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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 CDRI<sub>ε</sub> and are associated with quantitative differences in CD3<ε> 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.

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We found that differences in avidity for IGRP

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Δκd map to CDR1α 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, expression 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 naive T cell repertoire and do not account, at least in isolation, for systemic tolerance of high-avidity IGRP

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autoreactive T cells, the tolerogenic signals that result in selective deletion of high-avidity IGRP

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reactive CD8 T cells do not account, at least in isolation, for PRS exposure and Nck recruitment to CD3ε, as demonstrated elsewhere (13). TCR and CD8 expression were verified by flow cytometry using anti-β8 (MR5-2), anti-CD8 (33-6.7, anti–TCR-β (H57-597), or anti-CD3ε (145-2C11) mAbs. Pepsin-Avidity recognition by transfectants was measured by IL-2 production, using irradiated NOD splenocytes as APCs.

**Immunoprecipitation and Western blotting**

Transfectants were washed in serum-free medium and incubated with PE-conjugated NRP-V7/Kd 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, 0.5% NP40, 1 mM iodoacetamide, and a protease inhibitor mixture). Postnuclear lysates were immunoprecipitated with anti-PE mAb-conjugated beads (Miltenyi Biotech). 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 Biotech, Rockford, IL). In other experiments, FACS-sorted CD8+ T cells from TCR-TG mice (adjusted for NRP-V7/Kd tetramer- T cell content), TCR-transfectants or FACS-sorted eGFP+ CD8+ 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-phosphotyrosine Ab (4G10, Millipore) or anti-CD3ε and/or anti-Nck mAbs.

**Immunofluorescence**

TCR transfectants were incubated with NRP-V7/Kd-coated beads for 2-30 min at 37°C. Cells were then fixed using the Cytofix–Cytoperm kit (BD Pharmingen) for 10 min at room temperature, and stained with rabbit anti-CD8 mAb 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−/− mice also display significantly reduced expression of CD3δ and CD3ε, the retroviruses encoded wild type (WT), PRS-mutant, or ITAM-mutant CD3δ chains linked to WT CD3δ and CD3ε chains by self-cleaving 2A peptides, which give rise to near absolute cleavage and expression of each CD3 chain with proper stoichiometry. Bone marrow–derived stem cells from TCR-TG, CD3ε−/− mice were cocultured with the retroviral producer cell lines for 48 h. The nonadherent bone marrow cells (2-4 × 10^6 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+ CD8+ T cells (2 × 10^6 per well) from retrogenic bone-marrow chimeras were incubated with various combinations of TUM or NRP-V7 peptide in the presence of irradiated NOD splenocytes as APCs (10^5 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 τ, 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 \( V_\alpha \)17 and \( J_\alpha \)42 elements, is associated with changes in \( V_\alpha \)17 element usage (13). As mice aged, the islet-associated IGRP206–214-specific CD8+ T cells that expressed \( V_\alpha \)17.5 replaced those expressing the \( V_\alpha \)17.6 element. Studies with TCR transfectants confirmed that cells expressing a \( V_\alpha \)17.5-J\( \alpha \)42 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 \( V_\alpha \)17-J\( \alpha \)42 rearrangements using \( V_\alpha \)17.6 and, to a lesser extent, \( V_\alpha \)17.4 elements (13).

To ascertain the independent contribution of the amino acid substitutions distinguishing these three \( V_\alpha \)17 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 \( V_\alpha \)17.4-J\( \alpha \)42+ NY8.3 clone and CDR3-identical \( V_\alpha \)17.4, \( V_\alpha \)17.5, or \( V_\alpha \)17.6 TCR-α–chains, or single or multiple amino acid variants of a \( V_\alpha \)17.5-J\( \alpha \)42 TCR-α rearrangement in which the \( V_\alpha \)17.5 element was mutated toward \( V_\alpha \)17.6 (Fig. 1A, Supplemental Table I). Stable transfectants expressing comparable levels of CD3ε were tested for their ability to: 1) bind to IGRP206–214/K\( \delta \) and NRP-V7/K\( \delta \) 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.](http://www.jimmunol.org/)

Role of naturally occurring sequence variability in \( V_\alpha \)17 on avidity of IGRP206–214-reactive CD8+ T cells. (A) Predicted amino acid sequences of 8.3-like TCR-α chains using each of the three NOD mouse \( V_\alpha \)17 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 \( V_\alpha \)17.6, 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 TCR–transfectants expressing the 8.3 (17.4) or 8.3-like TCR-α chains on \( K_0 \) 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 mAbs (bottom panel). (D) Recruitment of Nck by CD3ε in naive TCRαβ-TG CD8 T cells expressing the \( V_\alpha \)17.4 or \( V_\alpha \)17.5 elements (along with the 8.3-β chain) in response to tetramer binding (30 min) as detected by CD3ε immunoprecipitation followed by Western blotting with anti-Nck mAb (upper 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 17.5/8.3-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\textsubscript{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\textsubscript{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\textsubscript{206-214}-reactive CD8\textsuperscript{+} T cells expressing different CDR3-invariant V\textsubscript{a}17.3\textsubscript{a}42 rearrangements were intrinsic to the germline-encoded E43K (V\textsubscript{a}17.4 and V\textsubscript{a}17.6 versus V\textsubscript{a}17.5) and F32Y plus D28E (V\textsubscript{a}17.6 versus V\textsubscript{a}17.5) amino acid differences, we sought to investigate the underlying mechanisms. 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\textsubscript{a}17.5 from V\textsubscript{a}17.4 and V\textsubscript{a}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\textsubscript{a}17.6 transfectants could not form caps, and that cap formation in V\textsubscript{a}17.4 transfectants was slower than that seen in V\textsubscript{a}17.5\textsuperscript{+} 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 presence of Nck in CD3ε molecules coimmunoprecipitated with CD3ε from NRP-V7/K\textsuperscript{d} tetramer-stimulated TCR transfectants (Fig. 1C) or naive TCR-TG T cells (adjusted for the open TD ensemble to contain the same absolute number of tetramer\textsuperscript{+} cells to compensate for differences caused by peripheral tolerance (13) (Fig. 1D). There was more Nck in the CD3ε precipitates of NRP-V7/K\textsuperscript{d} tetramer-stimulated V\textsubscript{a}17.5\textsuperscript{+} transfectants and naive TCR-TG T cells than in CD3ε precipitates of their V\textsubscript{a}17.4\textsuperscript{+} counterparts.

These data indicate that the amino acid differences among these three V\textsubscript{a}17 elements used by IGRP\textsubscript{206-214}-reactive CD8\textsuperscript{+} T cells somehow control the recruitment of Nck to CD3ε.

Enhanced Nck recruitment capacity of CD3ε in high-avidity 17.5a/8.3β-CD8\textsuperscript{+} T cells is imprinted in the thymus and is associated with TCR downregulation

We have shown previously that, in TCR-TG NOD mice, the 17.5a/8.3β-TCR undergoes partial central deletion as well as an age-dependent form of peripheral deletion that begins ∼4–7 d after birth and increases progressively afterward (13). In these mice, central deletion of TG CD8\textsuperscript{+} T cells is constitutive (occurs from birth) and is associated with positive selection of TCR-TG CD8\textsuperscript{+} thymocytes that express significantly lower levels of the TG-TCR, as compared with non–TCR-TG NOD mice or 17.4a/8.3β-NOD mice (Fig. 2A).

Because the CD3ε molecules of splenic 17.5a/8.3β–TCR-TG CD8\textsuperscript{+} T cells contained more Nck than their intermediate-avidity counterparts (17.4a/8.3β–CD8\textsuperscript{+}), even in non–antigen-stimulated cells (see Fig. 1D), we considered the possibility that tolerance in 17.5a/8.3β-NOD mice might be mechanistically linked to the increased Nck recruitment capacity of 17.5a/8.3β-TCR- associated CD3ε molecules. To investigate this, we first measured changes in CD3ε-PRS exposure during thymocyte development using the APA1/1 mAb. To obviate the potentially confounding effects of incomplete allelic exclusion at the TCR-α locus (i.e., dilution of TG TCRs by coexpression of endogenous TCR-α chains), and to be able to compare the magnitude of CD3ε exposure in TCR-αβ–TG thymocytes without the need to exclude endogenous T cells via pMHC tetramer staining, we performed these studies in TCR-TG NOD. Tera\textsuperscript{+}ε\textsuperscript{−} mice, which only export thymocytes expressing the TG TCR. We note that 17.5a/8.3β-NOD.Tera\textsuperscript{+}ε\textsuperscript{−} and 17.5a/8.3β-NOD. Tera\textsuperscript{−}ε\textsuperscript{+} mice have similar thymocyte profiles except for a lower frequency of pMHC-tetramer\textsuperscript{+} CD4\textsuperscript{+}CD8\textsuperscript{−} thymocytes in the former, as expected (Fig. 2A, Supplemental Fig. 1). As described previously for TCR-TG NOD.Tera\textsuperscript{+}ε\textsuperscript{−} mice (13) TCR-TG CD4\textsuperscript{+}CD8\textsuperscript{−} thymocytes from 17.5a/8.3β-NOD. Tera\textsuperscript{−}ε\textsuperscript{+} mice have a statistically significant and specific reduction in the size of the TCR\textsuperscript{a}ε\textsuperscript{−}NOD CD4\textsuperscript{+}CD8\textsuperscript{−} T cell subset compared with their 17.4a/8.3β–TCR-TG counterparts (Fig. 2A; data not shown). In addition, the CD8\textsuperscript{−}CD4\textsuperscript{+} thymocytes of 17.5a/8.3β-NOD. Tera\textsuperscript{−}ε\textsuperscript{+} mice express significantly lower levels of the TG TCR and bind cognate pMHC tetramers with significantly lower mean fluorescence intensity than 17.4a/8.3β- NOD. Tera\textsuperscript{−}ε\textsuperscript{+} mice (Fig. 2A).

Analyses of splenic and lymph node CD8\textsuperscript{+} 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\textsuperscript{+} 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 17.5a/8.3β–CD8\textsuperscript{−} thymocytes, splenocytes, and mesenteric lymph node T cells contain ∼3-fold higher levels of open CD3ε conformers than their 17.4a/8.3β–TCR-TG counterparts (Fig. 2G). Thus, the signals that trigger deletion of TCR\textsuperscript{a}ε\textsuperscript{−}NOD CD8\textsuperscript{−}CD4\textsuperscript{+} thymocytes increase the fraction of CD3ε molecules that expose their Nck-binding PRS.

Increased recruitment of Nck to the CD3ε-PRS in 17.5a/8.3β-CD8\textsuperscript{−} 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 17.5a/8.3β–CD8\textsuperscript{−} 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 CD3ε\textsuperscript{−}ε\textsuperscript{−} 17.5a/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 17.5a/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 contact residues of IGRP206–214 are mutated to encode Ala-targeted strain of NOD mice in which the codons encoding the two UWhitney transfer). The Absence of the cognate IGRP206–214 epitope did not abrogate the 8.3 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 Alanine (K209A and F213A) (NOD.G6pc2K209A/F213A KIKI) (25). As expected, both 17.4a/8.3β-NOD.G6pc2K209A/F213A KIKI and 17.5a/8.3β-NOD.G6pc2K209A/F213A KIKI mice developed a significantly lower incidence (and decelerated) form of diabetes than their G6pc2 WT counterparts (Fig. 4A, 4B). As was the case for 17.4a/8.3β-NOD.G6pc2K209A/F213A KIKI mice (25), and unlike 17.5a/8.3β-NOD mice, 17.5a/8.3β-NOD.G6pc2K209A/F213A KIKI mice did not recruit 17.5a/8.3β-CD8+ T cells into islets (Supplemental Fig. 2). The few TCR-TG NOD.G6pc2K209A/F213A KIKI mice that developed T1D probably did so by recruiting autoreactive T cells expressing endogenous TCRs and targeting other autoantigenic epitopes, such as the Insulin B15-23 epitope (Supplemental Fig. 2).

A similar phenomenon has been described by us and others in LCMV-Gp33-specific TCR-TG NOD mice (14, 37).

Remarkably, however, 17.4a/8.3β-NOD.G6pc2K209A/F213A KIKI and 17.5a/8.3β-NOD.G6pc2K209A/F213A KIKI mice displayed 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 17.5α/8.3β TCR in CD8+CD4+ and CD8+ CD4+ thyocytes (Fig. 4C) nor the interstrain differences in APA1/1+ CD3e recruitment to the TCR-CD3 complex that we saw in CD8+CD4+ thyocytes (Fig. 4E).

We have shown previously that 17.5α/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/Kd 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 APA1/1+ CD3e.

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 and, especially, splenic CD8+CD4+ T cell ratios as well as increased percentages of NRP-V7/Kd tetramer-positive CD8+ splenocytes as compared with age-matched 17.5a/8.3β-NOD mice, suggesting a role for endogenous IGRP206–214 in central and peripheral tolerance of high-avidity IGRP206–214-auto-reactive CD8+ T cells (Fig. 5). However, the absence of IGRP206–214 only delayed the kinetics of peripheral deletion in 17.5a/8.3β-NOD.G6pc2K209A/F213A KIKI versus 17.5a/8.3β-NOD mice (by ∼1–2 wk), without abrogating its development; both 17.5a/8.3β-NOD and 17.5a/8.3β-NOD.G6pc2K209A/F213A KIKI mice experience significant declines in the CD8:CD4 ratio and in the percentage of NRP-V7/Kd tetramer+ cells within the CD8+ T cell subset during the first 3 wk of life (Fig. 5). The delayed (∼1 wk) appearance of decreased NRP-V7/Kd tetramer reactivity relative to the reduction in the CD8:CD4 ratios (Fig. 5) occurs in both strains and is presumably due to progressive replacement of the dwindling IGRP206–214-reactive CD8+ T cell pool by T cells expressing endogenous TCRs.
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.

**FIGURE 4.** The developmental biology, but not the diabetogenic potential, of 17.5α/8.3β-TCR-TG T cells is dissociated from IGRP206-214 expression. (A and B) Incidence of T1D in 17.4α/8.3β-NOD. G6pc2-K209A/F213A KI/KI (n = 18) and 17.5α/8.3β-NOD.G6pc2-K209A/F213A KI/KI mice (n = 32) versus 17.4α/8.3β-NOD (n = 38) and 17.5α/8.3β-NOD mice (n = 88), respectively. (C and D) Thymic (C) and splenic (D) flow cytometry profiles of 17.4α/8.3β-NOD (n = 5; 8–16-wk-old), 17.5α/8.3β-NOD (n = 4; 8–17-wk-old) and 17.5α/8.3β-NOD.G6pc2-K209A/F213A KI/KI mice (n = 5; 8–15-wk-old). Values shown in each quadrant or histogram correspond to average percentages of cells ± SEM. Differences in mean fluorescence staining for TCR, Vß8, and NRP-V7/Kδ tetramer between 17.5-CD8+ T cells developing in 17.5α/8.3β-NOD versus 17.5α/8.3β-NOD.G6pc2-K209A/F213A KI/KI mice were statistically nonsignificant (Mann–Whitney U test; mean fluorescence intensity (mfi) values for CD8 single positive (SP) cells are shown on the right of each panel). (E and F) Representative APA1/1 staining histograms corresponding to thymocytes (E) and splenocytes (F) from 17.4α/8.3β-NOD.Tcrα2/2/G6pc2-K209A/F213A KI/KI, 17.5α/8.3β-NOD. Tcrα2/2/G6pc2-K209A/F213A KI/KI, and 17.5α/8.3β-NOD.Tcrα2/2 mice. N.S., not statistically significant (Mann–Whitney U test).
**Discussion**

We have shown previously that progressive replacement of low-avidity IGRP\textsubscript{206–214}-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 V\textalpha 17 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 IGRP\textsubscript{206–214}reactive T cells are both driven by peripheral and central recognition of IGRP\textsubscript{206–214}/K\textdagger. We find that differences in physical and functional avidity among IGRP\textsubscript{206–214}reactive CD8\textsuperscript{+} T cells are intrinsic to germline-encoded amino acid differences among these V\textalpha 17 elements mapping to the CDR1\alpha, and that these amino acid differences control the kinetics of CD3\textepsilon activation and Nck recruitment to CD3\textepsilon. Surprisingly, however, we find that tolerance of high-avidity IGRP\textsubscript{206–214}reactive CD8\textsuperscript{+} T cells and all the associated biochemical correlates, unlike their recruitment to pancreatic islets, are dissociated from IGRP\textsubscript{206–214}/K\textdagger 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 (17.5a/8.3b) IGRP\textsubscript{206–214}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 TCR\textsuperscript{low} thymic emigrants in the periphery. Although CD3\epsilon-PRS exposure is clearly a feature of the naive, steady-state T cell repertoire, the tolerogenic signals that result in selective deletion of TCR\textsuperscript{hi} 17.5a/8.3b–CD8\textsuperscript{+} thymocytes imprint onto their nondeleted TCR\textsuperscript{low} counterparts a permanent ∼3-fold increase in the number of CD3\epsilon open conformers, as compared with TCR\textsuperscript{hi} 17.5a/8.3b–CD8\textsuperscript{+} thymocytes. These data are consistent with the recent observation that antigenic challenge induces maximum PRS exposure and that open CD3\epsilon 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 CD3\epsilon PRS exposure weakens 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 17.5a/8.3b–TCR-TG NOD. CD3\epsilon\textsuperscript{−/−} donor mice, transduced with PRS or ITAM mutated versions of CD3\epsilon, were indistinguishable from those seen in chimeras expressing WT CD3\epsilon. This indicated that enhanced recruitment of Nck to CD3\epsilon-PRS and CD3\epsilon 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 CD3\epsilon’s ITAM, which is not affected in these CD3\epsilon mutant chimeras, may compensate for the lack of CD3\epsilon-PRS or CD3\epsilon-ITAM. These observations are compatible with previous findings in non–TCR-TG mice (27) as well as in CD3\epsilon-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 V\textalpha 17.5\textsuperscript{+} IGRP\textsubscript{206–214}reactive CD8\textsuperscript{+} T cells could not be accounted for by recognition of cognate IGRP\textsubscript{206–214}/K\textdagger complexes. TCR-TG NOD mice expressing a mutant form of IGRP, in which the two TCR-contact residues of IGRP\textsubscript{206–214} 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 IGRP\textsubscript{K209A-F213A} peptide cannot trigger the activation or elicit the cytotoxicity of IGRP\textsubscript{206–214}reactive CD8\textsuperscript{+} T cells (regardless of avidity), and does not impair, either in vitro or in vivo, their responsiveness to a subsequent challenge with IGRP\textsubscript{206–214} (25). Although comparison of the fate of TCR-TG T cells in IGRP\textsubscript{206–214}targeted versus WT 17.5a/8.3b–TCR-TG NOD mice showed that IGRP\textsubscript{206–214} does have a role in the tolerance of high-avidity autoreactive CD8\textsuperscript{+} T cells, this was only true in the neonatal period. That is, absence of IGRP\textsubscript{206–214} delayed but did not abrogate tolerance of this T cell specificity.

The source of IGRP\textsubscript{206–214} contributing to tolerance in the neonatal period is likely the β cell itself, because IGRP\textsubscript{206–214}induced tolerance was more pronounced in the periphery than in the thymus and 2) we cannot detect the presence of either of the two isomers of IGRP mRNA (or other possible splice variants) in unsorted NOD thymic stroma or purified UEA-1/AIRE\textsuperscript{+} 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 IGRP\textsubscript{206–214} cannot account for deletional tolerance of high-avidity IGRP\textsubscript{206–214}reactive CD8\textsuperscript{+} T cells in the postneonatal period, we reasoned that these T cells might undergo tolerance by cross-reacting with other pMHC complexes. Because this IGRP\textsubscript{206–214}dependent 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 antibiotic therapy reduces, but does not eliminate, the bacterial content of the gut.

An alternative possibility is that differences in tolerance susceptibility among IGRP\textsubscript{206–214}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 CDR1\alpha 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-A\textsuperscript{b} (MHC class II) restricted TCR (4.1) undergo deletion in NOD mice expressing one of several different antidiatricogenic 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-TG) 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 T1ID) 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 pMHC-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-Aκ chain in a molecular fashion (54). Likewise, most B9-23–specific TCRs use a rather conserved TCR-α chain, and the 1–Aκ complex targeted by type B insulin-reactive CD4+ T cell clones presumably exists at a unique conformation (55).

In summary, we have shown that germ-line-encoded variability in CDR1α 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 postnatal period. These observations demonstrate, we believe for the first time, that tolerance of pathogenic autoreactive T cells need not be triggered by the pMHC 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 MHC residue binding bias, and susceptibility to peptide-non-specific tolerance. Such a relationship might be related to an altered mode of binding to pMHC, whereby MHC residues on MHC compensate for the paucity of TCR-peptide and molecular contacts with pathogenic (non-tolerogenic) MHC molecules. Such a hypothetical mechanism would allow the immune system to eliminate potentially pathogenic TCRs (i.e., those having high avidity for self-MHC regardless of the bound peptide) without the need to express every self-protein in the thymus.

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