimuli or by lymphopenia, can result in activation of the residual populations of self-reactive T cells and subsequent development of autoimmune diseases (12–14).

The fate of T cells during T cell development in the thymus depends on the avidity of interaction between the TCR and MHC/self-peptide complexes (15). High avidity interactions with self-peptides usually result in clonal deletion of thymocytes by negative selection; however, another possible outcome of high avidity interactions with self-Ag is the generation of immunoregulatory CD4⁺CD25⁺ T cells (16, 17). A growing body of evidence indicates that the thymus may have a profound impact on organ-specific tolerance. A wide array of organ-specific Ags is expressed by a specialized population of thymic medullary epithelial cells (18), and the expression of many of these tissue-specific Ags is under the control of the Aire gene (19). Aire-dependent deletion and anergy may lower the frequency of highly pathogenic (high affinity), organ-specific T cell clones, as well as possibly enhance the production of CD25⁺ regulatory T cells that have the capacity to suppress the remaining repertoire of (low affinity) autoreactive T cells (20). However, some tissue-specific Ags are expressed by thymic epithelium in an Aire-independent manner (19).

Experimental autoimmune gastritis is an organ-specific autoimmune disease of the stomach that represents an excellent model of human pernicious anemia (21). The development of the gastric lesion is initiated by the CD4⁺ T response to the gastric H/K ATPase β subunit (H/Kβ), a subunit of the abundant H/K ATPase membrane protein in parietal cells (21, 22). The dominant gastritogenic epitope of the H/K ATPase β subunit in H-2d mice was mapped to residues 253–277 (H/Kβ253–277) (23). Immunization of H-2d mice with H/K ATPase or H/Kβ253–277 induces H/Kβ-specific T cells of low affinity (24), indicating that the anti-H/Kβ repertoire present in normal individuals is of low avidity. For example, T cells isolated from H/Kβ253–277 peptide-immunized mice required a high threshold level of peptide for activation (6, 24). TCR transgenic mice, expressing one of the low affinity, H/Kβ253–277-specific TCRs (1E4), spontaneously develop autoimmune gastritis at low incidence, confirming the pathogenic potential of the 1E4 T cells in vivo (25).

Abbreviations used in this paper: H/Kβ, H/K ATPase β subunit; H/Kα, H/K ATPase α subunit; H/Kβ253–277, residues 253–277 of H/K ATPase β subunit; HSA, heat-stable Ag; II, invariant chain.
Although transgenic animal models have been invaluable in establishing the fate of high avidity self-reactive T cells, there is less information available regarding the thymic selection events associated with low avidity, self-reactive T cells. In view of the fact that low avidity, self-reactive T cells may be responsible for the development of many organ-specific autoimmune diseases, knowledge concerning their thymic selection and tolerance is clearly important in understanding the regulation of autoimmune disease. To assess the affect of thymic expression of the gastric H/Kβ253–277 epitope on the selection and fate of low affinity, gastric-specific 1E4 T cells, we have used a strategy to deliver the gastric autoepitope to the MHC class II loading compartment of APCs (26). Namely, we have generated transgenic mice expressing a recombinant invariant chain (Ii) fusion protein (Ii-H/Kβ253–277) under the control of the Ii promoter. In one transgenic line (M1) where there was a low level of presentation of the H/Kβ253–277 epitope, thymic presentation of H/Kβ253–277 resulted not in negative selection, but, rather, in enhanced positive selection of 1E4 cells. An important question arising from these findings is whether differences in thymic levels of tissue-specific Ags influence the size of the repertoire of low avidity, self-reactive pathogenic T cells and the propensity to develop autoimmune disease.

Materials and Methods

Mice

Six- to 12-wk-old BALB/cCrSlc mice were obtained from Monash University central animal facility (Clayton, Australia). 1E4-TCR (25) and Ii-H/Kβ253–277 transgenic mice were backcrossed to BALB/cCrSlc mice a minimum of six or three times, respectively, and maintained as heterozygotes. All mice were housed under conventional conditions at Monash University Medical School animal facility and Department of Biochemistry and Molecular Biology, University of Melbourne, animal facility. All work with animals was performed with approval of the Monash University and University of Melbourne ethics committees.

Peptides and Abs

Peptides (23) were synthesized by Auspep (Melbourne, Australia). Conjugated mAbs, anti-CD4-allophycocyanin (GK1.5), anti-V8.8.3-PE (I3B.3), anti-CD25-FTTC (PC61), anti-CD44-FTTC (IM7), anti-CD2L (MEL-14), anti-CD11c-PE (HL3), anti-heat-stable Ag (anti-HSA)-biotin (M1/69), and anti-mouse I-A^d-FTTC (AMS-32.1) were obtained from BD PharMingen (San Diego, CA), and anti-CD8-FTTC (Ly-2) was purchased from Caltag Laboratories (Burlingame, CA); Rabbit anti-rat IgG-biotin and streptavidin-HRP were purchased from Vector Laboratories (Burlingame, CA), sheep anti-rabbit IgG-FTTC was purchased from SILENUS Labs (Boronia, Australia), porcine anti-rabbit IgH-HRP was obtained from DAKO (Copenhagen, Denmark), and goat anti-mouse IgG-biotin and streptavidin-biotinylated HRPs were purchased from Amersham (Sydney, Australia). Rat anti-mouse Ii mAb (clone In-1) (27) was purified by protein G-Sepharose chromatography. Anti-H/Kβ253–277 polyclonal serum was produced in a rabbit immunized three times with a synthetic peptide corresponding to the sequence of mouse H/Kβ253–277 and Abs purified by affinity chromatography. The specificity of the affinity-purified anti-H/Kβ253–277 polyclonal Abs was demonstrated by dot-blot analysis and immunoblotting of extracts from Ii-H/Kβ253–277 transgenic COS cells.

Generation of Ii-H/Kβ253–277 transgenic mice

A transgene was constructed consisting of the Ii promoter, Ii p31 cDNA encoding residues 253-277 from the H/K ATPase subunit cDNA encoding as 253–277 (Fig. 1A). Briefly, PCR with oligonucleotide primers 5'-ATAATTATCACAAGTTGGTGTCAGTCTGTG-3' and 5'-GGGAATTCACCTGTTGCTTACCTGTTTAAAG-3' was used to amplify the region of murine H/K ATPase subunit encoding residues 253–277 from the H/K ATPase subunit cDNA (26). The resulting fragment was inserted immediately after the last codon of the p31 isoform of murine Ii cDNA into the unique DraIII site (26), creating pEiI-H/Kβ253–277. The region spanning the Ii/H/Kβ epitope was confirmed by DNA sequencing. An 842-bp Ii-H/Kβ253–277 fragment (Ii-H/Kβ253–277) was then liberated from pEiI-H/Kβ253–277 with NsiI and EcoRI and ligated into NsiI/EcoRI-digested pG31, a plasmid containing murine Ii genomic DNA and promoter (29), resulting in replacement of the 1.81-kb Ii genomic DNA fragment, containing exons 5–8, with the Ii-H/Kβ253–277 cDNA fragment. The resulting vector was designated pEiI-H/Kβ253–277. The 6.6-kb Ii-H/Kb construct (Fig. 1A) was isolated from the pEiI-H/Kβ253–277 vector by digestion with SfiI and EcoRI and purified as previously described (30). The generation of transgenic mice, using fertilized C57BL/6 eggs, was conducted at the Walter and Eliza Hall Institute central microinjection facility (Melbourne, Australia). A number of Ii-H/Kβ253–277 transgenic founder mice were obtained, and lines were established by crossing to BALB/cCrSlc mice. Ii-H/Kβ253–277 transgenic mice were identified by PCR analysis of mouse tail genomic DNA using primers to amplify a 370-bp region between exon 4 and Ii-H/Kβ253–277 sequence.

Frozen tissue preparation and indirect immunofluorescence

Tissues were covered in OCT Tissue-Tek II embedding compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen-cooled isopentane. Sections (4 μm) were cut from frozen blocks at –30°C using a Tissue-Tek II cryostat microtome (Miles Laboratories, Elkhart, IN) and air-dried overnight at 4°C. Sections were treated with filtered 10% FCS/PBS/0.02% sodium azide for 20 min, washed with PBS0.05% Tween 20, then stained with Abs and examined by confocal microscopy as previously described (6).

FIGURE 1. Characterization of Ii-H/Kβ253–277 transgenic mice. A, The structure of the Ii-H/Kβ253–277 transgene, comprising mouse genomic DNA that includes the Ii promoter (■), exons 1–4 of the Ii gene (■), and Ii cDNA (■) fused with the cDNA encoding the H/Kβ253–277 (■). B, Immunoblot analysis was performed on extracts of thymus and spleen from Ii-H/Kβ253–277 transgenic (Tg) and nontransgenic (NTg) mice. Total membranes from the tissues were extracted in 1% Triton X-100, and 50 μg of protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblots were probed with anti-H/Kβ253–277 Ab, followed by anti-rabbit IgG-HRP or anti-li mAb and anti-rat IgG-biotin and streptavidin-HRP, as indicated. Bound secondary conjugate was detected by chemiluminescence. Controls using secondary Ab alone showed no reactivity (not shown).
Immunoblotting

Spleens and thymi were homogenized in PBS containing complete protease inhibitors (Roche, Mannheim, Germany), and the nuclei were pelleted. The supernatant was centrifuged at 100,000 × g for 1 h, and the membrane pellet was resuspended in 20–50 µl of 1% Triton X-100 in PBS. Extracts (50 µg protein), diluted in reducing sample buffer, were subjected to SDS-PAGE and immunoblotting as previously described (31).

Cell suspension preparations and dendritic cells

Spleen, thymus, and lymph node cell suspensions were prepared in 10% FCS in PBS as previously described (6). Bone marrow-derived dendritic cells were prepared as previously described (32).

Flow cytometry

One to three million cells were incubated with Abs and analyzed by flow cytometry on a FACScan or FACS Calibur (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences) as previously described (25).

In vitro and in vivo T cell proliferation assays

In vitro T cell proliferation assays were performed as previously described (6). For in vivo proliferation, lymph node cell suspensions (2.5 × 10^7 cells in PBS) from 1E4-TCR transgenic mice cells were labeled with 5 µM CFSE as previously described (33). Cells were pelleted, washed twice with PBS/FCS, and injected i.v. into mice.

Detection of autoantibodies and gastritis

Detection of Abs to the gastric H/K ATPase by ELISA and H&E staining

Previously we identified the immunodominant autoreactive epitope of the gastric H/K ATPase β subunit as residues 253–277 (23). To explore the consequences of selective expression of this H/K ATPase epitope in APCs, we have generated transgenic mice expressing this gastritogenic epitope fused to the luminal C terminus of the p31 isoform of Ii (Fig. 1A).

To specifically detect the Ii-H/Kβ253–277 product in transgenic mice, a polyclonal anti-H/Kβ253–277 Ab was generated as described in Materials and Methods. The affinity-purified anti-H/Kβ253–277 Ab was initially characterized using transfected COS cells as a source of Ii-H/Kβ253–277. Anti-H/Kβ253–277 Ab showed immunofluorescence and immunoblotting reactivity with COS cells transfected with Ii-H/Kβ253–277, but not with wild-type Ii, indicating that the Ab was specific for the H/Kβ253–277 peptide (not shown). The Ii-H/Kβ253–277 fusion protein was detected in frozen lymphoid tissue sections of the transgenic mice by immunofluorescent staining using the affinity-purified anti-H/Kβ253–277 Ab. In the thymus, strong staining was observed in the medulla, with more diffuse staining in the cortex (Fig. 2). The Ii-H/Kβ253–277 fusion protein was also detected in lymph node sections (Fig. 2). In both tissues the staining patterns were similar to those observed with anti-MHC class II mAb and anti-Ii mAb (data not shown). The thymus showed a 33-kDa band in the major peaks of Ii-H/Kβ253–277 fusion protein was located in the red pulp, and marginal zones with little staining were observed in B cell follicles (Fig. 2). No staining was observed in sections from nontransgenic mice (Fig. 2). These data demonstrate that the Ii-H/Kβ253–277 transgene is expressed in the thymus and periphery.

Immunoblotting of tissue extracts detected the Ii-H/Kβ253–277 fusion protein of the expected size (Fig. 1B). Total membrane extracts of tissues were prepared from age-matched transgenic and nontransgenic littermate mice, and the samples were probed with an anti-Ii mAb, specific for an N-terminal epitope of Ii. Anti-Ii mAb recognizes both endogenous Ii and Ii-H/Kβ253–277 fusion protein. In transgenic and nontransgenic samples, 31- and 41-kDa bands were identified in both thymus and spleen, corresponding to the sizes of endogenous p31 and p41. A comparison of the ratios of the p31/p41 isoforms of Ii in transgenic and nontransgenic samples showed an increased level of the p31 isoform in both thymus and spleen samples from transgenic mice. In addition, the broad band of ~31–33 kDa from tissue samples of transgenic mice was consistent with the presence of the 33-kDa Ii-H/Kβ253–277 fusion protein. Immunoblotting with affinity-purified anti-H/Kβ253–277 sera confirmed the presence of a specific 33-kDa component containing the gastric epitope (Fig. 1B).

APCs from the Ii-H/Kβ253–277 transgenic mice present the H/Kβ253–277 epitope

Bone marrow-derived dendritic cells were generated from transgenic and nontransgenic mice to analyze the presentation of the H/Kβ253–277 epitope. These cells were characterized as CD11c+ and MHC class IIhigh. Cultured dendritic cells from the Ii-H/
Kβ253-277 transgenic mice showed strong intracellular staining with the anti-H/Kβ253-277 Ab (not shown), and the 33-kDa li-H/Kβ253-277 fusion protein was detected by immunoblotting extracts of cultured dendritic cells derived from transgenic, but not non-transgenic, mice (Fig. 3A). These results directly demonstrate the expression of H/Kβ253-277 in MHC class II-positive APCs. Viable dendritic cells were analyzed by flow cytometry with the anti-H/Kβ253-277 Ab; however, no surface staining was observed. It is possible that the anti-epitope Ab does not recognize the MHC class II-H/Kβ253-277 complex.

To determine whether bone marrow-derived dendritic cells from li-H/Kβ253-277 transgenic mice can present the H/Kβ253-277 epitope, an in vitro T cell proliferation assay was performed using 1E4 T cells, which are specific for the H/Kβ253-277 peptide. 1E4 T cells from 1E4-TCR transgenic mice were challenged with LPS-treated dendritic cells alone or with dendritic cells in the presence of exogenous H/Kβ253-277 peptide. In the absence of exogenous peptide, dendritic cells from transgenic mice alone gave >2-fold higher T cell responses than those in assays using dendritic cells from nontransgenic mice (Fig. 3B). T cell proliferation was detected with relatively high ratios of dendritic cells to T cells, indicating that large numbers of dendritic cells were required to stimulate the low affinity H/Kβ253-277-specific T cells. As expected, 1E4 T cells proliferated strongly in assays containing the exogenous H/Kβ gastritogenic peptide regardless of the source of dendritic cells (Fig. 3B). Therefore, bone marrow-derived dendritic cells from the li-H/Kβ253-277 transgenic mice can present the endogenous H/Kβ253-277 gastritogenic epitope to MHC class II-restricted CD4+ T cells, albeit at low levels.

**H/Kβ253-277-specific T cells are positively selected in li-H/Kβ253-277 transgenic mice**

1E4-TCR is specific for the H/Kβ253-277 peptide and is MHC class II restricted (1-E8) (25). By crossing 1E4-TCR transgenic mice with li-H/Kβ253-277 transgenic mice, the presentation of H/Kβ253-277 by thymic APCs could be analyzed by examining the selection and maturation of Ag-specific 1E4 T cells (Vβ3+).

Analyses were performed on progeny (6–15 wk old) from li-H/Kβ253-277×1E4-TCR crosses. The total number of thymocytes was similar among the four resulting genotypes for mice 10–15 wk old: nontransgenic, 0.74×107±0.31 (n = 5); li-H/Kβ253-277, 0.82×107±0.2 (n = 5); 1E4-TCR, 0.9×107±0.28 (n = 9); and li-H/Kβ253-277/1E4-TCR, 0.84×107±0.49 (n = 5). Analyses of a group of younger mice (6–8 wk) also showed a similar total thymocyte count among the genotypes: li-H/Kβ253-277, 6.0×107±0.4; 1E4-TCR, 5.0×107±0.35; and li-H/Kβ253-277/1E4-TCR, 5.54×107±0.21. The proportion of CD4+CD8+ thymocytes was also similar in the four genotypes: nontransgenic, 79.0±3.3% (n = 6); li-H/Kβ253-277, 80.8±4.4% (n = 6); 1E4-TCR, 75.8±14% (n = 10); and li-H/Kβ253-277/1E4-TCR, 59.4±9.5% (n = 7; Table I). A representative FACS analysis is shown in Fig. 4. The proportion of single-positive CD4+CD8– thymocytes was considerable lower in 1E4-TCR mice (3.7±0.6%) compared with nontransgenic mice (11.3±1.2%), consistent with our previous findings (25). This marked reduction in the CD4+CD8– thymocyte population in 1E4-TCR transgenic mice may be due to inefficient positive selection (25). Surprisingly, the proportion of single-positive CD4+CD8– thymocytes was increased in li-H/Kβ253-277/1E4-TCR double-transgenic mice (18.0±4%) compared with 1E4-TCR single-transgenic mice (3.7±0.6%; Table I), suggesting that CD4+ 1E4 T cells may be positively selected after interaction with cells presenting H/Kβ253-277. In contrast, the proportion of single-positive CD4+CD8– thymocytes in li-H/Kβ253-277/1E4-TCR double-transgenic mice was similar to that in nontransgenic mice (Table I).

Further analyses were conducted to assess whether the major differences in thymocyte differentiation between single- and dual-transgenic mice were associated with positive selection. Firstly, the proportion of CD4+CD8– thymocytes, although higher than that in wild-type mice, was similar between the 1E4-TCR single-transgenic (18.6±14.1%; n = 10) and li-H/Kβ253-277/1E4-TCR double-transgenic (18.0±7%; n = 7) mice (Table I). Secondly, we examined the CD44 and CD25 expression of the double-negative thymocyte population. The proportions of double-negative thymocytes that stain for either CD44 and/or CD25 were very similar in single- and double-transgenic mice (not shown). Components of the TCR/CD3 complex are first expressed at the CD44+CD25+ (DN3) stage of thymic development (15). The DN3 population (CD44+CD25+) was 0.22% of the total thymocytes in the 1E4-TCR single-transgenic mice and 0.24% of the total thymocytes in the double-transgenic mice, proportions very similar to that detected in nontransgenic mice (0.14%). Therefore,

![Image](http://www.jimmunol.org/Downloadedfrom)
there was no detectable difference in thymocyte differentiation between single- and double-transgenic mice before the expression of TCR.

As thymocytes increase the expression of TCR and accessory molecules after positive selection (34), mature H/Kβ253-277-specific thymocytes were identified by high levels of CD4 and Vβ8.3 expression and lack of CD8 expression (CD4 bright Vβ8.3 bright CD8−). We identified the CD4 bright Vβ8.3 bright cells (Fig. 4A), then enumerated the CD8− cells within this population (Fig. 4B). The proportion of CD4 bright Vβ8.3 bright thymocytes in li-H/Kβ253-277 single-transgenic mice was low (0.7 ± 0.2%; n = 6) and similar to that in nontransgenic mice (0.7 ± 0.2%; n = 6; Fig. 4B and Table I). This is expected given that li-H/Kβ253-277 mice also express endogenous Li and are likely to have a diverse T cell repertoire. The proportion of CD4 bright Vβ8.3 bright CD8− T cells in li-H/Kβ253-277/1E4-TCR transgenic mice was 2.0 ± 1% (n = 10). Significantly, the proportion of CD4 bright Vβ8.3 bright CD8− thymocytes in li-H/Kβ253-277/1E4-TCR double-transgenic mice (13.4 ± 3.4%; n = 7) was increased 6-fold compared with that in 1E4-TCR single-transgenic mice (Fig. 4B and Table I), indicating that H/Kβ253-277-specific 1E4 thymocytes may be more efficiently positively selected in li-H/Kβ253-277/1E4-TCR double-transgenic mice as a consequence of encountering APCs presenting H/Kβ253-277.

To confirm that the CD4 bright Vβ8.3 bright CD8− population detected in the double-transgenic mice was mature thymocytes, as distinct from circulating peripheral T cells, we examined the expression of HSA. As peripheral T cells are HSA negative, staining for HSA can distinguish between mature thymocytes and peripheral T cells that may have re-entered the thymus (35). The majority of the CD4 bright Vβ8.3 bright thymocytes from 1E4-TCR single-transgenic and li-H/Kβ253-277/1E4-TCR double-transgenic mice were HSA positive (99% in both cases; Fig. 5). In contrast, and as expected, the peripheral population of Vβ8.3 CD4+ T cells in both lines was HSA negative (Fig. 5). This result clearly demonstrates that the CD4 bright Vβ8.3 bright CD8− cells detected in the thymus are mature thymocytes rather than T cells from the periphery that are recirculating through the thymus. Collectively these data show that the ~6.5-fold increase in the proportion of mature CD4+ single-positive thymocytes in double-transgenic mice compared with that in the TCR single-transgenic mice is due to enhanced positive selection.

Analysis of peripheral 1E4 T cells in li-H/Kβ253-277×1E4-TCR mice

Splenocytes were analyzed from the progeny of the li-H/Kβ253-277×1E4-TCR crosses to determine the status of the peripheral pool of 1E4 T cells. The number of splenocytes was similar in the resulting four genotypes (not shown). The proportion of CD4+ cells was also similar in the four genotypes (Table I). In contrast, CD4+ Vβ8.3+ splenocytes were drastically increased in both the 1E4-TCR and the li-H/Kβ253-277/1E4-TCR periphery compared with that in li-H/Kβ253-277 mice (li-H/Kβ253-277 3.7 ± 1.6 (n = 5); 1E4-TCR, 16.7 ± 3.2 (n = 9); li-H/Kβ253-277/1E4-TCR, 14.9 ± 7 (n = 6); Table I). Additionally, the majority of CD4+ T cells (~71%) in the periphery of li-H/Kβ253-277/1E4-TCR double-transgenic mice expressed Vβ8.3. These data clearly show that CD4+ Vβ8.3+ T cells migrate into the periphery of li-H/Kβ253-277/1E4-TCR double-transgenic mice. Even though the number of CD4+ CD8− thymocytes differed between 1E4-TCR single-transgenic mice and li-H/Kβ253-277/1E4-TCR double-transgenic mice, the similar numbers of CD4+ Vβ8.3+ T cells in the periphery of both single- and double-transgenic mice are not surprising in view of the homeostatic regulation of the size of the peripheral CD4 T cell population (36, 37).

The 1E4-TCR uses Vβ8.3 and Vα9. An Ab to Vα9 is currently unavailable. The CD4+Vβ8.3+ splenocytes in the li-H/Kβ253-277/1E4-TCR double-transgenic mice were not enriched for cells expressing endogenous TCRα chains, as the proportion of cells expressing either Vα2 or Vα8 (endogenous TCRα chains) was similar in li-H/Kβ253-277/1E4-TCR double-transgenic mice compared with that in 1E4-TCR mice (not shown). The lack of increased endogenous TCRα-chain usage argues against the rearrangement of additional TCRs for the maturation of CD4+ Vβ8.3+ thymocytes in the double-transgenic mice.

An in vitro T cell proliferation assay was performed with T cells from li-H/Kβ253-277×1E4-TCR double-transgenic mice to determine whether Vβ8.3+ T cells were responsive to H/Kβ253-277 (Fig. 6). Splenocytes from 1E4-TCR transgenic and li-H/Kβ253-277/1E4-TCR double-transgenic littermate mice were challenged with the H/Kβ253-277 peptide or a 14 mer within the H/Kβ253-277 peptide (H/Kβ253-274) to which the 1E4 clone can also respond (24). An equal number of CD4+ Vβ8.3+ T cells was used in each assay. An identical T cell response was observed for T cells from 1E4-TCR transgenic and li-H/Kβ253-277/1E4-TCR double-transgenic mice to peptides H/Kβ253-277 and H/Kβ253-274 (Fig. 6). T cells from neither mouse responded to an irrelevant peptide. As the splenic Vβ8.3+ T cells from the double-transgenic mice can respond to the H/Kβ gastritogenic peptide, these data show that peripheral CD4+ Vβ8.3+ T cells are clonotypic.

The majority of splenic H/Kβ253-277-specific T cells are naive

To determine whether the CD4+ Vβ8.3+ peripheral T cells in li-H/Kβ253-277/1E4-TCR double-transgenic mice were activated or naive, levels of the cell surface markers CD25, CD44, and CD62L were measured by flow cytometry.
were analyzed. Naive T cells express low levels of CD25 and CD44 and high levels of CD62L (38). The expression of these markers is reversed when the cells are activated. The majority of CD4\(^+\)/H11001 V\(\beta\)8.3\(^+\) T cells from all genotypes were CD25\(^-\) (nontransgenic, 88%; Ii-H/K\(\beta\)253–277, 93 ± 1.7%; 1E4-TCR, 91 ± 4.2%; Ii-H/K\(\beta\)253–277/1E4-TCR, 93 ± 2%), CD44 int (nontransgenic, 82%; Ii-H/K\(\beta\)253–277, 71 ± 5.6%; 1E4-TCR, 74 ± 12%; Ii-H/K\(\beta\)253–277/1E4-TCR, 63 ± 11%), and CD62L high (nontransgenic, 70%; Ii-H/K\(\beta\)253–277, 58 ± 7.2%; 1E4-TCR, 67 ± 5.8%; Ii-H/K\(\beta\)253–277/1E4-TCR, 56 ± 5%; **n** = 3 for all groups). A representative example is shown in Fig. 7. The CD4\(^+\)/V\(\beta\)8.3\(^+\) T cell population from Ii-H/K\(\beta\)253–277/1E4-TCR double-transgenic mice showed higher CD44 expression and lower CD62L expression than the population from 1E4-TCR single-transgenic mice; nonetheless, the majority of cells in the double-transgenic mice had a naive phenotype. Analysis of the CD4\(^+\)/V\(\beta\)8.3\(^+\) T cell population from peripheral lymph nodes, in particular draining lymph nodes of the stomach, showed that the CD4\(^+\)/V\(\beta\)8.3\(^+\) T cell population from either single- or double-transgenic TCR mice had similar expression levels of CD25, CD44, and CD62L compared with the CD4\(^+\)/V\(\beta\)8.3\(^+\) T cell population from the spleen (not shown).

**APCs in nongastric tissues of Ii-H/K\(\beta\)253–277 transgenic mice cannot activate mature 1E4 T cells in vivo**

Although bone marrow-derived dendritic cells from Ii-H/K\(\beta\)253–277 transgenic mice can activate 1E4 cells in vitro, the above data indicate that the majority of CD4\(^+\)/V\(\beta\)8.3\(^+\) peripheral T cells in Ii-H/K\(\beta\)253–277/1E4-TCR double-transgenic mice were naive. One explanation for these apparently contradictory findings is that the in vitro proliferative response of the Ii-H/K\(\beta\)253–277 expressing dendritic cells was very weak and was detected using a high ratio of dendritic cells to T cells. Although the in vitro assay demonstrates that the H/K\(\beta\) epitope is presented by APCs in transgenic mice, the conditions required to drive proliferation of 1E4 T cells may not be physiologically relevant. To explore this issue further, in vivo T cell proliferation assays were conducted to analyze presentation of the H/K\(\beta\) epitope in the periphery of Ii-H/K\(\beta\)253–277 transgenic mice. Lymph node cells from 1E4-TCR transgenic mice were labeled with CFSE and injected i.v. into Ii-H/K\(\beta\)253–277 transgenic mice.
immunization of mice with the H/K genic mice to activate naive 1E4 T cells in vivo. In contrast, after samples.

H/K peptide (H/K/H11021/H9252/H11003) transgenic mice are responsive in vitro. Splenocytes (5 × 10^6) from Ii-H/K/H9252/H11003/H12135/H18554 were pulsed after 48 h with [3H]thymidine and were harvested 16 h later. The majority of peripheral CD4 T cells from Ii-H/K/H9252/H11001/H11001 were killed and analyzed. Seventy-one percent (71%) of seven) of the Ii-H/K/H9252/H11001/H11001 mice developed a gastric gastritis in some of the Ii-H/K/H9252/H11001/H11001 mice also contained a large pool of peripheral 1E4 T cells. T cells in the periphery of double-transgenic mice are naive. Splenocytes (5 × 10^6) from Ii-H/K/H9252/H11001/H11001/H11001 mice were collected, and CFSE-labeled 1E4-TCR T cells were identified by labeling for CD4 and Vβ8.3. Flow cytometric analysis revealed that the H/Kβ253-277-specific T cells had not divided in either Ii-H/Kβ253-277 or nontransgenic mice (not shown); therefore, insufficient levels of H/Kβ epitope derived from the recombinant Ii were presented by APCs in the periphery of Ii-H/Kβ253-277 transgenic mice to activate naive 1E4 T cells in vivo. In contrast, after immunization of mice with the H/Kβ253-277 peptide, CFSE-labeled 1E4-TCR T cells proliferated strongly, demonstrating that the labeled 1E4 T cells could be stimulated in vivo given sufficient levels of Ag (not shown). Collectively the above data indicate that APCs in Ii-H/Kβ253-277 transgenic mice present low levels of the H/Kβ epitope, sufficient to influence thymic development, but below the threshold for activation of low avidity 1E4 T cells in non-gastric peripheral tissues.

**Discussion**

There have been limited studies analyzing the ability of peripheral autoantigens to induce positive selection of autoreactive T cells. In this study we have investigated the fate of low affinity, self-reactive T cells in mice expressing the cognate gastric epitope in MHC class II-positive cells. The self-reactive T cells we have used in this study, namely, H/Kβ-specific 1E4 T cells, are present in the periphery of normal mice (24). The original analysis of 1E4 TCR...
transgenic mice showed there was a marked reduction in the CD4+ thymocyte population compared with that in wild-type mice, possibly due to inefficient positive selection (25). Inefficient positive selection in TCR transgenic mice could result from a low abundance of the positive-selecting self-peptide(s) for the individual Ag specificity (40, 41). In this study we have shown a dramatic increase in positive selection of 1E4 self-reactive pathogenic T cells upon expression of the cognate epitope in MHC class II-positive cells within the thymus. Furthermore, the selected T cells were able to seed the periphery and initiate autoimmunity.

Our data show that the Ii-H/Kβ253-277 fusion protein was expressed in M1 transgenic mice, and the H/Kβ epitope could be processed and presented by MHC class II-positive cells. In particular, bone marrow-derived dendritic cells showed high levels of intracellular Ii-H/Kβ253-277 product and were able to stimulate naive 1E4 H/Kβ-specific CD4+ T cells in vitro. However, the level of H/Kβ253-277 presentation in the M1 transgenic line is clearly low, as, firstly, Ii-H/Kβ253-277-positive dendritic cells were only able to weakly stimulate 1E4 cells; secondly, splenic APCs could not activate CFSE-labeled 1E4 T cells in vivo; and thirdly, the majority of 1E4 T cells in the double-transgenic mice were naive. The low level presentation of the H/Kβ epitope in the APCs of transgenic mice may be due to either poor processing of the Ii fusion protein and/or weak affinity of the processed H/Kβ epitope for the IE3 molecule.

Based on our findings, we propose that the MHC class II presentation of the H/Kβ253-277 epitope from the transgene product is sufficient to allow thymic positive selection of 1E4 T cells, but is insufficient to trigger negative selection of 1E4 T cells or activate 1E4 T cells in the periphery. This scenario is consistent with current models of thymic selection (15), but highlights the influence of thymic expression on the selection of low avidity, disease-causing T cells. In other studies using model Ags, such as pigeon cytochrome c, agonist peptides have also been shown to mediate positive selection under certain experimental conditions (42). Our studies extend these earlier findings to include self-reactive T cells that are responsible for the induction of organ-specific autoimmune diseases.

Thymic expression of peripheral Ags can result in the development of CD25+CD4+ regulatory T cells (16, 43). However, thymic expression of the Ii-H/Kβ253-277 fusion protein did not result in a significant increase in the number of regulatory T cells bearing I1E4 TCR. Firstly, the number of CD25+CD4+Vβ8.3+ T cells in the periphery of double-transgenic mice is only slightly increased compared with that in I1E4-TCR single-transgenic mice, and secondly, the Ag-driven proliferative responses of the peripheral CD4+Vβ8.3+ T cells are identical in single- and double-transgenic mice. The generation of 1E4 regulatory T cells would be expected to result in reduced in vitro proliferative responses, using splenocytes as a source of APC (44, 45). The observation that enhanced positive selection in double-transgenic mice did not include an increased proportion of 1E4 regulatory T cells is consistent with the proposal that the thymic generation of CD25+CD4+ regulatory T cells is a consequence of high avidity, rather than low avidity, interactions between TCR and self-Ag (16).

A marked difference was observed in the incidence of autoimmune gastritis between 1E4 TCR single-transgenic and double-transgenic mice. The low level of spontaneous autoimmunity in single-transgenic mice illustrates that low avidity 1E4 T cells are pathogenic under certain circumstances; however, as the majority of mice do not spontaneously develop autoimmune gastritis, the presence of large numbers of peripheral 1E4 cells can be tolerated. One possible explanation is that under normal circumstances the level of presentation of H/Kβ in local gastric lymph nodes is insufficient to activate naive 1E4 T cells, and the development of gastritis in a few TCR single-transgenic mice may be associated with environmental factors that result in inflammation within the gastric environment leading to enhanced presentation of H/Kβ autoantigen and activation of naive 1E4 cells. In contrast, the increased incidence of spontaneous autoimmune gastritis in double-transgenic mice, compared with 1E4 single-transgenic mice, may be due to a higher level of presentation of the gastritogenic epitope by APCs within the stomach as a result of the combined contributions from Ii-H/Kβ253-277 and endogenous H/K ATPase β subunit. Analysis of a small number of mice from a pathogen-free environment has indicated that housing conditions have no impact on disease development in double-transgenic mice (our unpublished observations).

Analysis of CD4+Vβ8.3+ T cells in paragastric lymph nodes of double-transgenic mice did not show a dramatic activation of 1E4 T cells within the stomach environment. Perhaps this result is not surprising, as gastritis develops over many weeks, and it is likely that analysis of the bulk population is not sufficiently sensitive to detect low level activation of H/Kβ-specific T cells within paragastric lymph nodes. It may be that the level of presentation within the gastric environment of double-transgenic mice is just at the threshold for activation, and only small numbers of T cells are activated at any one time. Over a period of days to weeks these activated T cells, which would migrate into the gastric mucosa, would increase in number and eventually cause tissue damage. Of relevance to this suggestion is that other studies involving autoantigen-specific transgenic TCR mice have also noted difficulty in detecting activated T cells in the local draining lymph nodes of the target organ, even though autoimmune disease develops (46).

In conclusion, this study has made the surprising finding that thymic expression of tissue-specific Ags can enhance positive selection of low avidity, self-reactive T cells that can cause organ-specific autoimmunity. Our findings suggest that the precise level
of tissue-specific Ags in the thymus may be an important consid-
eration in protection against autoimmune disease.

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References
by a single MHC/peptide ligand: autoreactive T cells are low-affinity cells. Im-
munity 10:83.
the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones
4. de Visser, K. E., T. A. Cordaro, D. Kiossias, J. B. Haenen, T. N. Schumacher, and
A. M. Kruisbeek. 2000. Tracing and characterization of the low-affinity self-
7. Lacaille, S., S. Tanama, W. Hammarström, and P. A. Gleeson. 2002. The expres-
sion of a self-peptide in the thymus may be an important consid-
eration of a gastritogenic epitope of the H/K ATPase subunit. Immunology 96:145.
23:2688.
9. Thymic selection of CD4+

+CD45- suppressor T cells: more questions than answers. Nat. Rev.
Immunol. 5:2389.
S. Naylor, S. Vukanovnic, K. A. Houghing, and S. C. Jameson. 2002. Rare,
structurally homologous self-peptides promote thymocyte positive selection. Im-
munity 17:131.
self-tolerance maintained by CD4

+CD25

+ naturally anergic and suppressive T cells: induction of autoimmune
expression in medullary thymic epithelial cells mirrors the peripheral self. Nat.
Immunol. 2:1032.
an immunological self shadow within the thymus by the aire protein. Am. J.
Physiol. 279:G209.
15. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley,
an immunological self shadow within the thymus by the aire protein. Am. J.
Physiol. 279:G209.
an immunological self shadow within the thymus by the aire protein. Am. J.
Physiol. 279:G209.
17. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley,
an immunological self shadow within the thymus by the aire protein. Am. J.
Physiol. 279:G209.
18. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley,
an immunological self shadow within the thymus by the aire protein. Am. J.
Physiol. 279:G209.
19. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley,
an immunological self shadow within the thymus by the aire protein. Am. J.
Physiol. 279:G209.