Autoantigen Recognition Is Required for Recruitment of IGRP 206–214-Autoreactive CD8+ T Cells but Is Dispensable for Tolerance

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Autoantigen Recognition Is Required for Recruitment of IGRP<sub>206–214</sub>-Autoreactive CD8<sup>+</sup> T Cells but Is Dispensable for Tolerance

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The progression of autoimmune responses is associated with an avidity maturation process driven by preferential expansion of high avidity clonotypes at the expense of their low avidity counterparts. Central and peripheral tolerance hinder the contribution of high-avidity clonotypes targeting residues 206–214 of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP<sub>206–214</sub>) during the earliest stages of autoimmune diabetes. In this study, we probe the molecular determinants and biochemical consequences of IGRP<sub>206–214</sub>/K<sup>d</sup> recognition by high-, intermediate-, and low-avidity autoreactive CD8<sup>+</sup> T cells, and we investigate the effects of genetic IGRP<sub>206–214</sub> silencing on their developmental biology. We find that differences in avidity for IGRP<sub>206–214</sub>/K<sup>d</sup> map to CDR1α and are associated with quantitative differences in CD3ε proline-rich sequence exposure and Nck recruitment. Unexpectedly, we find that tolerance of high-avidity CD8<sup>+</sup> T cells, unlike their activation and recruitment into the pancreas, is dissociated from recognition of IGRP<sub>206–214</sub>, particularly in adult mice. This finding challenges the view that tolerance of pathogenic autoreactive T cells is invariably triggered by recognition of the peptide–MHC complex that drives their activation in the periphery, indicating the existence of mechanisms of tolerance that are capable of sensing the avidity, hence pathogenicity of autoreactive T cells without the need to rely on local autoantigen availability. The Journal of Immunology, 2012, 189: 2975–2984.

The preimmune T and B cell repertoire results from random rearrangements of highly diverse arrays of V, D, and J genes during lymphocyte ontogeny. Conformational flexibility and ligand promiscuity are features of the Ag-binding site of the Ag receptors encoded by these rearrangements, particularly those binding cognate Ag with low affinity, which are typically found in the preimmune B cell repertoire. Somatic mutation of BCRs and selection of Ag-reactivated B cells within germlinal centers afford significant increases in both the overall association rate of the Ag-binding reaction and in the structural rigidity of the receptor’s Ag-binding site (1, 2). In turn, this BCR affinity-maturation process enables dramatic improvements in the affinity and fidelity of Ag recognition by cognate B cells (3–7).

T cell responses undergo a similar avidity maturation process. In this case, however, changes in the Ag-specific T cell population’s ligand-binding avidity result from competitive survival of clones bearing high-affinity TCRs for peptide–MHC (pMHC) at the expense of clones bearing low-affinity TCRs for the same pMHC (8–11). Because T cells cannot somatically mutate their TCRs, the mechanisms underlying the recruitment of predominantly low-avidity T cells in primary immune responses, and their progressive replacement by high-avidity clonotypes upon Ag reencounters, remain poorly understood. We have previously provided developmental bases for a CD8<sup>+</sup> T cell avidity maturation response associated with the progression of spontaneous autoimmune diabetes in NOD mice (10, 12–14). We found that avidity maturation of the CD8<sup>+</sup> T cell population targeting residues 206–214 of islet-specific glucose 6 phosphate catalytic subunit-related protein (IGRP<sub>206–214</sub>) (15–17) is conditioned by selective tolerance (central and peripheral) of high-avidity clonotypes and is driven by local competition among high- versus low-avidity V<sub>α</sub>17-MRD-Jc<sub>42+</sub> clonotypes using different V<sub>α</sub>17 elements (13). Experiments in TCR-transgenic (TG) animals showed that whereas autoimmune inflammation preferentially fuels the local expansion of the high avidity T cell pool (13) and fosters the development of memory-like autoregulatory CD8<sup>+</sup> T cells from the low-avidity pool (14), mechanisms of peripheral tolerance selectively reduce the size of the former (13). Collectively, these results provide an explanation as to why there is prevalent recruitment of low-avidity clones in the primary autoimmune response, and why these clonotypes are progressively (yet slowly) replaced by their high-avidity counterparts as the animals age.

This study was initiated to probe the molecular determinants and biochemical consequences of IGRP<sub>206–214</sub>/K<sup>d</sup> recognition by high-, intermediate-, and low-avidity autoreactive CD8<sup>+</sup> T cells, and to investigate the effects of genetic IGRP<sub>206–214</sub> silencing on their developmental biology. Since central and peripheral tolerance of autoreactive T cells is thought to be invariably triggered by recognition of cognate pMHC complexes, be it on Aire-expressing medullary thymic epithelial cells (18–21), peripheral Aire-expressing cell types (22), or autoantigen-loaded dendritic cells (23), we expected that IGRP<sub>206–214</sub> silencing would abrogate the functional, biochemical, and developmental consequences of recognition of IGRP<sub>206–214</sub> by high-avidity autoreactive T cells.

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We found that differences in avidity for IGRP<sub>206-214</sub><sup>d</sup> map to CDR1ε residues involved in direct interactions with MHC and in guiding MHC restriction, and that these differences are associated with quantitative differences in CD8 coreceptor recruitment, exposure of CD3ε’s proline-rich sequence (CD3ε-PRS), and Nck recruitment at the intracellular level. Although we found that CD3ε-PRS exposure and Nck recruitment to CD3ε are a feature of the naïve T cell repertoire and do not account, at least in isolation, for systemic tolerance of high-avidity IGRP<sub>206-214</sub> autoreactive T cells, the tolerogenic signals that result in selective deletion of high-avidity IGRP<sub>206-214</sub>-reactive CD8 thymocytes imprint onto their nondeleted counterparts a permanent ~3-fold increase in the number of CD3ε molecules exposing their PRS. Surprisingly, we found that central and peripheral tolerance of high-avidity IGRP<sub>206-214</sub>-reactive CD8<sup>+</sup> T cells, including the associated increase in CD3ε conformational change, are completely dissociated from the recognition of IGRP<sub>206-214</sub>. This finding is in stark contrast to the absolute need for IGRP<sub>206-214</sub> expression for activation and recruitment of these T cells into pancreatic islets. These data challenge the generally held view that tolerance of pathogenic autoreactive T cells is invariably triggered by recognition of the pMHC complex that drives their activation in the periphery. A corollary of these observations is the implication that the immune system is equipped with an autoantigen-independent mechanism of tolerance capable of sensing the avidity, hence pathogenicity of autoreactive T cells targeting autoantigens like IGRP, which are not expressed in medullary thymic epithelial cells and only at relatively low levels in the periphery.

Materials and Methods

**Mice**

17.4a/8.3β (8.3-NOD) and 17.5a/8.3β-TCR-TG mice have been described (13, 24). The TCR transgenes were introgressed into NOD.129P2(C)-Tcrα<sup>+/+/</sup>/Mphi/Po<l>ol</l> (herein referred to as NOD.Tcrα<sup>−/−</sup>), NOD.G6pc2<sup>2</sup>κ<sub>2</sub>/κ<sub>1</sub>F213A proc/K<sup>1</sup>/K<sup>1</sup> and NOD.G6pc2<sup>2</sup>κ<sub>2</sub>/κ<sub>1</sub>F213A proc/K<sup>1</sup>/K<sup>1</sup> mice. NOD.G6pc2<sup>2</sup>κ<sub>2</sub>/κ<sub>1</sub>F213A proc/K<sup>1</sup>/K<sup>1</sup> mice, carrying an immunologically silent IGR<sup>P206-214</sup> epitope in which the two TCR contact residues were substituted with alanine, were from C. Terhorst (Harvard Medical School) (26). The cultures were pulsed with 1 μCi of [3H]-thymidine and 18 h later harvested and assayed for [3H]-thymidine incorporation. Values obtained with the negative control peptide TUM were subtracted. Statistical significance was assumed at <0.05.

**Peptides, pMHC tetramers, and Abs**

The peptides TUM (KYYQVTTL), IGR<sup>P206-214</sup> (VYLKTNVFL), NRP-A7 (KYKNANFL), NRP-V7 (KYKNANFLV), and Insulin B15-23 (LYLVCCGERG) and the corresponding tetramers (PE-labeled) were prepared as described (27, 28). Because Cd3ε<sup>−/−</sup> mice also display significantly reduced expression of CD3γ and CD3δ, the retroviruses encoded wild type (WT), PRS-mutant, or ITAM-mutant CD3ε chains linked to WT CD3γ and CD3δ chains by self-cleaving 2A peptides, which give rise to near absolute clavage and expression of each CD3 chain with proper stoichiometry. Bone marrow–derived stem cells from TCR-TG, Cd3ε<sup>−/−</sup> mice were cocultured with the retroviral producer cell lines for 48 h. The nonadherent bone marrow cells (2-4×10<sup>5</sup> in PBS containing 2% FBS and 20 U/ml heparin) were injected i.v. into sublethally irradiated (1100 rad) hosts. Mice were killed 8–12 wk later for phenotypic and functional studies.

**Flow cytometry**

Thymi, spleens, and lymph node cell suspensions were analyzed with three-color flow cytometry using combination of anti-CD8-PerCP (53-6.7), anti-CD4-FITC (IM7), and pMHC tetramer-PE or anti–V<sub>b</sub>-PRS–specific APA1/1 mAb and an Alexa 488-conjugated NRP-V7/K<sub>d</sub> tetramers for 30 min at 37°C. Cells were lysed with 1 ml of cold lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 0.3% NP40, 1 mM iodoacetamide, and a protease inhibitor mixture). Postnuclear lysates were immunoprecipitated with anti-PE mAb-conjugated beads (Miltenyi Biotec). Total lysates and immunoprecipitates were mixed with SDS sample buffer, boiled for 5 min, resolved in 10–12% SDS-PAGE, transferred to PVDF membranes, probed with CD3ε- or Nck-specific Abs, and developed with the SuperSignal West Pico Chemiluminescence kit (Pierce Biotec, Rockford, IL). In other experiments, FACS-sorted CD8<sup>+</sup> T cells from TCR-TG mice (adjusted for NRP-V7/K<sub>d</sub> tetramer<sup>+</sup> T cell content), TCR-transfectants or FACS-sorted eGFP<sup>+</sup> CD8<sup>+</sup> T cells from retrogenic bone-marrow chimeras were washed with serum-free medium and incubated with pMHC tetramers at 37°C for 5 min. Cells were lysed in 0.3% NP40-containing buffer and immunoprecipitated with anti-CD3ε mAb, electrophoresed in SDS-PAGE, and blotted with anti-phosphorytrosine Ab (4G10, Millipore) or anti-CD3ε and/or anti-Nck mAbs.

**Immunofluorescence**

TCR transfectants were incubated with NRP-V7/K<sub>d</sub>-coated beads for 2–30 min at 37°C. Cells were then fixed using the Cytofix–Cytoperm kit (BD Pharmedigm) for 10 min at room temperature, and stained with rabbit anti-CD3δ or the CD3ε-PRS–specific APA1/1 mAb and an Alexa 488-conjugated secondary Ab. The cells were examined with a Deltavision fluorescence microscope (Applied Precision, Issaquah, WA). Cells staining with the APA1/1 mAb or displaying focalized CD8 staining at the T cell–pMHC–coated particle contact site were scored as positive for staining and capsynapse formation, respectively; cells not staining with the APA1/1 mAb or displaying diffuse CD8 staining were scored as negative.

**Retroviral-mediated stem cell gene transfer**

Retroviral producer cell lines encoding wild type and mutant (PRS-deficient) Cd3εs 2A peptide-linked CD3 chains and an IRES-eGFP cassette were produced as described (27, 28). Because Cd3ε<sup>−/−</sup> mice also display significantly reduced expression of CD3γ and CD3δ, the retroviruses encoded wild type (WT), PRS-mutant, or ITAM-mutant CD3ε chains linked to WT CD3γ and CD3δ chains by self-cleaving 2A peptides, which give rise to near absolute clavage and expression of each CD3 chain with proper stoichiometry. Bone marrow–derived stem cells from TCR-TG, Cd3ε<sup>−/−</sup> mice were cocultured with the retroviral producer cell lines for 48 h. The nonadherent bone marrow cells (2×10<sup>5</sup> in PBS containing 2% FBS and 20 U/ml heparin) were injected i.v. into sublethally irradiated (1100 rad) hosts. Mice were killed 8–12 wk later for phenotypic and functional studies.

**Proliferation and cytokine secretion**

FACS-sorted eGFP<sup>+</sup> CD8<sup>+</sup> T cells (2×10<sup>5</sup> per well) from retrogenic bone-marrow chimeras were incubated with various concentrations of TUM or NRP-V7 peptide in the presence of irradiated NOD splenocytes as APCs (10<sup>5</sup> per well) for 48–72 h at 37°C in 5% CO2. Supernatants were assayed after 48 h of culture for IL-2 or IFN-γ content, or both, by ELISA (R&D systems, Cambridge, MA). The cultures were pulsed with 1 μCi of [3H]-thymidine and 18 h later harvested and assayed for [3H]-thymidine incorporation. Values obtained with the negative control peptide TUM were subtracted.

**Diabetes**

Diabetes was monitored by measuring urine glucose levels with Diastix (Miles, Ontario, Canada) twice weekly. Animals were considered diabetic after two consecutive readings of +++ or greater. The average blood glucose levels in mice diagnosed as diabetic using this criteria are 21.96 ± 3.8 mM (SEM).

**Statistical analyses**

Data were compared by Mann–Whitney U, Fisher t, or two-way ANOVA tests. Statistical significance was assumed at p < 0.05.
Results

Germline-encoded variability in the TCR-α–chains of IGRP206–214-reactive T cells controls the kinetics of CD8 recruitment to ligand-bound TCRs and Nck recruitment to CD3ε

We have previously shown that avidity maturation of the IGRP206–214-specific CD8+ T cell population in the islets of prediabetic NOD mice, which invariably uses CDR3-invariant TCR-α–chains using Vα17 and Jα42 elements, is associated with changes in Vα17 element usage (13). As mice aged, the islet-associated IGRP206–214-specific CD8+ T cells that expressed Vα17.5 replaced those expressing the Vα17.6 element. Studies with TCR transfectants confirmed that cells expressing a Vα17.5-Jα42 rearrangement in the context of at least three different TCR-β–chains cloned from IGRP206–214-reactive CD8+ T cells recognized cognate pMHC with higher physical and functional avidity than those expressing CDR3-identical Vα17-Jα42 rearrangements using Vα17.6 and, to a lesser extent, Vα17.4 elements (13).

To ascertain the independent contribution of the amino acid substitutions distinguishing these three Vα17 elements to the observed differences in avidity, we compared the physical and functional avidity of nine groups of transfectants (in a TCR-α–β–CD8+ T cell hybridoma) coexpressing the TCR-β rearrangement of the diabetogenic Vα17.4-Jα42+ NY8.3 clone and CDR3-identical Vα17.4-Jα42, Vα17.6-Jα42 TCR-α–chains, or single or multiple amino acid variants of a Vα17.5-Jα42 TCR-α rearrangement in which the Vα17.5 element was mutated toward Vα17.6 (Fig. 1A, Supplemental Table I). Stable transfectants expressing comparable levels of CD3ε were tested for their ability to: 1) bind to IGRP206–214/Kd and NRP-V7/K7 tetramers (the latter is a higher-avidity ligand of IGRP206–214-reactive TCRs); and 2) secrete IL-2 in response to peptide-pulsed NOD splenocytes (Supplemental Table I). With the exception of Q27R and A80T, all other substitutions (F32Y and D28E, with or without Q27R and/or A80T) resulted in a significant loss in tetramer-binding avidity, indicating a key contribution of residues 32 and 28 (in combina-

![FIGURE 1. Role of naturally occurring sequence variability in Vα17 on avidity of IGRP206–214-reactive CD8+ T cells.](http://www.jimmunol.org/2977)

A. Predicted amino acid sequences of 8.3-like TCR-α chains using each of the three NOD mouse Vα17 elements (17.6, 17.5, and 17.4) and a series of mutant sequences (17.5 → 17.6). Shaded amino acids highlight sequence differences versus Vα17.6, the element that is enriched in IGRP206–214-reactive CD8+ T cells isolated from islets of 9-wk-old NOD mice (low avidity) (13).

B. Effects of molecular differences between 8.3 b-TCR–transfectants expressing the 8.3 (17.4) or 8.3-like TCR-α chains on Kd values of tetramer binding (left panel) and on their ability to produce IL-2 against peptide-pulsed (1 μM; IGRP206–214 or NRP-V7) NOD splenocytes (right panel). *p < 0.05 (Mann–Whitney U test).

C. Recruitment of Nck by CD3ε in TCR-αβ transfectants in response to tetramer challenge (30 min). Lysates were immunoprecipitated with anti-CD3ε, run in an SDS-PAGE gel, and blotted with anti-Nck (upper panel) or anti-CD3ε (bottom panel). Nck blot.

D. Recruitment of Nck by CD3ε in naive TCRαβ-TG CD8 T cells expressing the Vα17.4 or Vα17.5 elements (along with the 8.3β–chain) in response to tetramer binding (30 min) as detected by CD3ε immunoprecipitation followed by Western blotting with anti-Nck mAb (upper panel) or anti-CD3ε (bottom panel). The number of CD8+ T cells was adjusted according to percentages of tetramer+ cells, to compensate for central and peripheral deletion of TG T cells in 17.5α/8.3β–TCR-TG mice.
tion) to the observed differences in avidity (Fig. 1B, left panel; Supplemental Table I). Measurements of IL-2 production revealed that the mutations that lowered the TCR’s tetramer-binding avidity also reduced the transfectant’s ability to produce IL-2 in response to peptide (Fig. 1B, right panel; Supplemental Table I), as expected. Although IGRP_{206–214} tetramers did not bind sufficiently well to some of these TCR mutants to allow reliable kinetic data to be obtained (Supplemental Table I), the pattern of IL-2 secretion induced by IGRP_{206–214} was comparable to that seen with NRP-V7 (Fig. 1B, right panel; Supplemental Table I), indicating that the above results were not a peculiarity of NRP-V7.

Having established that differences in physical and functional avidity between IGRP_{206–214}-reactive CD8+ T cells expressing different CDR3-invariant V_{17.3α}-42 rearrangements were intrinsic to the germline-encoded E43K (V_{17.4} and V_{17.6} versus V_{17.5}) and F32Y plus D28E (V_{17.6} versus V_{17.5}) amino acid differences, we sought to investigate the underlying mechanisms. Because TCR-pMHC interactions between T cells and APCs in immune synapse (in these transfectants upon binding of pMHC-tetramer− to the immune synapse) to the observed differences in avidity (Fig. 1B, left panel; Supplemental Table I). Measurements of IL-2 production revealed that the mutations that lowered the TCR’s tetramer-binding avidity also reduced the transfectant’s ability to produce IL-2 in response to peptide (Fig. 1B, right panel; Supplemental Table I), as expected. Although IGRP_{206–214} tetramers did not bind sufficiently well to some of these TCR mutants to allow reliable kinetic data to be obtained (Supplemental Table I), the pattern of IL-2 secretion induced by IGRP_{206–214} was comparable to that seen with NRP-V7 (Fig. 1B, right panel; Supplemental Table I), indicating that the above results were not a peculiarity of NRP-V7.

Because TCR-pMHC interactions between T cells and APCs involve coreceptors and result in TCR oligomerization and cluster formation (29, 30), we reasoned that the amino acid differences distinguishing V_{17.5} from V_{17.4} and V_{17.6} had qualitative or quantitative effects, or both, on at least some of these consequences of TCR ligation, and these consequences have a major role in defining the avidity of the pMHC-driven T cell–APC interaction. In fact, kinetic studies of CD8 cap formation (recruitment to the immune synapse) in these transfectants upon binding of pMHC-coated beads indicated that V_{17.6} transfectants could not form caps, and that cap formation in V_{17.4} transfectants was slower than that seen in V_{17.5} transfectants (data not shown).

TCR aggregation, immune synapse formation, and T cell activation require transduction of signals from the TCR to CD3 molecules and this is associated with a conformational change in the CD3ε chain that exposes a PRS that serves as a binding site for the first SH3 domain of the adaptor molecule Nck (31, 32). In T cells, Nck links the TCR-CD3 complex with molecules such as WASP and dynamin 2, which are necessary for the cytoskeletal rearrangements underlying T cell activation, proliferation, and cytokine secretion (33–36). We thus investigated whether differences in the functional avidity of these two TCRs might be associated with differences in CD3ε-PRS exposure and Nck recruitment to CD3ε. We compared the expression of Nck in CD3ε molecules coimmunoprecipitated with CD3ε from NRP-V7/K8 tetramer-stimulated TCR transfectants (Fig. 1C) or naive TCR-TG T cells (adjusted for differences in their developmental biology) (Fig. 2A; data not shown). In addition, the CD8+CD4– thymocytes of 17.5α/8.3β–TCR-TG mice express significantly lower levels of the TG TCR and bind cognate pMHC tetramers with significantly lower mean fluorescence intensity than 17.4α/8.3β–NOD. Tera2−/− mice (Fig. 2A). Most importantly, the CD8+CD4– thymocytes of 17.5α/8.3β–NOD. Tera2−/− mice express significantly higher levels of CD3ε open conformers (APA1/1+) than 17.4α/8.3β–NOD. Tera2−/− mice, despite expressing lower levels of total TCR (Fig. 2B, 2C). These differences were also seen in TG CD4+CD8+ thymocytes (Fig. 2A–C), suggesting that they are imprinted at the double-positive stage of thymocyte development, presumably by engagement of the 17.5α/8.3β–TCR by the deleting pMHC ligand.

Analyses of splenic and lymph node CD8+ T cells confirmed that the TCR downregulation and increased APA1/1 staining seen in thymocytes are inherited by their peripheral descendants (Fig. 2D–F). Although virtually all thymocytes and peripheral T cells express APA1/1+, CD3ε molecules regardless of TCR avidity, normalization of the mean fluorescence intensity of CD3ε-PRS staining by the corresponding total TCR values indicated that the TCR-CD3 complexes of 17.5α/8.3β–CD8+ thymocytes, splenocytes, and mesenteric lymph node T cells contain ~3-fold higher levels of open CD3ε than CD8+CD4– TCR-TG thymocytes (Fig. 2G). Thus, the signals that trigger deletion of TCR in 17.5α/8.3β–CD8+ thymocytes increase the fraction of CD3ε molecules that expose their Nck-binding PRS.

Increased recruitment of Nck to the CD3ε-PRS in 17.5α/8.3β–CD8+ T cells is associated with, but does not account for, differences in their developmental biology

The above observations prompted us to wonder whether increased recruitment of CD3ε open conformers to the TCR-CD3 complex in 17.5α/8.3β–CD8+ thymocytes might be responsible for their increased susceptibility to central or peripheral deletion. To investigate this, we reconstituted lethally irradiated NOD mice with hematopoietic stem cells from CD8ε−/− 17.5α/8.3β–TCR-TG marrow transduced with retroviruses encoding WT or PRS- or ITAM-mutant CD3ε molecules and IRES-eGFP (27, 28). Analyses of retrogenic mice 8–12 wk after stem cell transfer showed that neither the PRS nor the ITAM CD3ε mutations had any significant effects on the developmental biology of the 17.5α/8.3β–TCR (Fig. 3A–E). Specifically, the three different retro-
genic chimeras harbored similar percentages of GFP+ thymocyte and splenocyte T cell subsets (Fig. 3A, 3B), and their peripheral eGFP+ T cells secreted similar levels of IL-2 and IFN-γ and proliferated equally well in response to NRP-A7–pulsed splenocytes (Fig 3C–E). Biochemical analyses of Ag-stimulated CD8+ T cells from the different chimeras confirmed that CD3ζ signaling was not compromised in the absence of CD3ε-PRS or -ITAM (Fig. 3F). Therefore, increased functional avidity of the 17.5α/8.3β-
TCR promotes the formation of open conformers of CD3ε and the recruitment of Nck, but neither the PRS nor the ITAM motifs of CD3ε are necessary for the TG T cells’ increased susceptibility to central and peripheral tolerance.

**Tolerance of 17.5a/8.3β-CD8+ T cells is not triggered by recognition of IGRP206–214**

Clearly, the increased susceptibility of the 17.5a/8.3β-TCR to tolerance, coupled with its ability to recruit more APA1/1ε (and Nck-binding) CD3ε molecules, is consistent with its increased physical and functional avidity for IGRP206–214/Kd as compared with the 17.4a/8.3β-TCR. However, because the murine thymus is not thought to express IGRP, we reasoned that these phenotypes might not be triggered by IGRP206–214/Kd. To investigate this, we expressed the 17.5a/8.3β- and 17.4a/8.3β-TCRs in a G6pc2 gene-targeted strain of NOD mice in which the codons encoding the two TCR contact residues of IGRP206–214 are mutated to encode Ala-

We have shown previously that 17.5a/8.3β–TCR-TG CD8+ T cells undergo peripheral deletion with age (13). These mice exhibit progressive, age-dependent reductions in the CD8/CD4 T cell ratio, percentage of total CD8+ T cells, and percentage of NRP-V7/Kε tetramer-positive T cells within the CD8+ subset, that stabilize in adult mice (>8 wk old). As shown in Fig. 4D and 4F, the splenic flow cytometry profiles of 8–17-wk-old 17.5a/8.3β-NOD.G6pc2K209A/F213A KIKI mice were indistinguishable from those seen in age-matched 17.5a/8.3β-NOD mice, including downregulation of the TCR and upregulation of APA1/1ε CD3ε.

To ascertain whether the kinetics of peripheral 17.5a/8.3β-CD8+ T cell deletion were in any way affected by lack of IGRP206–214 expression, we compared the thymocyte and splenocyte flow cytometry profiles of 17.5a/8.3β-NOD versus 17.5a/8.3β-NOD.G6pc2K209A/F213A KIKI mice of 4, 7, 14, 21, and 28 d of age. One, 2, and 3-wk-old 17.5a/8.3β-NOD.G6pc2K209A/F213A KIKI mice displayed increased thymic, splenic, and lymph node flow cytometry profiles that were essentially identical to those seen in 17.4a/8.3β-NOD and 17.5a/8.3β-NOD mice, respectively (Fig. 4C, 4D; Supplemental Fig. 3). Absence of the cognate IGRP206–214 epitope did not abrogate the downregulation of the 17.5a/8.3β TCR in CD8+CD4+ and CD8+ CD4+ thymocytes (Fig. 4C) nor the interstrain differences in APA1/1ε CD3ε recruitment to the TCR-CD3 complex that we saw in CD8+CD4+ thymocytes (Fig. 4E).

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These data indicate that endogenous IGRP206-214 plays a role in central and peripheral tolerance of high-avidity IGRP206-214-reactive 17.5α/8.3β-CD8+ T cells, particularly in the neonatal period. However, whereas expression of IGRP206-214 is absolutely necessary for recruitment of these T cells to pancreatic islets, it is completely dispensable for tolerance.
Discussion

We have shown previously that progressive replacement of low-avidity IGRP206–214-specific clonotypes by their higher avidity counterparts during the transition of asymptomatic islet inflammation to overt diabetes in NOD mice is driven, at least in part, by Vα17 usage, and conditioned by tolerance of the high-avidity T cell pool, which limits its contribution during the earliest stages of disease development (13). In this study, we sought to investigate the molecular, biochemical, and developmental underpinnings of this pathogenic T cell avidity maturation response, and to formally demonstrate that recruitment and selective tolerance of high-avidity IGRP206–214-reactive T cells are both driven by peripheral and central recognition of IGRP206–214/Kd. We find that differences in physical and functional avidity among IGRP206–214-reactive CD8+ T cells are intrinsic to germline-encoded amino acid differences among three Vα17 elements mapping to the CDR1α, and that these amino acid differences control the kinetics of CD3ε activation and Nck recruitment to CD3ε. Surprisingly, however, we find that tolerance of high-avidity IGRP206–214-reactive CD8+ T cells and all the associated biochemical correlates, unlike their recruitment to pancreatic islets, are dissociated from IGRP206–214/Kd recognition, particularly in adult mice. This finding challenges the generally held view that tolerance of pathogenic autoreactive T cells is invariably driven by recognition of the pMHC complex that drives their activation and recruitment in the periphery.

A key feature in the developmental biology of high-avidity (17.5α/8.3β) IGRP206–214-reactive T cells is that they undergo partial deletion in the thymus (selectively sparing thymocytes expressing low levels of the TG TCR) as well as a form of near complete deletion of these TCRlow thymic emigrants in the periphery. Although CD3ε-PRS exposure is clearly a feature of the naive, steady-state T cell repertoire, the tolerogenic signals that result in selective deletion of TCRhi 17.5α/8.3β–CD8+ thymocytes imprint onto their nondeleted TCRlow counterparts a permanent ~3-fold increase in the number of CD3ε open conformers, as compared with TCRhi 17.4α/8.3β–CD8+ thymocytes. These data are consistent with the recent observation that antigenic challenge induces maximum PRS exposure and that open CD3ε conformers likely mark TCRs that have been engaged, outlasting TCR occupancy (38). These observations, as well as previous studies by others and us, have suggested that CD3ε PRS exposure enables a more efficient recruitment of Nck, which in turn would serve to amplify TCR signaling, particularly in response to weak agonists (28, 31, 32). Surprisingly, the T cell development and functional profiles of TCR-TG T cells in retrogenic bone marrow chimeras reconstituted with stem cells from 17.5α/8.3β–TCR-TG NOD. CD3ε+−/− donor mice, transduced with PRS or ITAM mutated versions of CD3ε, were indistinguishable from those seen in chimeras expressing WT CD3ε. This indicated that enhanced recruitment of Nck to CD3ε-PRS and CD3ε signaling via its ITAM does not account, at least in isolation, for the in vivo consequences of high avidity. This does not imply that these two motifs do not play a role; signaling through CD3ε’s ITAM, which is not affected in these CD3ε mutant chimeras, may compensate for the lack of CD3ε-PRS or CD3ε-ITAM. These observations are compatible with previous findings in non–TCR-TG mice (27) as well as in CD3ε-PRS knock-in mice where mutation of the PRS motif did not have any major effects on T cell function (39).

Our most surprising observation was that tolerance of Vα17.5+ IGRP206–214-reactive CD8+ T cells could not be accounted for by recognition of cognate IGRP206–214/Kd complexes. TCR-TG NOD mice expressing a mutant form of IGRP, in which the two TCR-contact residues of IGRP206–214 were replaced with alanines (K209A and F213A) (25), deleted this TCR (in both the thymus and periphery) with the same efficiency, albeit with delayed tempo, as compared with TCR-TG NOD mice expressing WT IGRP. We note that the IGRP209A-F213A peptide cannot trigger the activation or elicit the cytotoxicity of IGRP206–214-reactive CD8+ T cells (regardless of avidity), and does not impair, either in vitro or in vivo, their responsiveness to a subsequent challenge with IGRP206–214 (25). Although comparison of the fate of TCR-TG T cells in IGRP206–214-targeted versus WT 17.5α/8.3β–TCR-TG NOD mice showed that IGRP206–214 does have a role in the tolerance of high-avidity autoreactive CD8+ T cells, this was only true in the neonatal period. That is, absence of IGRP206–214 delayed but did not abrogate tolerance of this T cell specificity.

The source of IGRP206–214 contributing to tolerance in the neonatal period is likely the β cell itself, because 1) IGRP206–214-induced tolerance was more pronounced in the periphery than in the thymus and 2) we cannot detect the presence of either of the two isofoms of IGRP mRNA (or other possible splice variants) in unsorted NOD thymic stroma or purified UEA-1/AIRE+ cells by RT-PCR (13). One possible explanation is that these T cells become exposed to IGRP during the neonatal wave of physiologic β cell apoptosis that peaks at ~2 wk of age in both rodents (40, 41) and humans (42), a time when T cells are more prone to undergo tolerance than activation (43, 44). Based on this, it is tempting to speculate that such a physiologic event is meant to protect the host from diabetogenic autoimmunity.

Because IGRP206–214 cannot account for deletional tolerance of high-avidity IGRP206–214-reactive CD8+ T cells in the postneonatal period, we reasoned that these T cells might undergo tolerance by cross-reacting with other pMHC complexes. Because this IGRP206–214-independent form of tolerance occurs at a time when the gut is being progressively colonized by commensal bacteria (45), we considered the possibility that the cross-reactive epitope might derive from the gut flora. However, results of ongoing studies in TCR-TG mice treated with a mixture of broad-spectrum antibiotics argue against this hypothesis, with the caveat that antibiologic therapy reduces, but does not eliminate, the bacterial content of the gut.

An alternative possibility is that differences in tolerance susceptibility among IGRP206–214-reactive T cells is determined by differences in the affinities with which their TCRs engage MHC (as opposed to peptide) residues, in a peptide-nonspecific manner. The mapping of differences in avidity to CDR1α residues 28 and 32, which are usually involved in direct interactions with MHC residues and guiding MHC restriction (46–48), would be consistent with this interpretation. In fact, there are specific examples of several other pathogenic autoreactive TCRs that engage peptide-MHC in a configuration that favors contacts with MHC residues and affords an intrinsic high-affinity for MHC that, in some cases, manifests itself as MHC promiscuity. For example, thymocytes expressing a highly diabetogenic I-εβ (MHC class II) restricted TCR (4.1) undergo deletion in NOD mice expressing one of several different antidiabetogenic MHC class II alleles, by engaging such class II molecules on hematopoietic APCs, in a peptide-nonspecific manner (49–51). Likewise, when expressed as a transgene in humanized (HLA-TA) mice, an encephalitogenic TCR triggered disease in the context of HLA-A3 but underwent tolerance in the context of HLA-A2 (52). In addition, structural studies of five different autoreactive TCRs (unrelated to T1D) have shown that they make substantially fewer contacts with peptide residues than conventional TCRs, either because they bind cognate pMHC with a unique topology (versus foreign Ag-specific TCRs), or target peptides that only partially occupy the peptide-binding cleft of the MHC molecule (53). Furthermore, features of
other T1D-relevant TCRs further suggest that such pHMC-recognition anomalies of pathogenic autoreactive TCRs might be the rule rather than the exception. For example, the BDC2.5-TCR recognizes a chromogranin A-derived peptide that binds to the I-Aa6 molecule in an unusual fashion (54). Likewise, most B9-23-specific TCRs use a rather conserved TCR-a chain, and the l-Ag7 complex targeted by type B insulin-reactive CD4+ T cell clones presumably exists at a unique conformation (55).

In summary, we have shown that germline-encoded variability in CDR1a has a key role in defining the avidity, pathogenicity, and tolerance susceptibility of IGRP206–214-reactive CD8+ T Cells. However, whereas pathogenicity of IGRP206–214-reactive CD8+ T cells is clearly driven by recognition of IGRP206–214, as expected, we find that developmental tolerance of high-avidity IGRP206–214-reactive clonotypes, including all the associated phenotypic and biochemical correlates, is dissociated from IGRP206–214 engagement, particularly in the postneonatal period. These observations demonstrate, we believe for the first time, that tolerance of pathogenic autoreactive T Cells not need to be triggered by the pHMC complex that triggers their activation and recruitment. When considered in the context of functional, developmental, and structural studies of other autoreactive TCRs, these observations support the hypothetical existence of a relationship between the pathogenicity of at least some autoreactive TCRs, their pHMC residue binding bias, and susceptibility to peptide- nonspecific tolerance. Such a relationship might be related to an altered mode of binding to pHMC, whereby pHMC residues on MHC compensate for the paucity of TCR-peptide and molecular contacts with pathogenic (non-tolerogenic) pHMC molecules. Such a hypothetical mechanism would allow the immune system to eliminate potentially pathogenic TCRs (i.e., those having high affinity for self-MHC regardless of the bound peptide) without the need to express every self-protein in the thymus.

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