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Toxin-Coupled MHC Class I Tetrants Can Specifically Ablate Autoreative CD8+ T Cells and Delay Diabetes in Nonobese Diabetic Mice

Benjamin G. Vincent,* Ellen F. Young,* Adam S. Buntzman,* Rosemary Stevens,* Thomas B. Kepler,† Roland M. Tisch,* Jeffrey A. Frelinger,* and Paul R. Hess*†‡

There is compelling evidence that self-reactive CD8+ T cells are a major factor in development and progression of type 1 diabetes in animals and humans. Hence, great effort has been expended to define the specificity of autoimmune CD8+ T cells and to alter their responses. Much work has focused on tolerization of T cells using proteins or peptides. A weakness in this approach is that residual autoreative T cells may be activated and exacerbate disease. In this report, we use a novel approach, toxin-coupled MHC class I tetrants. Used for some time to identify Ag-specific cells, in this study, we use that same property to delete the Ag-specific cells. We show that saporin-coupled tetramers can delete islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-reactive T cells in vitro and in vivo. Sequence analysis of TCRv-chains of IGRP+ cells reveals the repertoire complexity in the islets is markedly decreased as NOD mice age and significantly altered in toxic tetramer-treated NOD mice. Further tetramer+ T cells in the islets are almost completely deleted, and, surprisingly, loss of tetramer+ T cells in the islets is long lasting. Finally, we show deletion at wk 8 of age of IGRP+ CD8+ T cells, but not dystrophia myotonica kinase- or insulin B-reactive cells, significantly delays diabetes in NOD mice. The Journal of Immunology, 2010, 184: 000–000.

Type 1 diabetes (T1D) is an autoimmune disease with a complex etiology. The disease in mice and humans is believed to be mediated by CD4+ and CD8+ T cells (1, 2). In both species, there is a progressive loss of insulin-producing β cells in the islets of Langerhans. Genome-wide association studies have shown that polymorphisms in the same genes of both species contribute to susceptibility to T1D, arguing that the fundamental processes are similar in mice and man (3). In NOD mice, the first evidence of insulitis is detected by wk 4 of age, and the majority of females develop frank diabetes by wk 20 of age.

The requirement of CD8+ T cells is well established. In the absence of CD8+ T cells, NOD mice do not develop T1D (4–6). Further, CD8+ T cells from diabetic mice are able to transfer disease (7). Finally, a single CD8+ T cell clone derived from the TCR transgenic NOD 8.3 mouse is also able to transfer disease into immunocompromised NOD SCID mice (8).

Although it is clear that CD8+ T cells can cause islet destruction, the normal pathogenesis is likely more complex. There is good evidence that an autoimmune response to insulin is also required to develop diabetes in mice transgenic for the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-reactive NY8.3 TCR (9, 10). Further, mice that have an altered insulin gene that abolishes the major epitope recognized in NOD mice do not develop diabetes (11). Additional experiments to induce tolerance to the insulin B (InsB) epitope at an early age also blocked the development of T1D, although induction of tolerance to IGRP is also effective in preventing progression to T1D (12). In contrast, clinical trials attempting to induce oral tolerance to insulin have been ineffective (13, 14). Finally, treatment with anti-CD3 Ab has been effective in both mice and humans, but comes with significant side effects (including cytokine production) that limit its utility (15).

The use of MHC class I (MHC I) tetrants has revolutionized the study of CD8+ T cells (16). The ability to bind to Ag-specific T cells could be used not only to identify Ag-specific T cells, but also to carry toxins and radionuclide to the cells for either imaging or deletion (17–19). In our laboratory, we have developed saporin (SAP)-coupled MHC I tetrants to kill Ag-specific T cells while sparing irrelevant T cells.

Several studies have shown that the repertoire of the islet-infiltrating T cells changes over time (20–22). There has been little information about whether the changes in the repertoire observed are the cause or the effect of disease progression. In this study, we show that deletion of IGRP-specific CD8+ T cells changes the islet repertoire and prolongs the disease-free interval of NOD mice.

Materials and Methods

Mice

NOD/ShiLtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen-free laboratory animal facility that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. NOD mice were used at 8–14 wk of age. In all experiments, NOD mice were defined as diabetic if two consecutive weekly blood glucose measurements were >250 mg/dl. Diabetes onset and incidence in unmanipulated mice is identical to that reported by The Jackson Laboratory (data not shown).
Toxin-Coupled MHC I Tetramers Can Remove CD8+ T Cells

NOD C57-Tg(TcrbCh/TcrbCh)1Shrm/Tisch (NOD CL4) mice, which bear transgenic CD8+ T cells that react to the influenza peptide hemagglutinin protein of influenza virus (HA) presented by H2Kd, were bred in-house (23). NOD C57-Tg(TcrbChVvVvVv1)Pesa/Dvsj (NOD 8.3) mice, which bear transgenic CD8+ T cells that react to H2Kd–IGRP and NOD.Cg-Prkd+scid (NOD scid) mice were purchased from The Jackson Laboratory.

Isolation of lymphocytes from islets
Pancreata were perfused with a 2 mg/ml solution of collagenase P (Sigma-Aldrich, St. Louis, MO), dissected, and incubated at 37°C for 20 min. Islets were purified using a Ficoll PM 400 (Sigma-Aldrich) gradient, handpicked, and counted, then cultured overnight in RPMI 1640 containing 10% FBS and 4 mg/ml recombinant murine IL-2 (Invitrogen, Carlsbad, CA). Infiltrating lymphocytes were collected and filtered through a 40-μm nylon filter. Each sample constituted all of the islet cells isolated from an individual mouse.

Tetramer
MHC I tetramers were prepared as previously described (24). Briefly, H2Kb or H2Dd and mouse β2-microglobulin were produced in Escherichia coli and refolded with peptide (from GenScript, Piscataway, NJ) in vitro. Refolded peptide-MHC monomer was purified by HPLC, biotinylated using biotinyl protein ligase, and assembled into tetramers by conjugation with UltraAvidin-PE (Leinco Technologies, St. Louis, MO). Peptides used were NRP-V7, KYNKANVFL; IGRP 201–214, VYLKTNVFL; InsB 15–23 (GV), LYLVCGERV; or dystophia myotonica kinase (DMK) 138–146, FQDENYLYL.

Toxic tetramer preparation and injection
NRP-V7-H2Kd tetramers conjugated to SAP were prepared as previously described for gp33-H2Dd tetramers (17). Eight-week-old NOD female mice received three injections of 200 pmol (2.82 μg) of H2Kd-IGRP-SAP, Kd-InsB-G9V-SAP, or Db-DMK-SAP in 200 μl PBS. Control NOD female mice received three injections of 200 μl PBS at the same time as the toxic tetramer injections. TCR analyses on CD8+ T cells in the spleen, pancreatic lymph nodes (PLNs), and islets were carried out either at 21 d posttreatment or at the time of diabetes diagnosis or 54 wk of age.

In vitro and in vivo depletion of 8.3 and CL4 T cells by toxic tetramer
As previously described, dissociated splenocytes from transgenic and wild-type NOD mice were either enriched for CD8+ T cells via negative selection or depleted of CD8+ T cells via positive selection using the appropriate immunomagnetic cocktails and LS columns on a QuadroMACS separator (Miltenyi Biotec, Sunnyvale, CA) (17). Enriched T cells were resuspended in PBS and injected i.v. via the lateral tail vein (2 × 105–5 × 107/200 μl/mouse). T cells were primed by i.p. injection of IGRP201-214 peptide (100 μg) 2 h posttransfer. Tetramers were diluted in PBS and injected i.v. [22 pmol (2.82 μg)/200 μl/mouse]. To assay cell survival, peripheral blood was collected from the superficial temporal vein using a Goldenrod lancet (MEDIpoint, Mineola, NY). Lymphocytes were enriched over a Lymphoprep (Aldrich, St. Louis, MO), dissected, and incubated at 37°C for 20 min. Islets were collected from the superficial temporal vein using a Goldenrod lancet and 4 ng/ml recombinant murine IL-2 (Invitrogen, Carlsbad, CA). Enriched T cells that are present and 2) the frequency of each individual clone. Entropy is greatest when there are many different T cell clones and when there are few clones that are highly represented in the population (i.e., few dominant clones). In pooled samples, entropy increases with decreased sequence sharing between individual samples in the pool (i.e., low frequencies of shared or public clones). Intuitively, this index represents the intrapopulation variability of the potential interactions available to T cells in the pool. If S is the total number of unique clonotypes in the pool, and pi is the proportion of the pool represented by clonotype i, the Shannon entropy H is defined as:

\[
H = - \sum_{i=1}^{S} p_i \log p_i
\]

where pi is the proportion of the pool represented by clonotype i, S is the total number of unique clonotypes in the pool, and H gives rise to sampling bias. The bias is itself estimable when the total number of unique clonotypes S is sampled in a population (31). In the present case, S is not known. To address this problem, we have developed a Bayesian method to estimate the Shannon entropy accounting for clonotypes in the population that are unique to the sample (T.B. Kepler, Vincent, and J.A. Frelinger, manuscript in preparation). Utilization of such a procedure is necessary because incomplete sampling could otherwise result in grossly underestimated entropy values and invalid comparisons between samples. Importantly, confidence intervals for the entropy estimation are also given by this method, which has been implemented in software and is available upon request.

Sequence sharing analysis
Sequences were defined as shared if they were present in samples taken from more than one mouse. Sequence sharing was calculated using a Python script.

Statistical analyses
Data were analyzed using Prism 4.0 (GraphPad, San Diego, CA). Mann-Whitney U tests were done to evaluate population differences in percentage of clonotypes shared, number of tetramer-positive cells per islet, and percentage of CD8+ T cells that were tetramer-positive. The Kruskal-Wallis test with Dunn’s posttests was used to evaluate population differences in TRBV 13-3 expression and graphical results displayed as dot plots with population mean indicated by horizontal bars. The Kaplan-Meier curve was used to determine the significance of the difference in diabetes incidence between treated and control mice. In all analyses, the significance level was 0.05.

TCR gene nomenclature
Gene names are given according to the ImmunoGeneTics nomenclature (32), with older nomenclature occasionally included parenthetically for clarity. A conversion chart between the various nomenclatures is available at http://imgt.cines.fr/textes/IMGTrepertoire/LocusGenes/#4 (33).
Results

TCRβ gene usage decreases in diversity over time in the islets, but not in the PLNs and spleen of 8- to 14-wk-old NOD mice

Previous work from our laboratory and others have suggested that the T cell repertoire in the periphery and the islets of prediabetic NOD mice is overlapping (20, 21). This suggests that the CD8+ T cells are generated in the periphery and migrate to the islets where they function. Further, if the complexity of the response in the islets decreases, as would be expected for selection, then deletion of those clones would be more feasible, because they would have a more homogenous avidity. We have extended previous studies to examine the clones expressed in the periphery and islets at times before 20 wk. By comparing three times, we can examine the trajectory of the changes in the complexity of the T cell repertoire and therefore better predict the outcome of deletion.

CD8+ NRP-V7+ T cells were sorted into individual wells and TCR usage determined for single cells. We began these experiments examining NRP-V7+ T cells because the authentic IGRP peptide was not available at the time, and many studies examining repertoire have already been done using NRP-V7+ T cells (34). We sequenced a total of 563 TCRβ-chains from single cells. Results of these experiments are summarized in Table I, and a list of these and other sequences recovered is presented in Supplemental Table I. Vβ gene usage was highly restricted in the islets at 12–14 wk of age (Fig. 1A). In all other tissues, Vβ usage was distributed among multiple Vβ families. TRBV 13-3 (old Vβ 8.1) was the dominant Vβ gene used in all tissues at all time points and increased in dominance in the islets over time (Fig. 1A), characterized by an increasing portion of the pool that expressed TRBV 13-3 as well as a decreasing total number of Vβ genes represented. Jβ gene usage was also restricted in the islets at 12–14 wk of age, with diversity in the islets at both ages less than that of the PLN and spleen. TRJB 2-4 and TRJB 2-7 were highly represented in all tissues at 8–10 wk of age, with TRJB 2-7 continuing to be highly represented at 12–14 wk of age in all tissues, in contrast to the frequency of TRJB 2-4 decreased in the PLN and spleen but increased in the islets. TRJB 1-2 rose in frequency in all tissues over time and was a dominant Jβ gene in the islets at 12–14 wk of age. These patterns of Vβ and Jβ usage are in agreement with prior work that showed dominance in the islets of TRBV 13-3 and TRJB 2-4 and 2-7 (12, 21, 25). The decrease in total Vβ and Jβ genes used in the islets indicates that the TCR repertoire becomes more restricted with age in NOD mice.

Although it is possible to examine these changes qualitatively, until recently, quantitative assessment of changes in repertoire have been challenging. We have recently adapted methodology from ecology to combine both the number and diversity of TCRβ sequences using entropy calculations. Entropy calculation takes into account both the number of different TCRβ sequences and the population distribution among the different sequences, an improvement over simple counting of the number of different sequences. Entropy in the islets at 12–14 wk was significantly less than at 8–10 wk of age (Fig. 1C). This decrease was determined by both a declining overall number of unique clones and an increased frequency of the most abundant clones (Supplemental Table I). In no other tissue was a significant difference seen between the two ages. In contrast to the pooled data, the nonpooled islet entropies showed no significant difference between the 8- to 10- and 12- to 14-wk-old samples, with the exception of a single 8–10 wk mouse that had an entropy value of 5.86 (Fig. 1D). This sample had 13 sequences recovered that were all unique clones (Supplemental Fig. 1), and this entropy value approaches maximal entropy. When this mouse is excluded from the 8- to 10-wk-old pool, the pooled entropy is still significantly different between the 8- to 10- and 12- to 14-wk-old pools. This implies that the frequency of shared clones between individual samples in the pools increases between 8–10 and 12–14 wk of age, thus contributing to a decrease in the pooled entropy without a corresponding decrease in individual sample entropies over time. Sequence sharing increased over time in the islets, but not in the PLN and spleen.

A major question remaining is: do the repertoires in the periphery and the islets evolve independently, or are they in equilibrium? In other words, are the effector T cells in the islets simply a sample of the periphery, or do the two anatomic sites have different selective pressures. If increased sequence sharing occurs only in the islets, this implies the islets have different selective pressures. In order to confirm more public use clonotypes (i.e., clones shared among NOD mice) found in the islets of 12- to 14- versus 8- to 10-wk-old animals contributed to the decrease in diversity over time, the sequence datasets were analyzed for the presence of shared clonotypes. Sequence sharing increased over time in the islets, but not in the PLN or spleen. This was the case regardless of whether unique clonotypes (Fig. 2A) or all T cell clones recovered (Fig. 2B) were considered. This suggests that the temporal decrease in pooled entropy seen in the islets was driven by increased sequence sharing, although the total number of unique clonotypes decreased and clonal dominance increased as well. The sequences of shared clonotypes and the samples from which they were recovered are reported in Supplemental Table II. Fourteen out of 18 shared clonotypes (78%) expressed TRBV 13-3, a markedly higher portion of clonotypes than at any age/tissue studied except for the 12- to 14-wk-old islet sample (Supplemental Fig. 1). Further, 8 out of 10 clonotypes recovered from more than two mice expressed TRBV 13-3. Of note, the most frequently shared clone (TRBV 13-3 TRJB 2-4 ASSDSQNTLY) differs from the pathogenic clone NY8.3 by only 1 aa, and 3 out of 18 shared clones used the CDR3 motif ASSDXNTLY.

Based on the result that pooled diversity decreased while sequence sharing increased in the islets but not other tissues, we reasoned that clonal selection was occurring selectively in the islet pool. The increased prevalence in all tissues and increasing dominance in the islets of the TRBV 13-3–bearing clones, along with the high frequency of

Table I. Summary of all TCRβ sequence data

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of Mice</th>
<th>No. of Sequences</th>
<th>No. of Unique Clonotypes</th>
<th>No. of Shared Clonotypes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islets 8–10 wk</td>
<td>6</td>
<td>97</td>
<td>34</td>
<td>7 (20)</td>
</tr>
<tr>
<td>Islets 12–14 wk</td>
<td>5</td>
<td>148</td>
<td>14</td>
<td>10 (71)</td>
</tr>
<tr>
<td>PLNs 8–10 wk</td>
<td>3</td>
<td>61</td>
<td>36</td>
<td>6 (17)</td>
</tr>
<tr>
<td>PLNs 12–14 wk</td>
<td>2</td>
<td>68</td>
<td>34</td>
<td>8 (24)</td>
</tr>
<tr>
<td>Spleen 8–10 wk</td>
<td>2</td>
<td>55</td>
<td>41</td>
<td>6 (15)</td>
</tr>
<tr>
<td>Spleen 12–14 wk</td>
<td>3</td>
<td>134</td>
<td>72</td>
<td>6 (8)</td>
</tr>
</tbody>
</table>

NRP-V7+–specific CD8+ T cells were single-cell sorted and their TCRβ genes amplified by RT-PCR and sequenced. Numbers of mice included, total sequences recovered, unique sequences recovered, and number of shared clones are listed for each group. A clonotype was defined as shared if it was recovered from more than one mouse. One mouse contributed more than one sample to this analysis (islets and PLNs taken from one 10-wk-old animal).

Otherwise, all mice contributed T cells derived from only one tissue.
using the estimate Entropy program, and reported with 95% confidence intervals represented by error bars. Each data point represents cells derived from a single mouse. In C, distribution.

FIGURE 1. Diversity of NRP-V7⁺CD8⁺ T cells in NOD mice decreases over time in the pancreatic islets, but not in the PLNs or spleen. NRP-V7⁻specific CD8⁺ T cells were single-cell sorted and their TCRβ genes amplified by RT-PCR and sequenced. A, Vβ gene-usage distribution. B, Jβ gene-usage distribution. C, Entropy of pooled samples. Each data point represents the entropy of samples pooled by age of mice and tissue of origin. D, Entropy of individual samples. Each data point represents cells derived from a single mouse. In C and D, Shannon entropy is used as an index of diversity, calculated using the estimate Entropy program, and reported with 95% confidence intervals represented by error bars.

TRBV 13-3 expression in the public clones, imply that the TRBV 13-3-bearing clones are selected. The presence of this defined public subset of dominant clones further suggests TCRβ repertoire status may be important in progression of islet autoimmunity.

Together, these data show that TCR diversity decreases over time and is consistent with either strong founder effects or selection in the islets, rather than equilibrium with the peripheral Ag-specific pool.

Kβ-NRP-V7-SAP tetramers deplete epitope-specific T cells in vitro and in vivo

The sequence analysis we presented suggests that the populations are relatively homogenous and, combined with data from Armani (34), suggests that elimination of high-affinity clones might be effective in preventing progression of diabetes. Further, the high prevalence of NRP-V7⁺ in the islets (22, 35) suggests that they are critical in the destruction of β cells and so would be good targets for removal.

Our previous work using toxin-coupled tetramers showed that in model systems, lymphocytic choriomeningitis virus gp33-specific P14 transgenic T cells were effectively deleted both in vitro and following adoptive transfer in vivo without significant toxicity to the mice (17). We hypothesized that treating NOD mice with Kβ-NRP-V7-SAP tetramers would alter the TCR repertoire of this diabetogenic population and potentially delay the onset of T1D in treated mice. We tested the ability of similar toxin-coupled tetramers assembled with NRP-V7 to remove 8.3 TCR transgenic CD8⁺ T cells. We cultured CD8⁺ T cells from the NOD 8.3 TCR transgenic mouse (which bears transgenic CD8⁺ T cells that react to H2Kβ-NRP-V7) and the NOD CL4 TCR transgenic mouse (which bears transgenic CD8⁺ T cells that react to the influenza peptide HA presented by H2Kβ) with cognate and noncognate toxic tetramers. Binding and cytotoxicity were specific for cognate tetramer relative to noncognate tetramer and free SAP (Fig. 3). Kβ-NRP-V7-SAP selectively depleted the 8.3 cells with very little toxicity to a nontargeted population (Fig. 4A).

FIGURE 2. Sequence sharing among NOD mice increases in the islets over time, but not in the PLNs or spleen. A sequence was defined as shared if it was recovered from more than one mouse, and the proportion of shared sequences in each group is shown. A, Sharing of unique clonotypes. The proportion of shared clonotypes in the 12–14 wk islet group and islet group and is consistent with either strong founder effects or selection in the islets, rather than equilibrium with the peripheral Ag-specific pool.

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FIGURE 2. Sequence sharing among NOD mice increases in the islets over time, but not in the PLNs or spleen. A sequence was defined as shared if it was recovered from more than one mouse, and the proportion of shared sequences in each group is shown. A, Sharing of unique clonotypes. The frequency of shared clonotypes in the 12–14 wk islet group was greater than in the 8–10 wk islet group. n = 6 mice in the 8–10 wk islet group, and n = 5 mice in the 12–14 wk islet group. *p = 0.026, one-tailed Mann-Whitney U test. B, Sharing of all T cell clones recovered. Again, the frequency of shared sequences was greater in the 12–14 wk islet group. n = 6 mice in the 8–10 wk islet group and n = 5 mice in the 12–14 wk islet group. *p = 0.041, one-tailed Mann-Whitney U test.
We then evaluated the ability of Kd-NRP-V7-SAP to selectively eliminate naive NOD.8.3 T cells in vivo (Fig. 4B). Purified 8.3 CD8+ T cells mixed with CD8-depleted NOD splenocyte helpers were transferred into NOD scid recipients, which in turn were treated with native IGRP206–214 peptide. Five days later, NOD scid mice received a single injection of PBS, NRP-V7, Kd-NRP-V7-SAP, or Kd-HA-SAP tetramers. After 7 d, 8.3 CD8+ T cells had modestly expanded in PBS-treated mice; as expected, this expansion was greatly enhanced by treatment with Kd-NRP-V7-SAP tetramers (upper panel). Mice were then injected i.v. with either Kd-NRP-V7 or Kd-NRP-V7-SAP tetramers. Three days posttreatment, splenocytes were stained with Kd-HA-PE, Kd-NRP-V7-Alexa Fluor647, 7-AAD, and an anti-CD8 mAb and analyzed by flow cytometry, showing the loss of NOD 8.3, but not NOD CL4 control, T cells following treatment with the Kd-NRP-V7-SAP tetramer (lower panel).

Kd-NRP-V7-SAP treatment decreases both the absolute number and frequency of NRP-V7+ T cells in the islet-infiltrating CD8+ T cell pool

We then assessed our ability to remove a heterogeneous pool of NRP-V7+ CD8+ T cell clonotypes from NOD mice as a preamble to testing the efficacy of direct killing of diabetogenic T cells to block progression to T1D. To test this, 13 NOD female mice were treated with Kd-NRP-V7-SAP or PBS. Islet-infiltrating NRP-V7+CD8+ T cells were isolated from treated and untreated mice at 3 wk posttreatment (11 wk of age) and analyzed by flow cytometry. Both the absolute number per islet and proportion of NRP-V7+ CD8+ T cells decreased with Kd-NRP-V7-SAP treatment (Fig. 4C, 4D). Fifty percent of the treated mice showed near complete depletion of NRP-V7+ CD8+ T cells, whereas the other half had substantial tetramer-positive T cells remaining, although nearly all were present at a lower frequency than the PBS control (p < 0.02, Fisher exact test).

NRP-V7+CD8+ T cells that express TRBV13-3 concentrate in the islets and are selectively depleted by Kd-NRP-V7-SAP treatment

It is possible that the depletion we observed was either stochastic or selected. If stochastic, we would expect the IGRP tetramer+ T cells would be drawn from the same distribution of TCRβ genes pre- and posttreatment. If the cells were selected by strength of tetramer binding, we expect to see a selective depletion of some classes. TRBV 13–bearing clones were present at a high frequency in all tissues at all times and increased in dominance and sharing in the islets in unmanipulated NOD mice. In order to determine the effect of Kd-NRP-V7-SAP treatment on TRBV 13–frequency in the NRP-V7+CD8+ T cell pool, TCRβ sequences were analyzed for frequency of TRBV 13-3 expression (Fig. 5). As indicated in Fig. 1,
Toxin-coupled tetramers selectively deplete epitope-specific T cells in vitro and in vivo. A, NOD 8.3 or CL4 splenocytes were cultured 3 d in RPMI 1640 containing 10% FCS with cognate or irrelevant toxic tetramer or without toxic tetramer, then stained with anti-CD8 and 7-AAD to measure viability. NRP-SAP refers to NRP-V7-SAP. Percent of tetramer-positive CD8+ T cells is shown. B, Kd-NRP-V7-SAP tetramer eliminates activated diabetogenic CD8+ T cells in vivo. Purified NOD-8.3 T cells (Thy1.2+) and CD8-depleted NOD splenocytes (Thy1.1+) were cotransferred into NOD scid hosts, followed by a priming injection of IGRP peptide. Five days later, PBLs were collected to verify equivalent transfer. NOD scid mice were then injected i.v. with the indicated tetramer (22 pmol) or PBS. Seven days posttreatment, PBLs were analyzed by flow cytometry, showing loss of NOD-8.3 T cells in mice that received the Kd-NRP-V7-SAP tetramer. Results are expressed as the mean percentage change versus treatment day 0. C, NRP-V7+CD8+ T cells are reduced in NOD mice treated with Kd-NRP-V7-SAP. CD8+ cells were isolated from the islets of Kd-NRP-V7-SAP-treated and control mice and analyzed by flow cytometry. One mouse from each group had less than two CD8+ T cells per islet (<5% of the average number) and only one tetramer-positive T cell in the sample. These NOD mice were considered to have not developed insulitis and were excluded from this analysis. Total number of tetramer-positive CD8+ T cells per islet is shown. p = 0.054, one-tailed Mann-Whitney U test. D, Proportion of CD8+ T cells that were tetramer-positive is shown. p = 0.036, one-tailed Mann-Whitney U test.

TRBV 13-3 expression was more prevalent in the NRP-V7+CD8+ T cell clones derived from the islets than from the PLN or spleen. At 11 wk in the PBS-injected NOD mice and 12–14 wk in untreated NOD mice, almost all of the tetramer-positive clones expressed TRBV 13-3. In the 8- to 10-wk-old islet pool, four out of six NOD mice showed the same exquisite dominance of TRBV 13-3. In NOD mice receiving Kd-NRP-V7-SAP, five mice showed complete absence of TRBV 13-3 expressing clones, one mouse had 1 out of 52 (1.9%) clones express TRBV 13-3, and another had 21 out of 39 (53.8%) express TRBV 13-3. In the islets of all but one treated mouse, the percentage of NRP-V7+specific CD8+ T cells that used TRBV 13-3 was less than in the spleen at either time point. These results demonstrate that TRBV 13-3–bearing NRP-V7+ CD8+ T cells are largely found in the islets and are depleted by Kd-NRP-V7-SAP treatment. Thus, the depletion by tetramer is not random, but selective.

Toxin-coupled tetramers produced long-term depletion of tetramer-specific CD8+ T cells in the islets

A critical question for the use of depleting agents is how long the effects of treatment persist. Treatment with depleting Abs, such as anti-CD3, is transient in humans (38) and nearly absent in NOD mice (39). Although the mechanism of action of anti-CD3 therapy is not clear, it is unlikely to function by lymphocyte depletion alone (15). Treatment with toxic tetramer, in contrast, does seem likely to function by removal of reactive cells.

To test whether altering the repertoire of pathogenic NRP-V7+ CD8+ T cells would affect T1D, 10 mice per group were injected with toxic tetramer or PBS and monitored for development of diabetes up to 54 wk of age. Islet-infiltrating NRP-V7+ CD8+ T cells were isolated upon development of overt diabetes (two blood sugar readings over 250 mg/dl) or at 54 wk of age. NOD mice treated with Kd-NRP-V7-SAP had significantly lower numbers of islet-infiltrating NRP-V7+ CD8+ T cells as measured by either the number of NRP-V7+ CD8+ T cells per islet (p = 0.002) or the percentage of NRP-V7+ cells in the CD8+ T cell population (p = 0.021) even up to 44 wk after the last treatment with Kd-NRP-V7-SAP (Fig. 6). The reduction in number of NRP-V7+ CD8+ T cells was not due to a reduction in overall numbers of CD8+ T cells at the time of measurement, because both Kd-NRP-V7-SAP– and PBS-treated mice had similar numbers of CD8+ T cells per islet (data not shown). In addition, the mean fluorescence intensity of the NRP-V7+ CD8+ T cells left in the islets

FIGURE 4. Toxic tetramers selectively deplete epitope-specific T cells

in vitro and in vivo. A, NOD 8.3 or CL4 splenocytes were cultured 3 d in RPMI 1640 containing 10% FCS with cognate or irrelevant toxic tetramer or without toxic tetramer, then stained with anti-CD8 and 7-AAD to measure viability. NRP-SAP refers to NRP-V7-SAP. Percent of tetramer-positive CD8+ T cells is shown. B, Kd-NRP-V7-SAP tetramer eliminates activated diabetogenic CD8+ T cells in vivo. Purified NOD-8.3 T cells (Thy1.2+) and CD8-depleted NOD splenocytes (Thy1.1+) were cotransferred into NOD scid hosts, followed by a priming injection of IGRP peptide. Five days later, PBLs were collected to verify equivalent transfer. NOD scid mice were then injected i.v. with the indicated tetramer (22 pmol) or PBS. Seven days posttreatment, PBLs were analyzed by flow cytometry, showing loss of NOD-8.3 T cells in mice that received the Kd-NRP-V7-SAP tetramer. Results are expressed as the mean percentage change versus treatment day 0. C, NRP-V7+CD8+ T cells are reduced in NOD mice treated with Kd-NRP-V7-SAP. CD8+ cells were isolated from the islets of Kd-NRP-V7-SAP-treated and control mice and analyzed by flow cytometry. One mouse from each group had less than two CD8+ T cells per islet (<5% of the average number) and only one tetramer-positive T cell in the sample. These NOD mice were considered to have not developed insulitis and were excluded from this analysis. Total number of tetramer-positive CD8+ T cells per islet is shown. p = 0.054, one-tailed Mann-Whitney U test. D, Proportion of CD8+ T cells that were tetramer-positive is shown. p = 0.036, one-tailed Mann-Whitney U test.

FIGURE 5. NRP-V7+CD8+ T cells that express TRBV 13-3 accumulate in the islets and are deleted by Kd-NRP-V7-SAP. T cells were isolated from the islets of NOD mice 3 wk post initial treatment with Kd-NRP-V7-SAP. T cells were isolated from the islets of NOD mice 3 wk after the posttreatment, PBLs were analyzed by flow cytometry, showing loss of NOD-8.3 T cells in mice that received the Kd-NRP-V7-SAP tetramer. Results are expressed as the mean percentage change versus treatment day 0. C, NRP-V7+CD8+ T cells are reduced in NOD mice treated with Kd-NRP-V7-SAP. CD8+ cells were isolated from the islets of Kd-NRP-V7-SAP-treated and control mice and analyzed by flow cytometry. One mouse from each group had less than two CD8+ T cells per islet (<5% of the average number) and only one tetramer-positive T cell in the sample. These NOD mice were considered to have not developed insulitis and were excluded from this analysis. Total number of tetramer-positive CD8+ T cells per islet is shown. p = 0.054, one-tailed Mann-Whitney U test. D, Proportion of CD8+ T cells that were tetramer-positive is shown. p = 0.036, one-tailed Mann-Whitney U test.

FIGURE 6. Kd-NRP-V7-SAP caused long-term depletion of tetramer-specific CD8+ T cells in the islets. Female NOD mice were given three i.v. injections of Kd-NRP-V7-SAP or PBS over 2 wk beginning at 8 wk of age. At onset of diabetes or 54 wk, NRP+ CD8+ CD3+ T cells were isolated from the islets and analyzed by flow cytometry. Absolute number of tetramer-positive CD8+ T cells per islet (p = 0.02, Mann-Whitney U test) (A) and proportion of CD8+ T cells that were tetramer positive (p = 0.002, Mann-Whitney U test) (B) are shown. PBS treatment group includes female NOD mice from two different toxic tetramer trials.

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of the K<sup>d</sup>-NRP-V7-SAP–treated NOD mice was also lower, 151 ± 103 versus 372 ± 242 for the PBS-treated NOD mice.

**Treatment with toxin-coupled tetramers can specifically delay the onset of T1D**

Notably, the onset of overt diabetes was significantly delayed in NOD mice treated with K<sup>d</sup>-NRP-V7-SAP versus the control group (24.5 and 16.5 wk of age, respectively; p = 0.04) (Fig. 7A). Thus, treatment with toxic tetramer resulted in the long-term depletion of NRP-V7<sup>d</sup> CD<sup>8+</sup> T cells in the islets, as well as significantly delayed the onset of overt diabetes in vivo. This result is consistent with the observation that NRP-V7<sup>d</sup> CD<sup>8+</sup> T cells are able to induce T1D and are important effector cells in disease progression.

Treatment with NRP-V7-SAP did not completely block progression to T1D, and we wondered whether there were other IGRP<sup>e</sup> CD<sup>8+</sup> T cells that were not eliminated with NRP-V7-SAP tetramers. NRP-V7 was originally identified as a mimotope that would activate 8.3 T cells both in vitro and in vivo (40). More recently, IGRP has been described as the natural ligand of 8.3 cells. We therefore examined the similarity of staining patterns of K<sup>d</sup>-IGRP and -NRP-V7 tetramers on NOD CD<sup>8+</sup> cells isolated from the islets (Fig. 7B). We found that although all of the K<sup>d</sup>-NRP-V7 staining CD<sup>8+</sup> T cells also bound K<sup>d</sup>-IGRP, a substantial population of K<sup>d</sup>-IGRP<sup>e</sup> CD<sup>8+</sup> T cells was K<sup>d</sup>-NRP-V7 negative. This demonstrates that there are potentially diabetogenic CD<sup>8+</sup> T cells specific to IGRP that will not be removed by NRP-V7-SAP. Alternatively, one could reason that it is better to deplete this high-affinity diabetogenic IGRP<sup>e</sup> population and leave the low-affinity IGRP cells expand to fill the space previously occupied by the high-affinity T cells because deletion of all of the IGRP<sup>e</sup> CD<sup>8+</sup> T cells would allow other high-affinity diabetogenic cell of other epitopes to fill in the gap (10, 12).

We then tested the ability of K<sup>d</sup>-IGRP tetramers to delay onset of T1D, because we thought that they might be more effective than K<sup>d</sup>-NRP-V7-SAP. Treatment with K<sup>d</sup>-IGRP-SAP was found to be more effective than K<sup>d</sup>-NRP-V7-SAP (Fig. 7C). Indeed, 50% of the IGRP-treated mice did not develop diabetes after 30 wk compared with 100% of the controls. Mean onset of diabetes was 32 wk for K<sup>d</sup>-IGRP-SAP compared with 24.5 wk for K<sup>d</sup>-NRP-V7-SAP treated animals and 16.5 wk with PBS. Thus, although treatment with K<sup>d</sup>-IGRP-SAP was more efficacious than K<sup>d</sup>-NRP-V7 when begun at 8 wk, it did not completely prevent the progression to T1D.

Several other class I epitopes have been described in NOD mice (41). These include peptides from DMK and InsB chain. InsB-G9V was used to increase the MHC I stability with minimal effect on TCR-MHC I interaction (42). Because these epitopes also likely contribute to the pathogenesis of diabetes, we chose to examine the impact of deleting these CD<sup>8+</sup> T cells. We prepared SAP-coupled tetramers loaded with each of these peptides and compared their ability to delay the development of T1D in NOD mice. We compared these treatments with tetramers complexed with NRP-V7 and IGRP peptides.

Neither K<sup>d</sup>-InsB nor D<sup>a</sup>-DMK tetramers were able to delay the onset of T1D. This was surprising given that NOD mice genetically tolerant to InsB do not progress to T1D (43). The age of treatment was not a major factor because a similar result was obtained when NOD mice were treated at 4 wk of age with the K<sup>d</sup>-InsB-SAP (data not shown). This result also demonstrates the specificity of deletion. It shows that toxin-coupled tetramer alone, irrespective of specificity is not sufficient to delay the onset of T1D.

**FIGURE 7.** K<sup>d</sup>-NRP-V7-SAP and K<sup>d</sup>-IGRP-SAP delayed onset of diabetes but not K<sup>d</sup>-InsB-SAP or D<sup>a</sup>-DMK-SAP. A, C, D. Ten NOD mice per group were given three i.v. injections of the indicated toxic tetramer over 2 wk beginning at 8 wk of age. A Kaplan-Meier curve is shown above with diabetes incidence as the dependent variable. A, K<sup>d</sup>-NRP-V7-SAP treatment significantly reduced diabetes incidence (log-rank test, p = 0.04). Mean time to onset: PBS = 16.5 wk and K<sup>d</sup>-NRP-SAP = 24.5 wk. B, NRP-V7-specific CD<sup>8+</sup> T cells are a subset of the IGRP-specific CD<sup>8+</sup> T cells. Lymphocytes were isolated from the islets of a NOD mouse and stained with anti-CD8 PerCP, anti-CD3 Alexa Fluor 488, and anti-CD19 Pacific Blue Abs in addition to two tetramers, K<sup>d</sup>-NRP-V7-SA-AF647 and K<sup>d</sup>-IGRP-SA-PE. Cells were analyzed by flow cytometry. The CD<sup>8+</sup>CD<sup>3+</sup> T cells are shown in the dot plot. C, K<sup>d</sup>-IGRP-SAP but not K<sup>d</sup>-DMK-SAP significantly delayed onset of diabetes (log-rank test, p = 0.02). Mean time to onset: PBS = 18 wk, K<sup>d</sup>-IGRP-SAP = 32 wk, and D<sup>a</sup>-DMK-SAP = 16 wk. D, K<sup>d</sup>-InsB-SAP did not reduce diabetes incidence. Mean time to onset: PBS = 14 wk, K<sup>d</sup>-InsB-SAP = 16.5 wk. Blood alanine aminotransferase levels (indicative of liver damage) measured after each dose of toxic tetramer did not rise above 350, consistent with mild transient effects.
Discussion

In this paper, we show that toxin-coupled tetramers significantly alter the T cell repertoire of NOD mice. The TCRβ repertoire of Kd-NRP-V7+CD8+ T cells in NOD mice is known to be highly restricted at 20 wk of age and to preferentially use TRBV 13-3 and TRBJ 2-4 or 2-7 (21). Our study represents the first dissection of the TCRβ repertoire in younger NOD mice. In agreement with earlier observations, we found dominance of TRBV 13-3 and TRBJ 2-4/2-7 to be true for 8- to 14-wk-old NOD mice as well. We show in this study for the first time that diversity of the Kd-NRP-V7+CD8+ T cell pool decreases in the islets over time but not in the PLN or spleen. Although this decrease in diversity is characterized by a declining number of unique clonotypes and an increase in frequency of the dominant clones, the primary driver is an increase in sequence sharing among clones present in the islets at 12–14 wk of age. This suggests a progressive selective pressure in the islets that is conserved among NOD mice. It also implies that selection of NRP-V7–specific CD8+ T cell clones over time is not uniform in all tissue compartments. Rather, selection in the islets is more robust for this T cell pool.

We further show that a dominant subset of NRP-V7+CD8+ T cell clones, those expressing TRBV 13-3, is enriched and increasingly public in the islets over time. Shared clonotypes were more likely than nonshared clonotypes to use TRBV 13-3, which suggests that the predominance of these clones in the shared pool is not primarily a consequence of their increased overall frequency in the total pool. Further, the TRBV 13-3+ NRP-V7+CD8+ T cell population was selectively depleted from the NRP-V7+CD8+ T cell pool in the islets by treatment with Kd-NRP-V7 tetramer conjugated to the ribosomal toxic SAP. It is intriguing that Kd-NRP-V7-SAP–mediated depletion of epitope-specific CD8+ T cells was incomplete 2 wk posttreatment, whereas the depletion of Kd-NRP-V7+CD8+ T cells measured up to 44 wk later was more complete (Figs. 4C, 6A). Prior work by our group has shown that CD8+ T cells expressing TCRs exhibiting decreased binding avidity for toxic tetramer are relatively resistant to toxic tetramer-mediated depletion (17). Thus, we demonstrated a potential for a therapeutic benefit where deletion of a single specificity can alter the outcome of disease progression.

Peptide-MHC tetramers assembled with the SAP are promising agents for direct epitope-specific depletion of T cells. This study presents the first evidence of a beneficial effect of toxic tetramer administration. Toxic tetramer assembled with IGRP was nearly as effective at decreasing diabetes incidence as low-affinity peptides in earlier studies (12, 20). In addition, peptide treatment was only effective when initiated at 4 wk of age, and disease progression was unaltered when peptide treatment was initiated in 10-wk-old NOD female mice. In this study, however, a marked effect on T1D was detected when 8-wk-old NOD female mice were treated with toxic tetramer, when islet infiltrates are well established. Further, in earlier experiments, continued peptide injections were necessary, whereas in our experiments, NOD mice were treated only three times over a 10-d period. We believe this technology represents a new strategy for in vivo immunomodulation in contexts where repeated administration of peptide may be undesirable or where a stable platform for direct rather than APC-mediated depletion is needed.

It is not surprising that toxic tetramer treatment eliminated NRP-V7–specific CD8+ T cells from the islets and slowed the progression of diabetes, but most NOD mice still progressed to diabetes without NRP-V7+CD8+ T cells. This adds more evidence that there are multiple driving epitopes in β cell autoimmunity. Others have depleted or tolerated NRP-V7-reactive CD8+ T cells in NOD mice without causing delay of T1D (10, 12). This is consistent with human data that show the preproinsulin epitope in many but not all A2+ patients arises before other detectable epitopes (44).

Interestingly, tolerization to proinsulin 2 prevents both T1D and development of IGRP-reactive CD8+ T cells (10) and mice transgenic for an insulin gene that produces an altered InsB chain also do not develop T1D (11). This suggests that the response to proinsulin and insulin epitopes is crucial as direct effectors are a critical check point in epitope spreading and precede IGRP-directed autoimmunity. We were surprised to find that the progression of diabetes was unaffected when 4-wk-old NOD mice were treated with Kd-InsB-SAP (data not shown). There are several explanations for our results. We might not have sufficiently depleted the InsB-specific CD8+ T cells, allowing them to play a critical role in initiating the autoimmune response. Alternatively, depletion at 4 wk may still be too late; if progression to T1D would already have passed the InsB checkpoint, there would be no effect. On the other hand, proinsulin or preproinsulin CD8+ T cell epitopes may be better candidates for intervention. Finally, our study does not address the role of the CD4 response to insulin, which is known to be diabetogenic (43). The inclusion of toxin-coupled class II tetramers with specificity for diabetogenic CD4+ T cells might well enhance the efficacy of treatment.

The magnitude of protection in our study (30%) was similar to that offered by low-affinity NRP-I4 peptide (12). One possible implication of this is that Kd-NRP-V7-SAP tetramer treatment depletes the same population of high-avidity clones that are depleted by peptide. Mean fluorescence intensity values for islet-infiltrating tetramer-positive T cells were significantly lower in the treated mice. This supports the idea that nondepleted clonotypes, presumably of lower avidity, expand and in turn affect disease progression. In light of this, it will be interesting to examine depletion efficacy, repertoire changes, and decrease in diabetes incidence posttreatment with toxic tetramers assembled with IGRP and other altered peptide ligands of this peptide, as NRP-V7 is not the optimal choice for depletion of diabetogenic T cells. The treatment with IGRP deletes both high- and moderate-affinity clones, resulting in better protection. Our data show that IGRP-assembled toxin tetramers are superior to NRP-V7. Somewhat surprisingly, we saw no ability of tetramers assembled with either InsB peptide or with DMK peptide to prolong the disease-free interval. This might not be surprising because the frequency of both Kd-InsB– and Dd-DMK–specific CD8+ T cells is low, even in untreated NOD mice. This result also suggests that the best targets are clonotypes found at a high frequency, which in turn likely reflects a key role in driving β cell autoimmunity. It further argues that if epitope spreading is a critical event in pathogenesis, both DMK and InsB are dependent on IGRP, although data from others would argue the reverse (11). Alternatively, the responses to these three epitopes might be independent of each other and required to interact in a complex way to produce diabetes. Regardless, peptide-MHC tetramers assembled with SAP are promising agents for direct epitope-specific depletion of T cells.

Toxin-coupled tetramers represent a new strategy for in vivo immunomodulation. We show epitope-specific depletion of CD8+ T cells using SAP-coupled MHC I tetramers. This approach is advantageous over current approaches, such as tolerization with peptide or use of T cell-depleting Abs, because peptide treatments can cause proliferation of T cells and deletion of a broad swath of T cells, leaving the risk of broad immunosuppression. In addition, this study offers a first look into the clonotype dynamics in young, prediabetic NOD mice and examines the increasingly public islet-infiltrating clonotypes with IGRP specificity. Finally, we demonstrate that toxic tetramer depletion of IGRP-reactive CD8+ T cells is long term and beneficial in delaying T1D.
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Disclosures

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References