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Toxin coupled MHC class I tetramers can specifically ablate autoreactive CD8+ T cells and delay diabetes in NOD mice[1]

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Abstract

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 residual autoreactive T cells may be activated and exacerbate disease.

In this report we use a novel approach - toxin coupled MHC class I tetramers. Used for some time to identify antigen specific cells, here we use that same property to delete the antigen specific cells. We show saporin coupled tetramers can delete IGRP reactive T cells *in vitro* and *in vivo*. Sequence analysis of TCRβ 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 8 weeks of age of $IGRP^+$ $CD8^+$ T cells, but not DMK or InsB reactive cells, significantly delays diabetes in NOD mice.

Introduction

Type 1 diabetes (T1D)[2] 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,

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Supplementary Figure 1. Percentage of TRBV 13-3 $NRP\text{-}V7^+CD8^+$ cell T clonotypes. TRBV 13-3 usage is highest for the shared clonotypes and the 12–14 week islet pools. For each tissue/age pool and for the pool of shared clonotypes, the percentage of clonotypes that expressed TRBV 13-3 is shown.

there is a progressive loss of insulin producing β cells in the islets of Langerhans. Genome-wide association studies have shown that polymorphism 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 4 weeks of age and the majority of females develop frank diabetes by 20 weeks 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).

While 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 IGRP reactive NY8.3 T cell receptor (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 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 antibody 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 tetramers has revolutionized the study of $CD8^+$ T cells (16). The ability to bind to antigen specific T cells could be used not only to identify antigen specific T cells, but also to carry toxins and radionuclide to the cells for either imaging or deletion (17–19). In our lab we have developed saporin coupled MHC class I tetramers to kill antigen 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. Here we show that deletion of IGRP-specific $CD8^+$ T cells changes the islet infiltrating T cell repertoire and prolongs the disease free interval of NOD mice.

Materials and Methods

Mice

NOD/ShiLtJ mice were purchased from Jackson Laboratories and housed in a specific pathogen–free laboratory animal facility that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). NOD mice were used at 8–14 weeks of age. In all experiments, NOD mice were defined as diabetic if two consecutive weekly blood glucose measurements were greater than 250 mg/dl. Diabetes onset and incidence in unmanipulated mice is identical to that reported by Jackson Laboratories (data not shown).

NOD.Cg-Tg($Tcra^{Cl4}$,$Tcrb^{Cl4}$)1Shrm/Tisch (NOD CL4) mice, which bear transgenic $CD8^+$ T cells that react to the influenza peptide HA presented by $H2K^d$, were bred in-house (23). NOD.Cg-Tg($TcraTcrb^{NY8.3}$)1Pesa/DvsJ (NOD 8.3) mice, which bear transgenic $CD8^+$ T cells that react to $H2K^d$-IGRP, and NOD.Cg-$Prkdc^{scid}$ (NOD scid) mice were purchased from Jackson Laboratories.

[2]Abbreviations: Type I Diabetes, TID; pancreatic lymph node, PLN; islet-specific glucose-6-phosphatase catalytic subunit-related protein, IGRP; Insulin B, InsB; dystophia myotonica kinase, DMK; streptavidin, SA; saporin, SAP

Isolation of Lymphocytes from Islets

Pancreata were perfused with a 2 mg/ml solution of collagenase P (Sigma), dissected and incubated at 37°C for 20 min. Islets were purified using a Ficoll PM 400 (Sigma) gradient, handpicked and counted then cultured overnight in RPMI 1640 containing 10% FBS and 4 ng/ml recombinant murine IL-2 (Invitrogen). 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 class I tetramers were prepared as previously described (24). Briefly, $H2K^d$ or $H2D^d$ and mouse β_2-microglobulin were produced in *E. coli* and refolded with peptide (from GenScript) *in vitro*. Refolded peptide-MHC monomer was purified by HPLC, biotinylated using Biotin Protein Ligase, and assembled into tetramers by conjugation with UltraAvidin-PE (Leinco). Peptides used were NRP-V7, KYNKANVFL; IGRP 201–214, VYLKTNVFL; InsB 15–23 (G9V), LYLVCGERV; or DMK 138–146, FQDENYLYL

Toxic Tetramer Preparation and Injection

NRP-V7-$H2K^d$ tetramers conjugated to saporin were prepared as previously described for gp33-$H2D^b$ tetramers (17). Eight week-old NOD female mice received 3 intravenous (i.v.) injections in the lateral tail vein, 6 d apart, beginning at 8 weeks of age. Injections contained 34 pmoles (4.36μg) of K^d-NRP-V7-SAP, K^d-IGRP-SAP, K^d-InsB-G9V–SAP or D^b-DMK-SAP in 200μl of PBS. Control NOD female mice received three injections of 200μl PBS at the same time as the toxic tetramer injections. TCR analysis of $CD8^+$ T cells in the spleen, pancreatic lymph nodes (PLN), and islets was carried out either at 21 d post-treatment or at the time of diabetes diagnosis or 54 weeks 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 ($2 \times 10^5 – 5 \times 10^6$ / 200 μl / mouse). T cells were primed by intraperitoneal (i.p.) injection of $IGRP_{206–214}$ peptide (100 μg) 2 h post-transfer. Tetramers were diluted in PBS and injected i.v. (22 pmol, (2.82μg) / 200 μl / mouse). To assay cell survival, peripheral blood was collected from the superficial temporal vein using a Goldenrod lancet (MEDIpoint, Inc., Mineola, NY). Lymphocytes were enriched over a Lympholyte-M gradient, stained with tetramers and antibodies for 1 h at 4°C, and analyzed on FACSCalibur flow cytometer (BD Biosciences, San Diego, CA).

Flow Cytometry and Single-cell Sorting

Lymphocytes from NOD mice were examined using flow cytometry. For sorting experiments, cells were stained with a cocktail of Alexa Fluor 488-anti-CD3 (Invitrogen), Pacific Blue-anti-CD4 (Invitrogen), APC-anti-CD8 (eBiosciences), PE-Cy7-anti-CD19 (BioLegends), and PE-ultraavidin(Leinco)-NRP-V7-K^d tetramer. For single-cell sorting, NRP-$V7^+CD3^+CD8^+$ $CD4^-CD19^-$ T cells were sorted by a MoFlo highspeed sorter (DakoCytomation) at 1 cell/well into a 96-well PCR plate (USA Scientific), each well containing 4 μl buffer of 0.5x PBS, 10 mM DTT, and 8 U RNaseOUT RNase inhibitor (Invitrogen Life Technologies). Plates were kept frozen at −20°C or −80°C. For tetramer-specific cell analyses on non-sorted samples, cells were stained with a cocktail of APC-Cy7-anti-CD3 (BD Biosciences, San Jose, CA), Pacific Orange-anti-CD4 (Invitrogen, Carlsbad, CA), PerCP-anti-CD8 (BD), Pacific Blue-anti-CD44 (Invitrogen), PE-Texas Red-anti-CD62 (Invitrogen), PE-Cy7-anti-CD19 (BioLegends, San

Diego, CA) and PE-ultra-avidin (Lenco, St. Louis, MO)-NRP-V7-K^d tetramer. Antibody concentrations used were determined by preliminary titrations. Cells were analyzed on a Dako (Beckman-Coulter) Cyan-ADP(Colorado Springs, CO). "Minus one" controls were performed in each experiment to ensure that the fluorescence measured originated from the correct stain. Staining controls used syngeneic spleen cells. Flow cytometry data was analyzed using Summit 4.3 (Dako).

Single-cell PCR and Sequencing

TCR usage was analyzed by a single-cell RT-PCR protocol (21,25). For TCR β-chain analysis, a panel of primers specific for all known TCR β-chain variable regions in combination with a β-chain constant region primer was used (26). RT-PCR amplicons were used as templates for a second round of PCR amplification using a panel of nested TCR β-chain-specific primers. PCR products were treated with Exonuclease I (NEB Biolabs) and shrimp alkaline phosphatase (Roche) and sequenced by the UNC Sequencing Core Facility. TCR sequence gene-usage identifications were performed using the SoDA software tool (27). Sequences were defined as identical if they shared the same Vβ and Jβ gene usage along with identical β-CDR3 regions.

Diversity Analysis

The Shannon entropy was chosen as the index of diversity because it is the only member of the family of valid diversity indices where the calculation derives equal weight from species richness (number of different clones recovered) and species frequency distribution (number of copies of each clone recovered); as such, the calculated diversity favors neither especially abundant nor especially rare species (as do the Simpson's index and simple species richness, respectively) (28). This index has been used widely in ecology and has been used to quantify the diversity of hepatitis C viral quasispecies and MHC class II regulatory gene segments (28–30). The Shannon entropy of a T cell population is determined by two parameters: 1) the number of different T cell clones 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 intra-population 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 p_i is the proportion of the pool represented by clonotype i, the Shannon entropy H is defined as:

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

In practice, the proportions p_i are not known, however, and must be estimated from finite samples. Simple substitution of these estimates into the definition of H gives rise to sampling bias. The bias is itself estimable when the total number of unique clonotypes S in the sampled population is known (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 unseen in the sample (Kepler, 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 Software, 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 post-tests 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 is diabetes incidence between treated and control mice. In all analyses, the significance level was 0.05.

T Cell Receptor Gene Nomenclature

Gene names are given according to the IMGT 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 pancreatic lymph nodes and spleen of 8–14 week old NOD mice

Previous work from our lab 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, since 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 weeks. 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 table S1. Vβ gene usage was highly restricted in the islets at 12–14 weeks 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 weeks 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 weeks of age, with TRJB 2–7 continuing to be highly represented at 12–14 weeks of age in all tissues, in contrast 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 weeks 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.

While is it 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 weeks was significantly less than at 8–10 weeks 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 (table S1). In no other tissue was a significant difference seen between the two ages. In contrast to the pooled data, the non-pooled islet entropies showed no significant difference between the 8–10 and 12–14 week old samples, with the exception of a single 8–10 week mouse that had an entropy value of 5.86 (Fig. 1d). This sample had 13 sequences recovered that were all unique clones (Fig. S1), and this entropy value approaches maximal entropy. When this mouse is excluded from the 8–10 week-old pool, the pooled entropy is still significantly different between the 8–10 and 12–14 week-old pools. This implies that the frequency of shared clones between individual samples in the pools increases between 8–10 and 12–14 weeks 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 isets 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 an increased number of public use clonotypes (i.e. clones shared among NOD mice) found in the islets of 12–14 versus 8–10 week-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 table S2. Fourteen out of eighteen shared clonotypes (78%) expressed TRBV 13-3, a markedly higher portion of clonotypes than at any age/tissue studied except for the 12–14 week-old islet sample (Fig. S1). Further, eight out of ten 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 one amino acid, and three out of eighteen shared clones used the CDR3 motif ASSDXXNTLY.

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 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 this data shows that TCR diversity decreases over time and is consistent with either strong founder effects or selection in the islets, rather than equilibrium with the periphery antigen specific pool.

K^d-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 LCMV 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 K^d-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 $H2K^d$-NRP-V7) and the NOD CL4 TCR transgenic mouse (which bears transgenic $CD8^+$ T cells that react to the influenza peptide HA presented by $H2K^d$) with cognate and non-cognate toxic tetramers. Binding and cytotoxicity were specific for cognate tetramer relative to non-cognate tetramer and free saporin (Fig. 3). K^d-NRP-V7-SAP selectively depleted the 8.3 cells with very little toxicity to a non-targeted population (Fig. 4a).

We then evaluated the ability of K^d-NRP-V7-SAP to selectively eliminate naïve 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 $IGRP_{206–214}$ peptide. Five days later, NOD scid mice received a single injection of PBS, NRP-V7, K^d-NRP-V7-SAP, or K^d-HA-SAP tetramers. After seven days, 8.3 $CD8^+$ T cells had modestly expanded in PBS-treated mice; as expected, this expansion was greatly enhanced by exposure to non-toxic cognate tetramer (36,37). In contrast, treatment with K^d-NRP-V7-SAP decreased 8.3 $CD8^+$ T cells in the blood by >75%, similar to the depletion noted in the spleens of lymphoreplete mice, showing that activated diabetogenic transgenic $CD8^+$ T cells are depleted by cytotoxic tetramer. Thus these experiments demonstrate diabetogenic T cells can be removed in vivo.

K^d-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 K^d-NRP-V7-SAP or PBS. Islet-infiltrating NRP-$V7^+CD8^+$ T cells were isolated from treated and untreated mice at 3 weeks post treatment (11 weeks of age) and analyzed by flow cytometry. Both the absolute number per islet and proportion of NRP-$V7^+$ $CD8^+$ T cells decreased with K^d-NRP-V7-SAP treatment (Fig. 4c–d). Fifty percent of the treated mice showed near complete depletion of NRP-$V7^+$ $CD8^+$ T cells, while the other half had substantial tetramer-positive T cells remaining, although nearly all were present at a lower frequency than the PBS control ($P<.02$ Fisher exact test).

NRP-V7$^+$CD8$^+$ T cells that express TRBV 13-3 concentrate in the islets and are selectively depleted by K^d-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 before and after treatment. If the cells were selected by strength of tetramer binding, we expect to see a selective depletion of some classes. TRBV 13-3 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 K^d-NRP-V7-SAP treatment on TRBV 13-3 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 Figure 1, TRBV 13-3 expression was more prevalent in the NRP-V7$^+$CD8$^+$ T cell clones derived from the islets than in those from the PLN or spleen. At 11 weeks in the PBS-injected NOD mice and 12–14 weeks in untreated NOD mice, almost all of the tetramer-positive clones expressed TRBV 13-3. In the 8–10 week-old islet pool, 4/6 NOD mice showed the same exquisite dominance of TRBV 13-3. In NOD mice receiving K^d-NRP-V7-SAP, 5 mice showed complete absence of TRBV 13-3 expressing clones, one mouse had 1/52 (1.9%) clones express TRBV 13-3, and another had 21/39 (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 K^d-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 antibodies 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 on the other hand 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 weeks of age. Islet infiltrating NRP-V7$^+$ CD8$^+$ T cells were isolated upon development of overt diabetes (2 blood sugar readings over 250 mg/dl) or at 54 weeks of age. NOD mice treated with K^d-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 percent of NRP-V7$^+$ cells in the CD8$^+$ T cell population ($p=0.021$) even up to 44 weeks after the last treatment with K^d-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, since both K^d-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 of the K^d-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^d-NRP-V7-SAP versus the control group (24.5 and 16.5 weeks of age respectively, $p=0.04$) (Fig. 7a). Thus treatment with toxic tetramer resulted in the long-term depletion of NRP-V7$^+$ CD8$^+$ 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$^+$ CD8$^+$ 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$^+$ CD8$^+$ 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^d-IGRP and -NRP-V7 tetramers on NOD CD8$^+$ cells isolated from the islets (Fig. 7b). We found that while all of the K^d-NRP-V7 staining CD8$^+$ T cells also bound K^d-IGRP, a substantial population of K^d-IGRP$^+$ CD8$^+$ T cells was K^d-NRP-V7 negative. This demonstrates that there are potentially diabetogenic CD8$^+$ 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$^+$ population and leave the low affinity IGRP cells expand to fill the space previously occupied by the high affinity T cells since depletion of all of the IGRP$^+$ CD8$^+$ 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^d-IGRP tetramers to delay onset of T1D, since we thought that they might be more effective that K^d-NRP-V7-SAP. Treatment with K^d-IGRP-SAP was found to be more effective than K^d-NRP-V7-SAP (Fig. 7c). Indeed 50% of the IGRP treated mice did not develop diabetes after 30 weeks compared to 100% of the controls. Mean onset of diabetes was 32 weeks for K^d-IGRP-SAP compared to 24.5 weeks for K^d-NRP-V7-SAP treated animals and 16.5 weeks with PBS. Thus although treatment with K^d-IGRP-SAP was more efficacious than K^d-NRP-V7 when begun at 8 weeks, 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 MHCI stability with minimal effect on TCR-MHCI interaction (42). Since these epitopes also likely contribute to the pathogenesis of diabetes, we chose to examine the impact of deleting these CD8$^+$ T cells. We prepared saporin 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^d-InsB nor D^b-DMK tetramers were able to delay the onset of T1D. This was surprising given that that NOD mice genetically tolerant to Ins-B do not progress to T1D (43). The age of treatment was not a major factor since a similar result was obtained when NOD mice were treated at 4 weeks of age with the K^d-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.

Discussion

In this paper we show that toxin coupled tetramers significantly alter the T cell repertoire of NOD mice. The TCRβ repertoire of K^d-NRP-V7$^+$ CD8$^+$ T cells in NOD mice is known to be highly restricted at 20 weeks of age and to preferentially use TRBV 13-3 and TRJB 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 8–14 week-old NOD mice as well. We show here for the first time that diversity of the K^d-NRP-V7$^+$CD8$^+$ T cell pool decreases in the islets over time but not in the PLN or spleen. Though 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 weeks of age. This suggests a progressive selective pressure in the islets which 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 non-shared 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 K^d-NRP-V7 tetramer conjugated to the ribosomal toxin saporin. It is intriguing that K^d-NRP-V7-SAP mediated depletion of epitope-specific CD8$^+$ T cells was incomplete two weeks post-treatment while the depletion of K^d-NRP-V7$^+$ CD8$^+$ T cells measured up to 44 weeks later was more complete (Figure 4c, Figure 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 saporin 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 weeks of age and disease progression was unaltered when peptide treatment was initiated in 10 week-old NOD female mice. Here, however, a marked effect on T1D was detected when 8 week-old NOD female mice were treated with toxic tetramer, when islet infiltrates are well established. Further, in earlier experiments continued peptide injections were necessary while in our experiments, NOD mice were treated only 3 times over a 10 day 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 tolerized NRP-V7 reactive CD8$^+$ T cells in NOD mice without causing delay of T1D (10,12). This is consistent with human data that shows 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 insulin B chain, also do not develop TID (11). This suggests that the response to proinsulin and insulin epitopes are crucial as direct effectors or 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 week old NOD mice with treated with K^d-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 weeks may still be too late; if progression to TID would already have passed the InsB check point, 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 K^d-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 non-depleted 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 after treatment with toxic tetramers assembled with IGRP and other altered peptide ligands of this peptide, as NRP-V7 is not be 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 since the frequency of both K^d-InsB and D^b-DMK specific $CD8^+$ T cells are 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 the saporin 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 saporin coupled MHC Class I tetramers. This approach is advantageous over current approaches such as tolerization with peptide or use of T cell-depleting antibodies since 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, pre-diabetic 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 TID.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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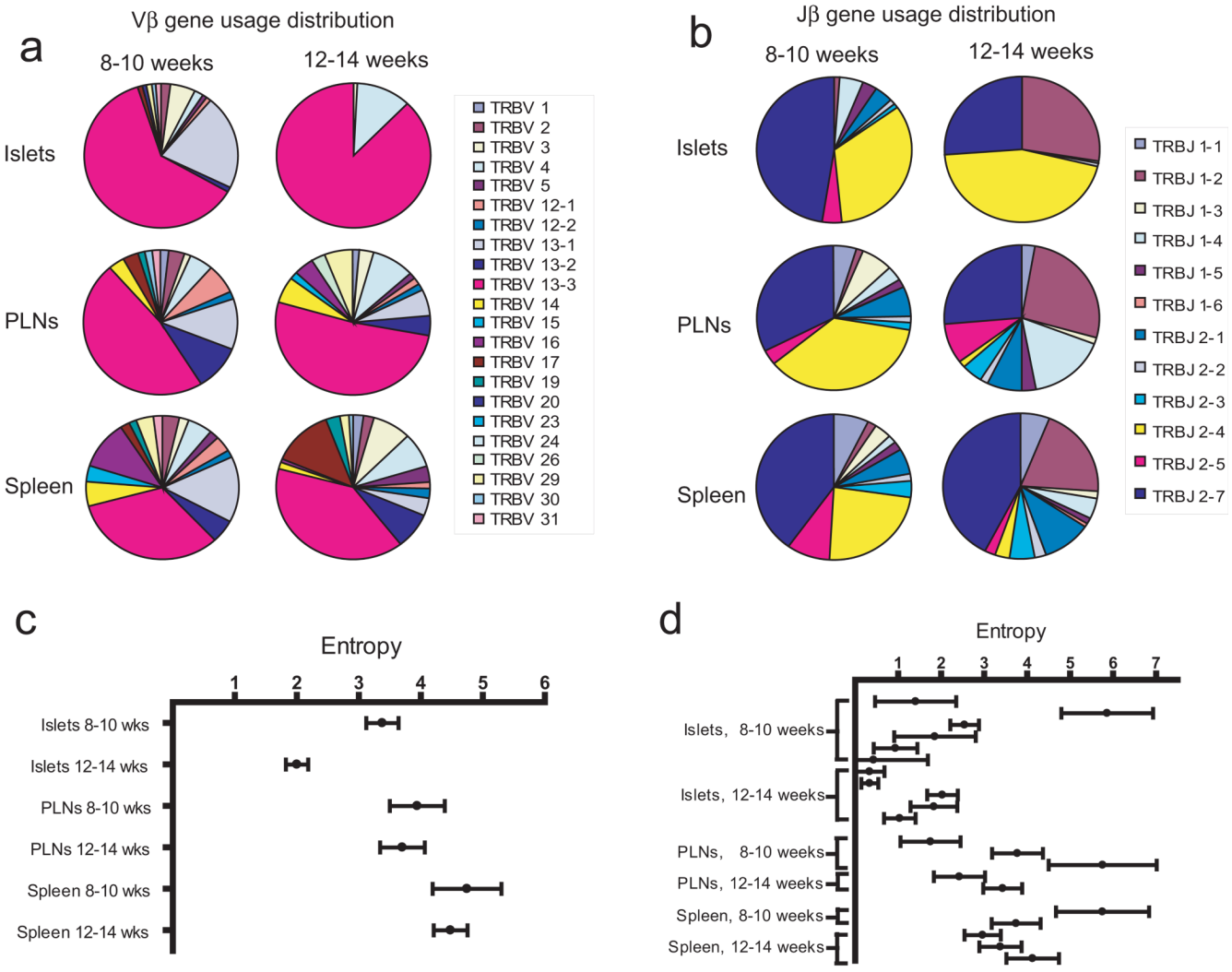

Figure 1.

Diversity of NRP-V7^{+}CD8^{+} T cells in NOD mice decreases over time in the pancreatic islets, but not in the pancreatic lymph nodes 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 estimateEntropy program, and reported with 95% confidence intervals represented by error bars.

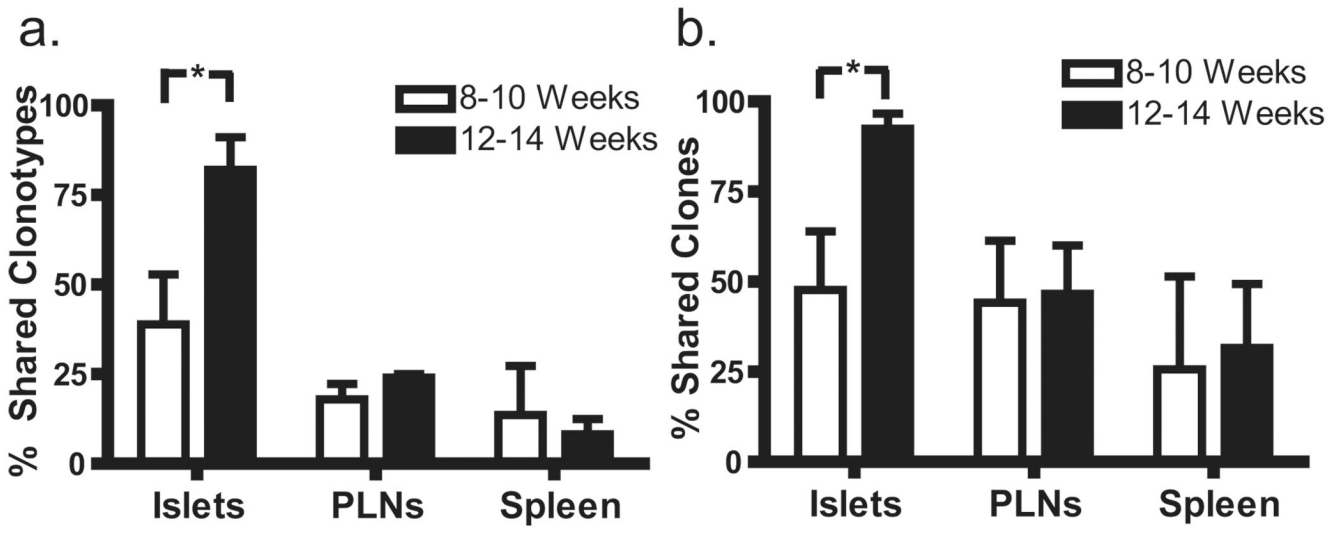

Figure 2.

Sequence sharing among NOD mice increases in the islets over time, but not in the PLN 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 week islet group was greater than in the 8–10 week islet group. n = 6 mice in the 8–10 week islet group and 5 mice in the 12–14 week islet group. * P = 0.026, One-tailed Mann-Whitney. (b) Sharing of all T cell clones recovered. Again, the frequency of shared sequences was greater in the 12–14 week islet group. n = 6 mice in the 8–10 week islet group and 5 mice in the 12–14 week islet group. * P = 0.041, One-tailed Mann-Whitney.

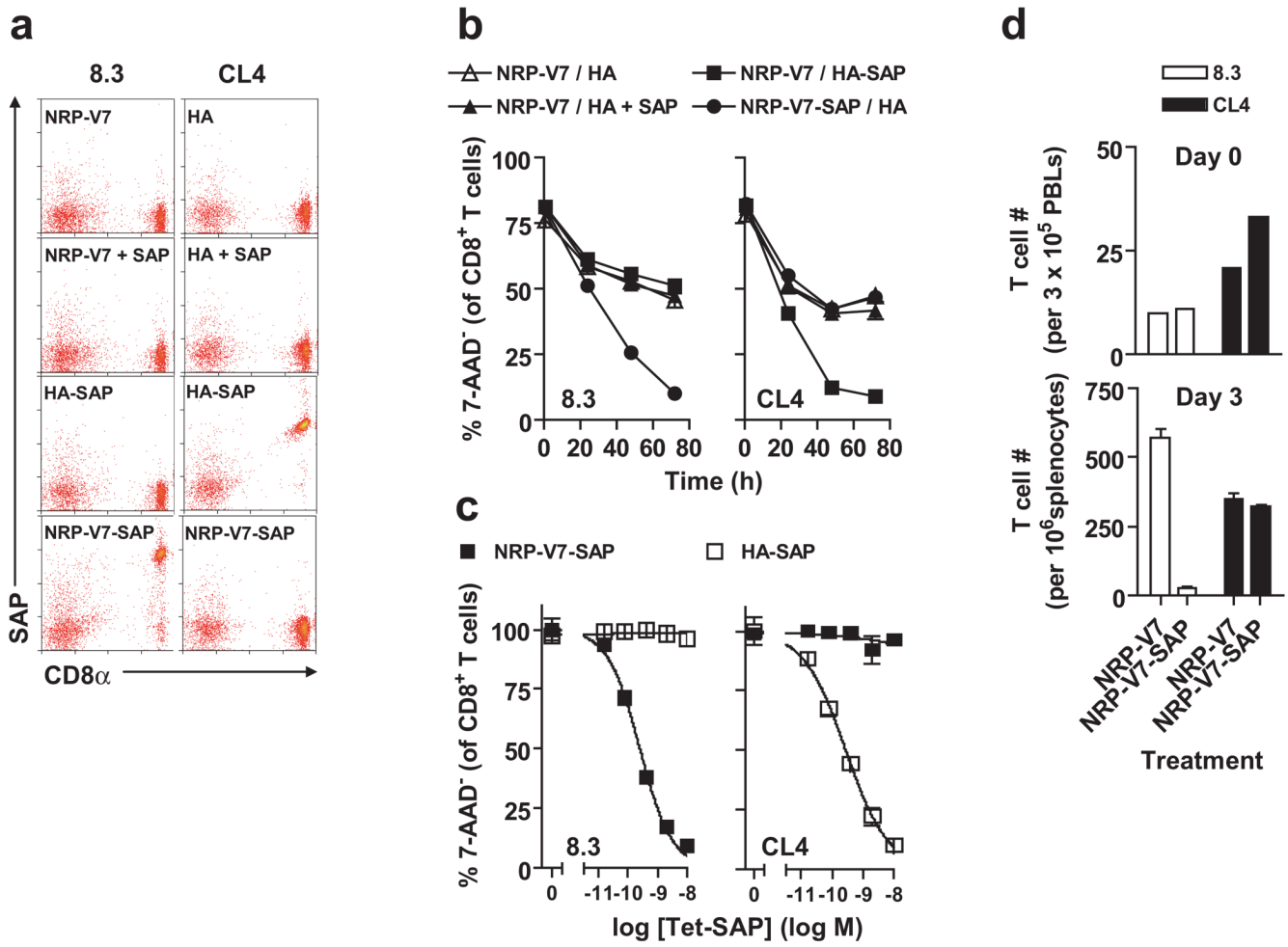

Figure 3.

Validation of specific binding and killing of $CD8^+$ T cells by SAP-coupled K^d tetramers in vitro and in vivo. (a) K^d tetramers prepared with SA-SAP retain binding specificity, and free SAP does not bind $CD8^+$ T cells. Peripheral blood lymphocytes from NOD 8.3 and NOD CL4 mice were incubated with tetramers at 4°C for 1 h, washed extensively, and probed for surface SAP binding with polyclonal anti-SAP Abs. (b) T cells are killed by cognate SAP-coupled tetramers in 72 h. Cultured T cells were harvested at 1, 24, 48 or 72 h. (c) T cells are killed by cognate SAP-coupled tetramers in dose-dependent fashion. T cells were harvested at 72 h; results are normalized to percent survival with non-toxic tetramer treatment alone. The EC50 values for killing of NOD 8.3 T cells were 0.254 and 0.564 nM in two independent experiments. In (b) and (c), T cells were incubated with tetramers at 37°C for 1 h, then washed and cultured in medium alone for the indicated times; harvested cells were analyzed by flow cytometry after staining with anti-CD8 mAb and 7-AAD. (d) The K^d-NRP-V7-SAP tetramer can eliminate naïve cognate $CD8^+$ T cells in vivo. Purified $Thy1.2^+$ NOD 8.3 and NOD CL4 T cells (1 × 106 each) were mixed and transferred i.v. into NOD $Thy1.1^+$ hosts. To verify equivalent transfer, PBLs were collected from recipient mice 1 day later and analyzed for $Thy1.2^+$ $tetramer^+$ $CD8^+$ T cells (upper panel); mice were then injected i.v. with either K^d-NRP-V7 or K^d-NRP-V7-SAP tetramers. Three days after treatment, splenocytes were stained with K^d-HA-PE, K^d-NRP-V7-AlexaFluor647, 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 K^d-NRP-V7-SAP tetramer (lower panel).

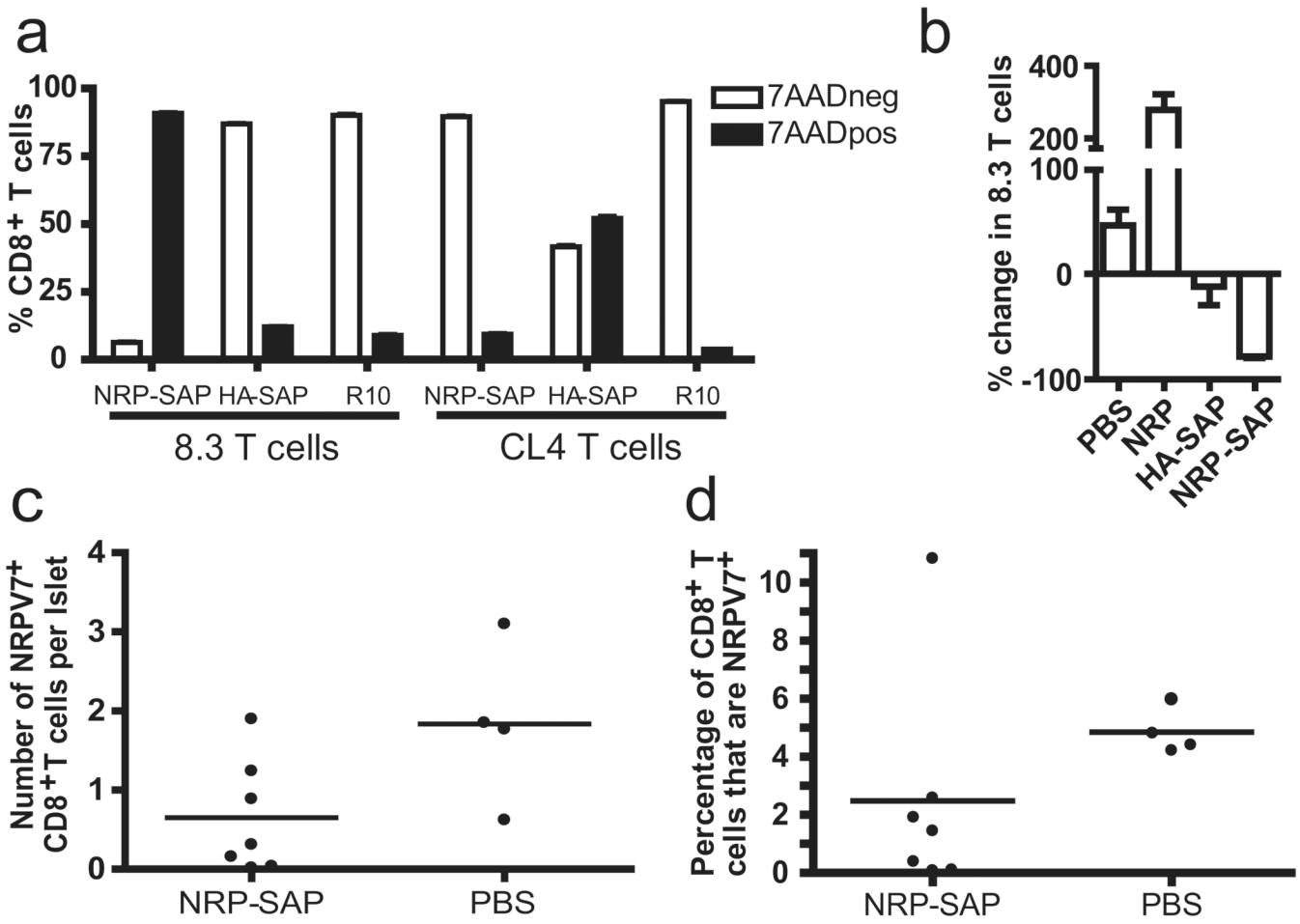

Figure 4.

Toxic tetramers selectively deplete epitope-specific T cells in vitro and in vivo. (a) NOD 8.3 or CL4 splenocytes were cultured 3 days in RPMI containing 10% FCS with cognate or irrelevant toxic tetramer or without toxic tetramer, then stained with anti-CD8 and 7AAD to measure viability. NRP-SAP refers to NRP-V7-SAP. Percent of tetramer-positive $CD8^+$ T cells is shown. (b) K^d-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 co-transferred 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 after treatment, PBLs were analyzed by flow cytometry, showing loss of NOD-8.3 T cells in mice that received the K^d-NRP-V7-SAP tetramer. Results are expressed as the mean percentage change vs. treatment day 0. (c) NRP-$V7^+CD8^+$ T cells are reduced in NOD mice treated with K^d-NRP-V7-SAP. $CD8^+$ cells were isolated from the islets of K^d-NRP-V7-SAP treated and control mice and analyzed by flow cytometry. One mouse from each group had less than 2 $CD8^+$ T cells per islet (less than 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. (d) Proportion $CD8^+$ T cells that were tetramer-positive is shown. P = 0.036, One-tailed Mann-Whitney.

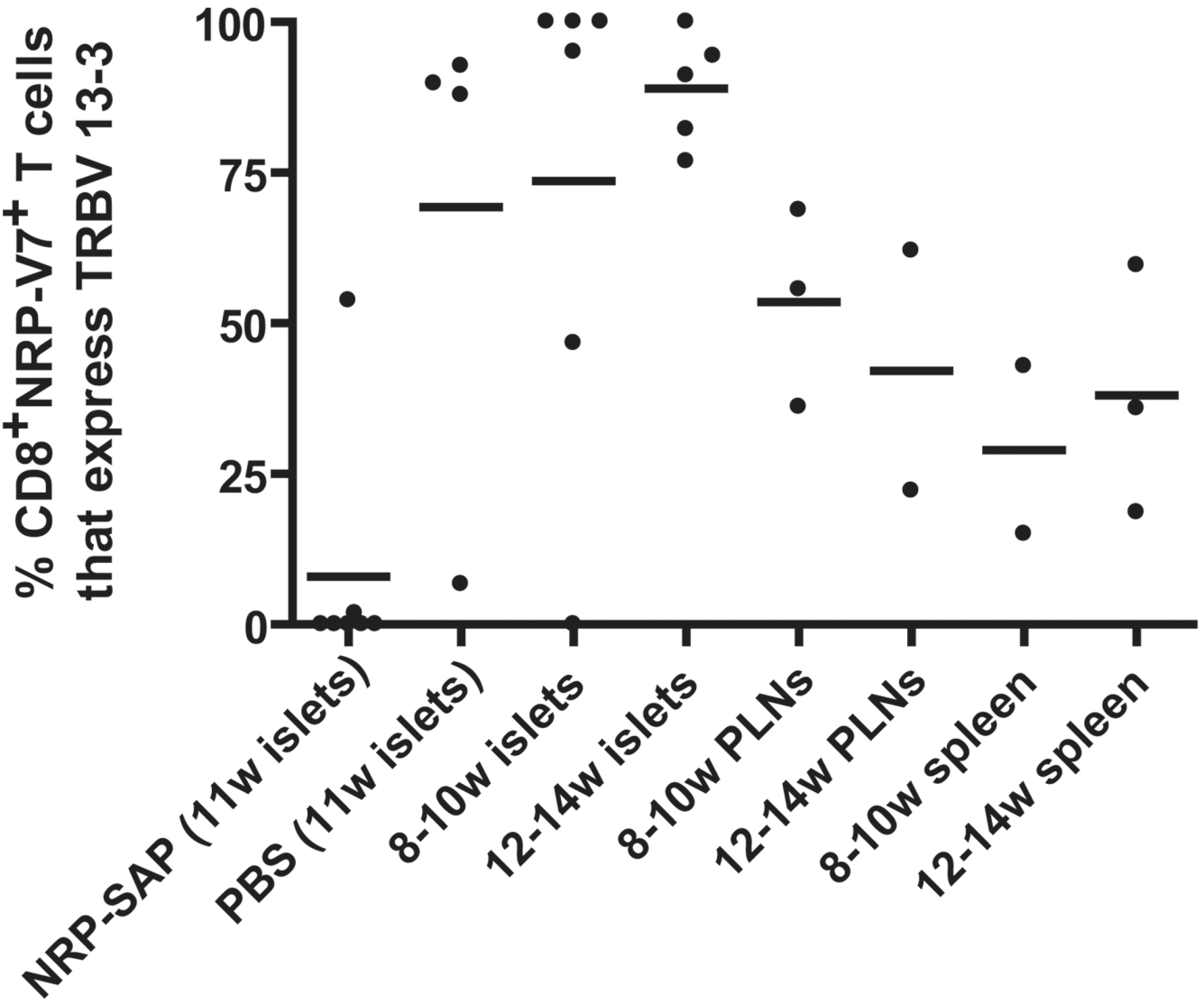

Figure 5.

NRP-V7$^+$CD8$^+$ T cells that express TRBV 13-3 accumulate in the islets and are deleted by K^d-NRP-V7-SAP. T cells were isolated from the islets of NOD mice 3 weeks post-initial treatment with K^d-NRP-V7-SAP, 3 weeks post-treatment with PBS, or from the islets, PLN, and spleen of unmanipulated NOD mice 8–14 weeks of age. Each data point represents proportion of islet-infiltrating NRP-V7$^+$CD8$^+$ T cells from a single mouse that expressed TRBV 13-3. One mouse from the K^d-NRP-V7-SAP treated group and one mouse from the PBS group had less than 2 CD8$^+$ T cells per islet (less than 5% of the average number) and only one tetramer-positive T cell in the sample. These mice were considered to have not developed insulitis and were excluded from this analysis. Data were analyzed using the Kruskal-Wallis test with Dunn's post-tests. Significant differences were found between the K^d-NRP-V7-SAP and PBS treated NOD mice ($P < 0.05$), 8–10 week islets ($P < 0.01$), and 12–14 week islets ($P < 0.001$).

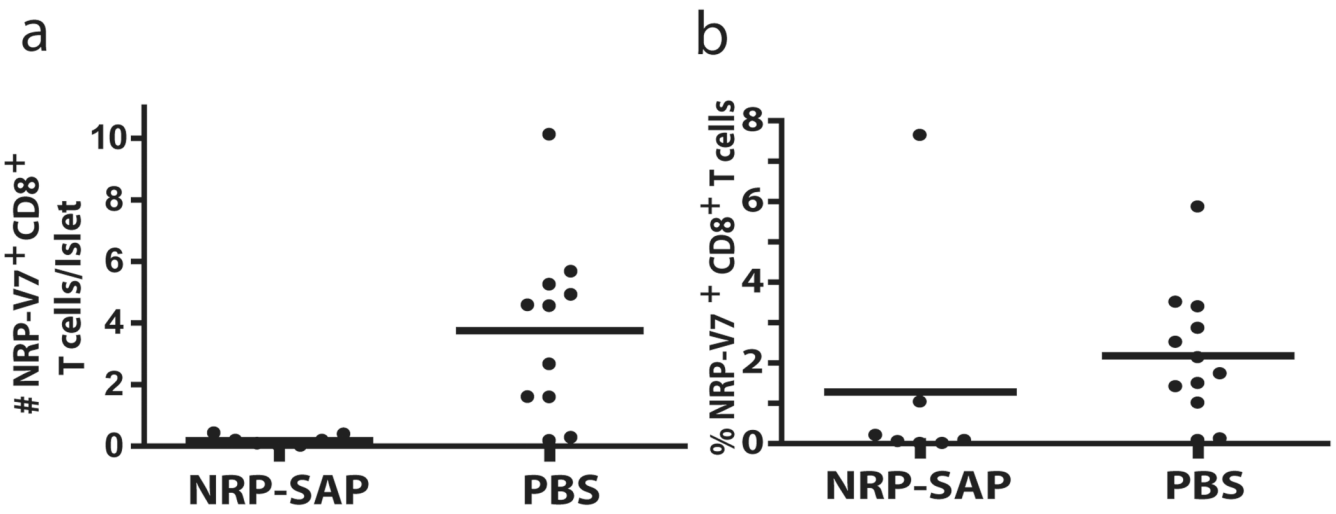

Figure 6.

K^d-NRP-SAP caused long-term depletion of tetramer-specific $CD8^+$ T cells in the islets. Female NOD mice were given 3 i.v. injections of K^d-NRP-SAP or PBS over 2 weeks beginning at 8 weeks of age. At onset of diabetes or 54 weeks, 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 (a) and proportion of $CD8^+$ T cells that were tetramer positive (b) are shown. PBS treatment group includes female NOD mice from 2 different toxic tetramer trials. ** P = 0.002, One-tailed Mann-Whitney. * P = 0.021, One-tailed Mann-Whitney.

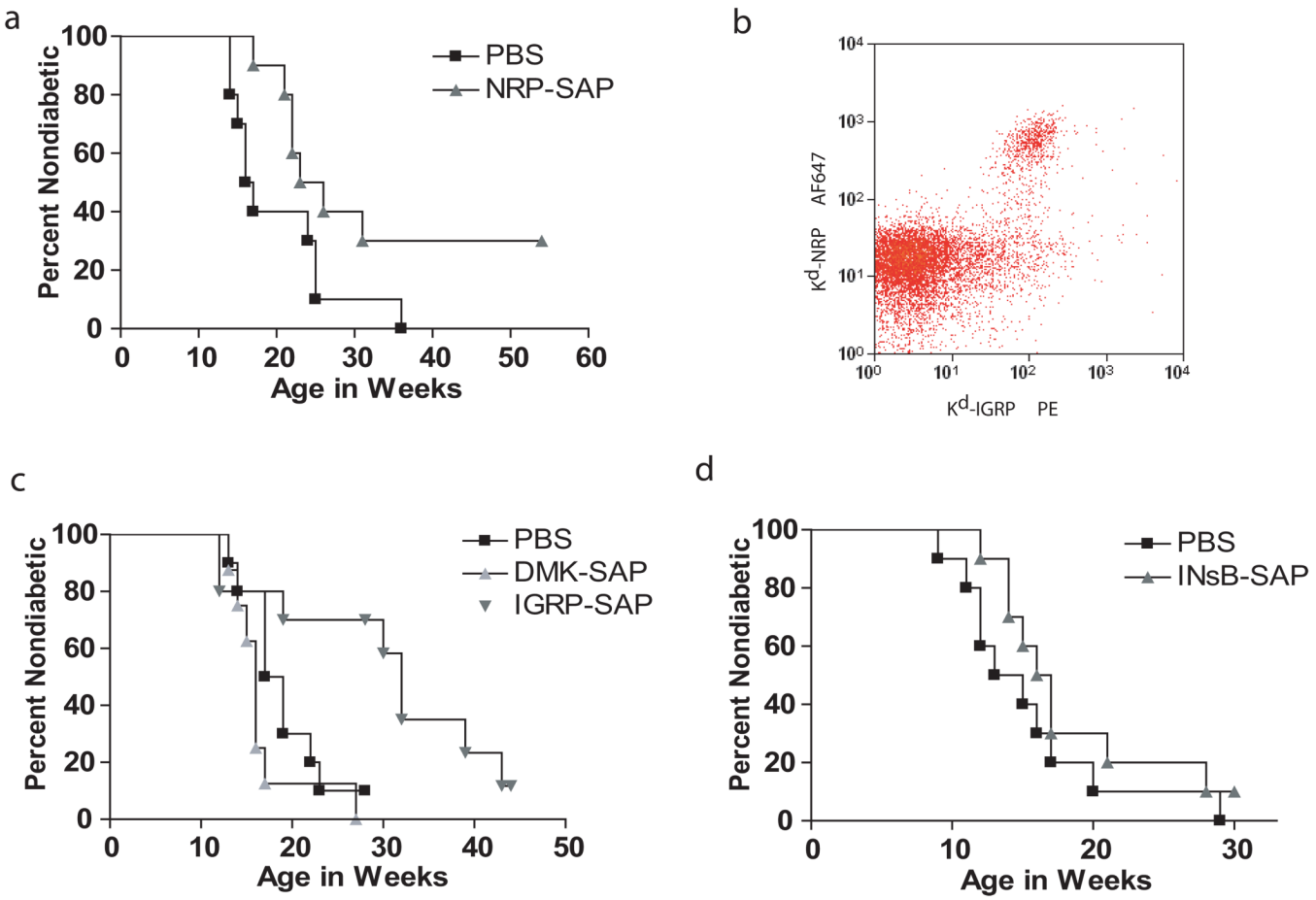

Figure 7.

K^d-NRP-V7-SAP and K^d-IGRP-SAP delayed onset of diabetes but not K^d-INsB-SAP or D^d-DMK-SAP. (a,c,d) Ten NOD mice per group were given 3 i.v. injections of the indicated toxic tetramer over 2 weeks beginning at 8 weeks of age. A Kaplan-Meier curve is shown above with diabetes incidence as the dependent variable. (a) K^d-NRP-SAP treatment significantly reduced diabetes incidence (logrank test, P = 0.04). Mean time to onset, PBS = 16.5 weeks, K^d-NRP-SAP = 24.5 weeks (b) NRP-V7-specific $CD8^+$ T cells are a subset of the IGRP-specific $CD8^+$T cells. Lymphocytes were isolated from the islets of a NOD mouse and stained with anti-CD8 PerCp, anti-CD3 AF488 and anti-CD19 Pacific Blue antibodies in addition to two tetramers, K^d-NRP-V7-SA-AF647 and K^d-IGRP-SA-PE. Cells were analyzed by flow cytometry. The $CD8^+CD3^+$T cells are shown in the dot plot. (c) K^d-IGRP-SAP but not K^d-DMK-SAP significantly delayed onset of diabetes (logrank test, P = 0 .02). Mean time to onset, PBS = 18 weeks, K^d-IGRP-SAP = 32 weeks, and D^d-DMK-SAP = 16 weeks (d) K^d-INsB-SAP did not reduce diabetes incidence. Mean time to onset, PBS = 14 weeks, K^d-INsB-SAP = 16.5 weeks. Blood ALT(alanine aminotransferase) levels (indicative of liver damage) measured after each dose of toxic tetramer did not rise above 350, consistent with mild transient effects.

Table I

Summary of Sequence Data

Summary of all TCRβ sequence data. 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 week old animal). Otherwise all mice contributed T cells derived from only one tissue.

Sample	# mice	# sequences	# unique clonotypes	# shared clonotypes
Islets 8–10 weeks	6	97	34	7 (20%)
Islets 12–14 weeks	5	148	14	10 (71%)
PLNs 8–10 weeks	3	61	36	6 (17%)
PLNs 12–14 weeks	2	68	34	8 (24%)
Spleen 8–10 weeks	2	55	41	6 (15%)
Spleen 12–14 weeks	3	134	72	6 (8%)