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Restricted Autoantigen Recognition Associated with Deletional and Adaptive Regulatory Mechanisms

John A. Gebe,* Betty B. Yue,* Kelly A. Unrath, † Ben A. Falk,* and Gerald T. Nepom*‡

Autoimmune diabetes (T1D) is characterized by CD4+ T cell reactivity to a variety of islet-associated Ags. At-risk individuals, genetically predisposed to T1D, often have similar T cell reactivity, but nevertheless fail to progress to clinically overt disease. To study the immune tolerance and regulatory environment permissive for such autoreactive T cells, we expressed TCR transgenes derived from two autoreactive human T cells, 4.13 and 164, in HLA-DR4 transgenic mice on a C57BL/6-derived “diabetes-resistant” background. Both TCR are responsive to an immunodominant epitope of glutamic acid decarboxylase 65555–567 peptide which is identical in sequence between humans and mice, is restricted by HLA-DR4, and is a naturally processed self Ag associated with T1D. Although both TCR use the identical Vα and Vβ genes, differing only in CDR3, we found stark differences in the mechanisms utilized in vivo in the maintenance of immune tolerance. A combination of thymic deletion (negative selection), TCR down-regulation, and peripheral activation-induced cell death dominated the phenotype of 164 T cells, which nevertheless still maintain their Ag responsiveness in the periphery. In contrast, 4.13 T cells are much less influenced by central and deletional tolerance mechanisms, and instead display a peripheral immune deviation including differentiation into IL-10-secreting Tr1 cells. These findings indicate a distinct set of regulatory alternatives for autoreactive T cells, even within a single highly restricted HLA-peptide-TCR recognition profile. The Journal of Immunology, 2009, 183: 59–65.

Central and peripheral mechanisms maintaining T cell tolerance to self Ags are variable in degree of completeness, and autoreactive T cells populate the peripheral immune system. Central tolerance in the thymus is largely governed through the interaction of the TCR with self-peptide-MHC complexes, in which high-avidity T cells are eliminated through apoptosis (1–3) or potentially differentiated into CD4+CD25+Foxp3-expressing regulatory T cells (Treg)4 (4, 5). Strategies by which autoreactive T cells may escape central tolerance to self Ags include down-modulation of receptor or costimulatory molecules (6) and skewing of CD4/CD8 coreceptor expression (7, 8). These mechanisms are incomplete, however, such that self reactivity by some peripheral T cells is an intrinsic property of normal immunity, perhaps required to enable the immune repertoire to respond to the diverse nature of foreign Ags (9).

Once in the periphery, several additional mechanisms operate as checkpoints to limit T cell activation to self Ags, including functional inactivation or anergy of the T cell (10, 11), activation-induced T cell deletion (12–14), generation of suppressive cytokine-secreting T cells (Tr1 and Th3) (15, 16), and differentiation of uncommitted T cells into Foxp3-expressing regulatory T cells (17, 18).

While several TCR transgenic mice have been developed to study tolerance to self Ags, the vast majority of studies use either alloreactive T cells or a foreign Ag-reactive T cell expressed as a TCR transgene along with the foreign Ag as a second transgene (4, 19, 20). In human type 1 diabetes (T1D), HLA-DR4 subjects commonly carry peripheral T cells reactive to a variety of islet-associated self Ags, including the immunodominant glutamic acid decarboxylase (GAD)65555–567 peptide, a naturally processed epitope of glutamic acid decarboxylase (21–24). Interestingly, recognition of this epitope displays a biased TCR repertoire, with prevalent use of Vβ5.1/Vα12.1, although CDR3 regions are variable (22). To study tolerance mechanisms associated with this dominant autoreactive specificity, we introduced transgenic TCR from two human CD4+ T cells specific for GAD65555–567, which differ only in their CDR3 regions, intercrossed into HLA-DR4 transgenic mice. Despite the close structural features of these two autoreactive TCR, stark differences in both central and peripheral tolerance mechanisms were elicited.

Materials and Methods

Mice

DR4041-IE mice (DR4) were obtained from Taconic. These C57BL/6 1-Ah600 mice express a human-mouse chimeric class II molecule in which the TCR-interacting and peptide-binding domains of mouse I-E (domains αi and βi, exon 2 in both genes) have been replaced with the αi and βi domains from DRA1*0101 and DRB1*0401, respectively. Retention of the murine αi and βi domains allows for the cognate murine CD4-murine MHC interaction (25).

TCR sequences for generation of the two T cell transgenic mice were obtained from human CD4+ T cell clones 164 (26) and 4.13 (22). Both human T cells are responsive to the same self Ag GAD65555–567 and both use human Vα12.1/Vβ5.1 T cell receptors. The 164 T cell was cloned from peripheral blood from an HLA DRA1*0101/B1*0401 diabetic individual (22). Human-mouse chimeric TCR transgenes were constructed by subcloning PCR amplified regions encoding rearranged Vaαs and VBβs from the human clones into pTcass and pTβcass TCR transgenic vectors, respectively (27). TCR transgenic vectors pTcass and pTβcass contain the natural mouse TCR α
and β promoter/enhancer elements and mouse Cα and Cβ constant regions, respectively, DNA injection into C57BL/6-Abβtrans (164 TCR) or F1-B6/C3H (4.13 TCR) mouse embryos was performed at the University of Washington (Seattle, WA) in the Comparative Medicine Animal Facility. Founder mice containing the human TCR transgenes were then crossed onto DR0401-IE mice to generate DR4/164 and DR4/4.13 mice. Additional crosses were made onto Rag2−/− mice. A founder mouse was also identified that contained only the 164 TCR β transgene. This was also crossed onto DR0401-IE mice. The 4.13 TCR transgenic mice generated in F1-B6/C3H were crossed for nine generations into DR0401-IE mice. All animal work was approved by the Benaroya Research Institute Animal Care and Use Committee and animals were housed in the Benaroya Research Institute American Association of Laboratory Animal Care-accredited animal facility.

**Tissue processing and flow cytometry**

Thymus, spleen, and lymph node tissues were processed into single-cell suspensions by gently passing through 0.40-µm cell strainers (BD Falcon; ref. no. 352340) using the rubber end of a 1-ml tuberculin syringe in DMEM-10 media (Invitrogen; catalog no. 11965-092) supplemented with 10% FBS (HyClone), 100 µg/ml penicillin, 100 µ/ml streptomycin, 50 µM 2-ME, 2 mM glutamine, and 1 mM sodium pyruvate (Invitrogen). Cell suspensions were centrifuged at 200 x g for 10 min, aspirated, and either 1) resuspended in DMEM-10 media (lymph node and thymus) or 2) RBC were lysed (for spleens) using 1 ml of ACK lysis buffer (28) for 5 min at 37°C at which time 30 ml of media was added and cells spun down (200 x g), aspirated, and resuspended in DMEM-10 media. The following chro- mophore-labeled Abs were used in flow cytometric analysis: anti-mouse CD4 (clone RM4-5), CD8 (clone 53-6.7), CD25 (clone PC61), CD62L (Mel-14), anti-human active caspase-3 (polyclonal, catalog no. 557091), CD44 (IM7), anti-human Vβ1-PE (clone IMMU 157; Immunotech/Coulter), and Vα12.1-FITC (clone 6d6; Endogen). FACS samples in media were prestained with Fc block for 10 min on ice and then stained with specific Abs on ice for 45 min, washed once, and resuspended in FACS stain buffer (PBS containing 1% FBS, 0.1% Na azide) before being run on a FACSCalibur or LSR II flow cytometer (BD Biosciences). Intracellular staining kit (catalog no. 88-8823-88; eBioscience). IL-10 (clone JES5-16E3; eBioscience) was performed using eBioscience anti-mouse IL-10 (clone 2A3; eBioscience) with the biotinylated Ab and PE-labeled streptavidin (eBioscience) in flow cytometric analysis. Tissue processing and flow cytometry was conducted according to the manufacturer’s instructions. Intracellular staining for active caspase-3, mouse anti-IFN-γ (XM1G12; eBioscience), and anti-IL-10 (clone JES5-16E3; eBioscience) was performed using biotinylated streptavidin kit (catalog no. 88-8823-88; eBioscience).

**Proliferation assays**

In lymph node or purified CD4+ T cell proliferation assays 1 x 10^6 lymph node cells were cultured with 2 x 10^5 (3000 rad) Cs-gamma-irradiated splenocytes (final volume 150 µl). Supernatants for cytokine analysis were taken (50 µl) at 48 h, and 1 µCi/well [3H]thymidine was added at 72 h. Thymidine incorporation was assayed at 96 h using liquid scintillation counting and performed using a Microbeta TriLux 1450 scintillation counter (Wallac-PerkinElmer Life Sciences). Spleenocyte responses were measured in the same manner using 5 x 10^5 spleenocytes per well. CD4 and CD8 single-positive cells were obtained using Mitenyn Biotech beads with purity of 90% or greater or by Ab labeling with CD4 and CD8 and sorting by flow cytometry.

**Cytokine analysis**

Cytokines IL-2, IL-4, IL-5, TNF-α, and IFN-γ were assayed using a mouse Th1/Th2 cytokine CBA kit (BD Biosciences; catalog no. 551287). IL-10 was assayed using a BD OptEIA mouse IL-10 ELISA set and mouse TGF-β1 was measured using a human/mouse TGF-β1 ELISA Ready-SET-Go! kit (BD Biosciences; catalog nos. 555252 and 88-7344, respectively). Supernatants from triplicate proliferation wells (50 µl/well) were combined for cytokine analysis, with 50 µl used for CBA analysis and 50 µl for IL-10 ELISA.

**Results**

**Thymic selection of autoreactive T cells**

Utilizing TCR from two structurally related DRB1*0401 (DR4) restricted human CD4+ T cell clones reactive to the autoantigen GAD65, human TCR transgenic mice were generated to investigate differential modes of T cell tolerance to the naturally processed GAD65555–567 autoantigen. The human CD4+ T cell clones 164 and 4.13 (obtained from two different subjects) are structurally related in that they both use TCR with human Vα12.1 (hVα12.1) and Vβ5.1 (hVβ5.1) gene sequences, which differ only in their CDR3 regions (Table I). Both of the human T cells recognize GAD65555–567 (22, 26), a region within the naturally processed and presented GAD65555–567 epitope (21, 23). The sequence of the DR4-binding minimal stimulating epitope GAD65555–567 is identical for GAD65 and GAD67 in both human and mouse and thus serves as a naturally processed self Ag T cell epitope in both species (29). Both 164/Rag2−/− and 164/Rag2−/− mice display reduced thymus cellularity (Fig. 1A), with 164 mice exhibiting a profound reduction in CD4 CD8+ double-positive cells (Fig. 1B). The reduction in cellularity and a decrease in double-positive cells is indicative of negative selection (20, 30, 31). While positively

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**Table I. Comparison of 164 and 4.13 TCR**

<table>
<thead>
<tr>
<th>Clone</th>
<th>CDR3 Region sequence</th>
</tr>
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<tbody>
<tr>
<td>164</td>
<td>A L S E E G G G A N S K L T</td>
</tr>
<tr>
<td>4.13</td>
<td>A L S E N R G G G T A S K L T</td>
</tr>
</tbody>
</table>

a Gray highlighted areas denote differences between 164 and 4.13 TCR CDR3 sequences: boldface, nonpolar-to-nonpolar amino acid changes; underlined residues, polar-to-polar changes; and parentheses, charge changes.

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**FIGURE 1.** Thymic lymphocyte profiles in 164 and 4.13 human TCR transgenic HLA-DR4 mice. Thymus cellularity of 8- to 10-wk-old mice (n = 3) (A), CD4/CD8 profiles (B), and TCR hVb5.1 vs hVα12.1 expression on CD4+CD8− gated cells (C). Human Vb5.1 expression on CD4+CD8+ thymocytes from 164 Vb only TCR mice (D).
Peripheral lymphocyte profiles in 8- to 10-wk-old 164 and 4.13 TCR transgenic DR4 mice. Peripheral spleen and lymph nodes (inguinal and para-aortic combined) cellularity (n = 3) (A). CD4 vs CD8 profile and TCR human Vα12.1 and Vβ5.1 expression on CD4^+CD8^- gated cells from spleen (B). Histogram in B shows CD3e expression on CD4^+CD8^- gated cells from 164 mice (black line), 4.13 mice (gray line), and isotype control (dotted line). Splenocyte Ag dose response in 164 (gray) and 4.13 (black) TCR transgenic DR4 mice on a Rag2^-/- background (C). For CD4^+CD8^- and CD4^+CD8^+ T cell stimulation (D) cells were sorted by flow and tested for proliferation in response to GAD65_555-567 or control HA Ag. All experiments were repeated at least three times with similar results.

Peripheral skewing of autoreactive T cells

4.13/Rag2^+/- and 164/Rag2^+/- mice both show reduced cellularity in the spleen (Fig. 2A), but only 164 mice show a reduction in peripheral lymph nodes. The 4.13 T cells in the spleen as in the thymus are heavily skewed toward CD4^+CD8^- lineage, reflecting their class II restriction (Fig. 2B). The 164/Rag2^+/- mice have fewer cells in the spleen, and <1% of CD4^+CD8^- T cells are hVα12.1^- and hVβ5.1-positive (Fig. 2B). Coinciding with the weak TCR expression in 164/Rag2^+/- mice is also a low expression of CD3e on CD4^+CD8^- gated cells (Fig. 2B, histogram). In contrast to the near absence of CD4^+CD8^- cells in 4.13/Rag2^+/- mice, 164/Rag2^+/- mice have nearly one-third of their T cells as CD8^-CD4^+ cells, which is also greater than that seen in non-TCR transgenic mice (Fig. 2B). The percentages of CD4 cells among all T cells (CD4/(CD4 + CD8)) (average of three mice) are 98 ± 1% in 164 mice and 73 ± 2% in 164 mice compared with 90 ± 1% in non-TCR transgenic mice, indicating that 4.13 T cells are strongly selected toward their MHC class II restriction, while T cell selection in 164/Rag2^+/- mice is skewed toward the CD8 compartment, similar to what is observed in the thymus. The stronger central tolerance in 164/Rag2^+/- mice is also reflected in the periphery by the greater expression of endogenous mouse mVα and mVβ T cell receptors (supplemental Fig. S1).4 In assays for Ag specificity we used splenocytes from Rag2^-/- TCR transgenic mice to ensure that all α/β T cells only express the hVα12.1 and hVβ5.1 transgenes. Splenocytes from both 4.13/Rag2^-/- and 164/Rag2^-/- mice respond to GAD65_555-567 in an Ag-specific manner, confirming their specificity for the GAD65 epitope (Fig. 2C). Because of the skewing of 164 T cells from 164/Rag2^-/- mice (also seen in 164/Rag2^-/- mice) into a CD8^-CD4^- pathway, we sorted 164/Rag2^-/- T cells into CD4^-CD8^- and CD4^-CD8^+ fractions and stimulated these fractions with irradiated splenocytes and peptide. We find that both populations are Ag specific, with the CD8 164 cells having a lower proliferative response (lower functional avidity) (Fig. 2D).

4 The online version of this article contains supplemental material.
Peripheral tolerance mediated by apoptosis

As with the low expression of the transgenic TCR on 164/Rag2+/+ thymocytes in the thymus (Fig. 2B), the TCR expression on 164/Rag2+/+ T cells in the periphery is also nearly absent (also true in 164/Rag2+/+ mice). This suggested that perhaps the ligand inducing negative selection in the thymus is also activating these cells in the periphery, and thus the extremely low level of TCR expression in the periphery is in part the result of constant activation of 164 cells in the periphery. By surface phenotyping we found that most peripheral 4.13/Rag2o/o CD4+ cells, like CD4+ cells from non-TCR/Rag2+/+ transgenic mice, are of a naive nature expressing high levels of CD62L and intermediate levels of CD44 (CD62LhighCD44int) (Fig. 3A). In contrast, ~40% of peripheral spleen CD4+ cells from 164/Rag2o/o mice are CD62LlowCD44high compared with ~10% in 4.13 and non-TCR transgenic mice, indicating an activated phenotype (Fig. 3A and B). A similar activation profile of 164/Rag2o/o CD4+ T cells was observed in other lymph nodes (pancreatic and inguinal, data not shown) and also in Rag2+/+ mice (supplemental Fig. S3). Therefore, we tested whether the low numbers of T cells in the peripheral tissues of 164/Rag2o/o mice could be the result of constant peripheral activation and subsequent activation-induced cell death. As shown in Fig. 3C, peripheral CD4+ 164/Rag2o/o T cells compared with 4.13/Rag2o/o and non-TCR transgenic cells stain with the apoptotic marker annexin V and additional staining indicated that the CD4+ 164/Rag2o/o T cells are also activated caspase-3+ (Fig. 3D). Peripheral CD4+ 4.13/Rag2o/o cells were negative for both annexin V and activated caspase-3 staining. Surface staining on CD4+ 164/Rag2o/o T cells indicated that a significant portion of these cells are also CD95+, suggesting that apoptotic signaling may occur through CD95 (Fig. 3E).

FIGURE 4. Foxp3 expression on spleen CD4+CD8+ T cells from non-TCR transgenic, 164, and 4.13 GAD TCR transgenic mice. Eight- to 12-wk-old DR4 mice on a Rag2o/o background were surface stained with CD4, CD25, and then intracellularly for Foxp3. Example of staining is shown in (A) on non-TCR transgenic DR4 splenocytes. Foxp3 expression in Rag2+/+ mice as a percentage of CD4+ cells is shown in (B) (average of three mice). Foxp3 expression on CD4+ T cells from 164 and 4.13 TCR transgenic mice on a Rag2o/o background is shown in C.

FIGURE 5. Cytokine profile of 164 and 4.13 T cells to GAD65 552–572. Splenocytes from DR4/164/Rag2o/o and DR4/4.13/Rag2o/o mice were stimulated with 100 μg/ml GAD65 or control peptide for 96 h. Supernatants were collected at 48 h and TNF-α, IFN-γ, IL-2, IL-4, and IL-5 were measured using a mouse Th1/Th2 kit, and IL-10 was measured by ELISA. Experiment was done three times with similar results.
stimulated for 4 days with GAD65 552–572 and then cultured with PMA/ionsomycin for 4 h with brefeldin A during the last 2 h. Experiment was done three times with similar results.

Both 164 and 4.13 mice show an enhanced selection of peripheral Foxp3+ cells

CD4+CD25+ cells that express Foxp3 participate in immune regulation, and the selection of these Treg can be mediated in foreign Ag-specific TCR transgenic mice by expression of the stimulatory Ag as a neo-self peptide driven by tissue-specific promoters (4, 32). It has also been shown that increasing avidity of the TCR for the peptide-MHC correlates with a propensity to develop along the thymic-derived Foxp3 Treg pathway (4). In our setting involving endogenous self Ag recognition, we find that peripheral CD4+ T cells from both autoreactive 4.13/Rag2+/− and 164/Rag2+/− TCR transgenic mice express increased numbers of Foxp3 cells, and that the percentage of CD4+ cells that express Foxp3 is highest in 164 mice compared with 4.13 mice, and both are greater than that seen in non-TCR transgenic mice (Fig. 4B). However, upon crossing TCR transgenic mice onto a Rag2-deficient background, peripheral Foxp3+ cells were near undetectable levels in either 164 or 4.13 mice (Fig. 4C), consistent with the induction of Treg populations in the nontransgenic fraction of endogenous T cells.

Peripheral 4.13 CD4+ T cells exhibit Th1 and Tr1 profiles

Cytokine analysis on in vitro-stimulated cells from both Rag2−/− mice transgenic for HLA-DR4, the human class II-restricting element for GAD65, an important islet Ag associated with autoimmune diabetes. On a C57BL/6 “diabetes-resistant” background transgenic for HLA-DR4, the human class II-restricting element for these TCR, very potent in vivo tolerance mechanisms were observed. The 164 TCR was associated with strong deletional events, both in the thymus and in the periphery, and surviving 164 T cells down-modulated TCR expression and/or switched from CD4 to CD8 phenotype, even as they maintained specific Ag reactivity. In marked contrast, the 4.13 TCR had less sensitivity to negative selection and no CD4-to-CD8 skewing, but instead used a predominant pathway of immunomodulation, skewing toward an IL-10 phenotype.

Discussion

Limiting pathogenic autoreactivity is of the utmost importance for a successful immune system, and several mechanisms provide functional checkpoints for this control. These mechanisms broadly fit into three categories: those that involve deletion of autoreactive cells, centrally and/or peripherally; those that involve down-modulation of activation molecules or receptors, changing activation thresholds; and those that involve active immune regulation. In this study we evaluated central and peripheral tolerance mechanisms using two TCR transgenic mice containing structurally similar receptors specific for a naturally processed self Ag. These TCR were derived from autoreactive CD4+ T cells present in humans with immunity to GAD65, an important islet Ag associated with autoimmune diabetes. On a C57BL/6 “diabetes-resistant” background transgenic for HLA-DR4, the human class II-restricting element for these TCR, very potent in vivo tolerance mechanisms were observed. The 164 TCR was associated with strong deletional events, both in the thymus and in the periphery, and surviving 164 T cells down-modulated TCR expression and/or switched from CD4 to CD8 phenotype, even as they maintained specific Ag reactivity. In marked contrast, the 4.13 TCR had less sensitivity to negative selection and no CD4-to-CD8 skewing, but instead used a predominant pathway of immunomodulation, skewing toward an IL-10 phenotype.

Both 164 and 4.13 T cells use Vα12.1/Vβ5.1 TCR and differ only in CDR3, a region that conventionally interacts primarily with the peptide in the Ag-binding MHC (33). Based on the higher thymic cellularity in 4.13 mice compared with 164 mice and the

FIGURE 6. Internal cytokine staining for IL-10 and IFN-γ from 164 (A) and 4.13 (B) mice were intracellularly stained with IL-10 and IFN-γ directly conjugated Abs. Spleen cells from Rag2−/− 164 and 4.13 mice were stimulated for 4 days with GAD65 552–572 and then cultured with PMA/ionsomycin for 4 h with brefeldin A during the last 2 h. Experiment was done three times with similar results.

FIGURE 7. IFN-γ and IL-10 production from stimulated sorted CD4+CD8− cells from DR4/4.13/Rag2−/− mice. CD4+CD8− cells from spleen, lymph node, and thymus were FACs sorted from tissues taken from 8- to 12-wk-old mice and stimulated with irradiated APC and either CD3/CD28 Ab (A) or GAD65 552–572 (B).
absence of differentiation toward the CD4+CD8+ pathway, it appears that the 164 TCR is of a higher avidity to peptide-MHC complexes in the thymus. As T cell CD4 avidity interaction with the $\beta_2$ domain of the MHC class II has been shown to contribute positively to thymic T cell selection (21, 34), the differentiation of immature CD4+CD8+ double-positive 164 thymocytes into CD4+CD8+ mature cells would presumably lower the TCR overall avidity to the MHC complex and enable escape from negative selection. This skewing toward a CD4+CD8+ expression pathway and away from a CD4-CD8+ pathway occurred despite the class II restriction of the original human 164 T cell clone. Consistent with this interpretation is our observation that peripheral CD4+CD8+ 164/Rag2−/− T cells have less functional avidity to GAD65555–572 stimulation than do CD4+CD8− 164/Rag2−/− T cells. The skewing of class II-restricted self Ag-reactive T cells toward a CD8 lineage has been observed in other TCR transgenic models, also in the context of strong negative selection (7, 8).

In addition to thymic deletion and CD4-to-CD8 skewing, T cells surviving in the 164 TCR mice showed significant down-regulation of the TCR molecule itself. This also is consistent with a strategy invoked for lowering avidity, and correlated in the mice with evidence of a very strong activation-induced cell death pathway. The end result of all these simultaneous high-avidity tolerance checkpoints was the presence in the peripheral circulation of a low number of autoreactive T cells, which nevertheless displayed strong Ag-specific proliferative and Th1 characteristics. Considering that both 164 and 4.13 TCR use Vα12.1 and Vβ5.1 and are responsive to the same Ag, it was remarkable that 4.13 T cells showed a completely different tolerance induction profile. A more modest central tolerance for 4.13 T cells was reflected in less thymic deletion and normal CD4+CD8+ maturation, and similarly no evidence for peripheral activation-induced cell death or receptor down-modulation was observed. A likely explanation for the absence of peripheral activation of 4.13 T cells was the peripheral generation of IL-10-producing Tr1 regulatory cells in these mice. IL-10 is a potent regulatory cytokine and has been shown to be important in regulating colitis and autoimmunity in experimental autoimmune encephalomyelitis and collagen-induced arthritis models (35–38). The absence of IL-10 from sorted CD4+CD8− T cells from the thymus upon stimulation with either CD3/CD28 or Ag-specific GAD65555–572 peptide also indicates that generation of these IL-10-secreting T cells was a peripheral differentiation event. It is interesting to speculate that T cell-generated IL-10 in 4.13 mice could be preventing the activation of 4.13 T cells in the periphery, which contrasts with the activated phenotype in peripheral 164 mice. This hypothesis is currently being tested by crossing DR4/4.13/Rag2−/− mice onto IL-10-deficient mice. While both 164 and 4.13 peripheral T cells are specific for GAD65555–567, because many TCR are degenerate in peptide recognition (39), we cannot exclude the possibility that cross-reactivity with other unknown ligands might contribute to the differences in functional profiles.

In the periphery, both 4.13 and 164 mice show an increase in Foxp3+ cells, which is consistent with that seen in quasi-self Ag models (4, 40). The larger increase in the percentage of Foxp3+ cells in 164 mice relative to 4.13 mice correlates with the increase in negative selection (higher avidity TCR) in the thymus. However, upon crossing to Rag2-deficient mice we did not detect peripheral CD4+CD25+ (Foxp3+) cells from either 164 or 4.13 mice. This is in contrast to HA-specific and OVA-specific TCR transgenic mice on a Rag-deficient background where the Ag is expressed as a neo-self Ag (40–42). In these models up to half of peripheral T cells are CD25+ and have a regulatory function. However, in TCR transgenic mice where the T cell-responsive Ag is endogenously expressed, CD4+CD25+ (Foxp3+) Treg do not develop on a Rag-deficient background. This includes a myelin basic protein-specific TCR (43) and the BDC2.5 TCR (44). It has been suggested that a high-avidity interaction between T cells and APC in the thymus is required for Treg development (45). Considering the strong negative selection in the thymus of both TCR mice suggesting a high functional avidity of the TCR for MHC-Ag, we were surprised to not find CD4+CD25+Foxp3+ cells in the periphery on Rag2−/− mice. A possible explanation for a lack of Foxp3+ Treg in these mice may be that both of these TCR are of high enough avidity that they are beyond the threshold for Foxp3 differentiation (5).

Peripheral tolerance methods of anergy (10, 11), deletion (12–14), or the generation of Tr1(15) and Th3(16) cells are a second line of defense against T cell autoimmunity. Once in the periphery 164 cells displayed a strong activation phenotype in both spleen and lymph nodes resulting in continued down-modulation of their TCR and concomitant activation-induced cell death through an activated caspase-3 pathway. Consistent with this is the expression of CD95 (FAS) on 164 T cells through which signaling has been shown to mediate deletion-induced peripheral tolerance (46, 47). The 4.13 T cells, which populate the periphery to a greater extent, do not undergo this type of peripheral tolerance, most likely due to their apparent lower overall pMHC avidity.

Autoreactive cells, such as those used to derive the 164 and 4.13 TCR in this study, occur frequently in humans with autoimmune disease, in people who are genetically at risk of autoimmune disease, and in normal HLA-matched individuals (48–52). Nevertheless, overt autoimmune disease is relatively rare, reflecting the importance of tolerance checkpoints in normal immune function. Our study, using human autoimmune TCR and human MHC transgenic mice, directly demonstrates multiple mechanisms that, sometimes simultaneously, elicit both central and peripheral tolerance. Indeed, the two structurally similar TCR used, derived from human HLA-DR4 subjects, with specificity for the same Ag and restriction element and differing only in their CDR3 regions, revealed stark differences in deletional, compensatory, and immunomodulatory mechanisms. That such distinction occurs even with closely related autoreactive TCR underscores the importance of understanding the contribution of this variation to disease susceptibility, pathogenic pathways, and response to therapy.

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Disclosures
The authors have no financial conflicts of interest.

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