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Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide

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Antigen therapy may hold great promise for the prevention of autoimmunity; however, most clinical trials have failed, suggesting that the principles guiding the choice of treatment remain ill defined. Here, we examine the antidiabetogenic properties of altered peptide ligands of CD8$^+$ T cells recognizing an epitope of islet-specific glucose-6-phosphatase catalytic subunit–related protein ($IGRP_{206–214}$), a prevalent population of autoreactive T cells in autoimmune diabetes. We show that islet-associated CD8$^+$ T cells in nonobese diabetic mice recognize numerous IGRP epitopes, and that these cells have a role in the outcome of protocols designed to induce $IGRP_{206–214}$-specific tolerance. Ligands targeting $IGRP_{206–214}$-reactive T cells prevented disease, but only at doses that spared low-avidity clonotypes. Notably, near complete depletion of the $IGRP_{206–214}$-reactive T-cell pool enhanced the recruitment of subdominant specificities and did not blunt diabetogenesis. Thus, peptide therapy in autoimmunity is most effective under conditions that foster occupation of the target organ lymphocyte niche by nonpathogenic, low-avidity clonotypes.

Administration of autoantigenic proteins or peptides in solution can blunt the initiation and/or progression of autoimmunity in experimental models of autoimmune disease[1–5], but limited clinical trials in humans using similar strategies have almost invariably met with failure[6–12]. This suggests that the principles guiding the choice and conditions of treatment are poorly defined and, as a result, inadequate for human application.

Unlike their experimental counterparts, spontaneous organ-specific autoimmune disorders result from complex responses against numerous epitopes in multiple antigens that arise spontaneously in a stochastic and often unpredictable sequence. This complexity is compounded by the fact that lymphocyte clones recognizing identical epitopes engage antigen–major histocompatibility complex (MHC) molecules within a broad range of avidities, the strength of which correlates with pathogenic potential[13–15]. Consequently, the outcome of any immunization strategy for the prevention of autoimmunity is likely to be influenced by the choice of autoantigen(s), dose and periodicity of treatment as well as route and form of administration. Unfortunately, our current understanding of the independent contribution of these variables to treatment outcome is extremely limited.

Type 1 diabetes (T1D) in both humans and nonobese diabetic (NOD) mice is an autoimmune disease that results from selective destruction of pancreatic beta cells by T lymphocytes recognizing a growing list of autoantigens[16]. Although initiation of T1D clearly requires the recruitment of autoreactive CD4$^+$ T cells, there is compelling evidence that initiation and progression of T1D is dependent on CD8$^+$ T cells[14,15]. We and others have shown that a large fraction of all islet-associated CD8$^+$ cells in NOD mice use highly homologous T-cell receptor-alpha (TCRα) chains (Vα17-Jα42)[17–20] and recognize the same peptide sequence (NRP-A7) in the context of the MHC molecule K^d (ref. 21). These T cells are already a significant component of the earliest NOD islet CD8$^+$ infiltrates[20–22], are diabetogenic[18,19], target a peptide from IGRP ($IGRP_{206–214}$, similar to NRP-A7)[23] and are unusually frequent in the periphery (>1/200 circulating CD8$^+$ cells)[23,24]. Notably, progression of insulitis to diabetes in NOD mice is invariably accompanied by cyclic expansion of the circulating $IGRP_{206–214}$-reactive CD8$^+$ T-cell pool[24] and by avidity maturation of its islet-associated counterpart[13]. When considered together, these data strongly support the idea that $IGRP_{206–214}$-reactive CD8$^+$ T cells have a key role in mouse T1D. Notably, the human *G6PC2* gene, which encodes IGRP and maps to chromosome 2q28-32 (ref. 25), overlaps a T1D susceptibility locus, IDDM7 (2q31)[26], raising the possibility that IGRP may also be target of the human diabetogenic response.

Administration of soluble peptides (without adjuvant) is an effective way of inducing antigen-specific T-cell tolerance[27,28]. Previously, we showed that repeated treatment of prediabetic NOD mice with soluble NRP-A7 blunted avidity maturation of the $IGRP_{206–214}$-reactive CD8$^+$ subset by selectively deleting clonotypes expressing TCRs with the highest affinity for peptide MHC[13]. These observations suggested that avidity maturation of pathogenic T-cell populations is a key event in the progression of benign inflammation

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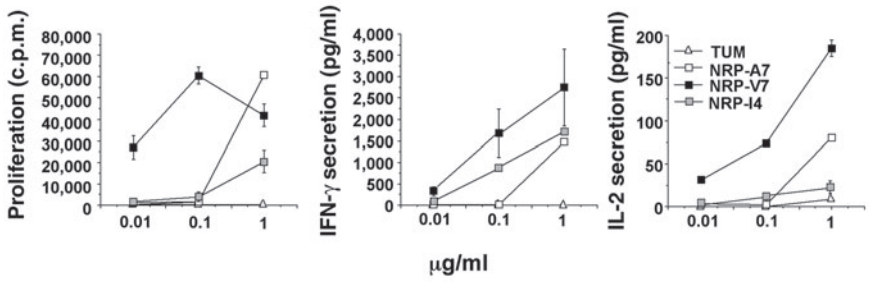

Figure 1 Agonistic activity of APLs on 8.3-CD8$^+$ T cells. Naive splenic 8.3-CD8$^+$ T cells (2×10^4) were incubated with peptide-pulsed γ-irradiated NOD splenocytes for 2 or 3 d (cytokine secretion and proliferation assays, respectively). None of the peptides induced secretion of IL-4 secretion (data not shown). Data are mean ± s.e.m. and are representative of two or three different experiments. *x*-axis refers to peptide concentrations.

to overt disease in autoimmunity. They also raised the possibility that NRP-A7's antidiabetogenic activity was mediated not only by deletion of high-avidity cells, but also by occupation of the 'high-avidity clonotype niche' (emptied by treatment with NRP-A7) by 'low-avidity' (and potentially antidiabetogenic) clonotypes. To test this hypothesis, here we identified altered peptide ligands (APLs) with partial, full or super agonistic activity for IGRP$_{206–214}$-reactive CD8$^+$ T cells and compared their antidiabetogenic activity *in vivo* over a wide range of doses.

RESULTS

APLs for IGRP$_{206–214}$-reactive CD8$^+$ T cells

We searched for APLs capable of engaging a transgenic IGRP$_{206–214}$-reactive TCR (8.3) with lower or higher affinity than NRP-A7 (ref. 22). NRP-I4 was chosen as a potential 'low-avidity' APL candidate because it behaved as a partial agonist (**Fig. 1**). NRP-V7 was chosen as a potential 'very high–avidity' APL because it had superior agonistic activity on 8.3-CD8$^+$ T cells than NRP-A7, an agonist (**Fig. 1**). To confirm that 8.3-CD8$^+$ cells recognized these peptides with different avidity (they all bound to K^d with similar affinity; data not shown), we compared the ability of peptide-K^d tetramers to stain 8.3-CD8$^+$ T cells. The intensities of tetramer staining were consistent with the functional avidities of the peptides (**Figs. 1** and **2a**) as well as with the peptides' ability to delete 8.3-CD8$^+$ T cells *in vivo* (**Fig. 2b**). These results were not a peculiarity of cells expressing the 8.3-TCR: whereas the NRP-I4 tetramer could not stain T cells derived from islets of nontransgenic NOD mice (data not shown), the NRP-V7 tetramer did so with higher intensity (**Fig. 2c**) and lower K_d (higher avidity; **Fig. 2d**) than the NRP-A7 tetramer. Thus, IGRP$_{206–214}$-reactive CD8$^+$ cells recognize NRP-I4, NRP-A7 and NRP-V7 with increasing avidity.

Anti-diabetogenic activity of IGRP$_{206–214}$ APLs

To investigate whether the above APLs had antidiabetogenic activity, we treated cohorts of female NOD mice with repeated injections of TUM (negative control), NRP-I4, NRP-A7 and NRP-V7 (in phosphate-buffered saline) over a wide range of doses. Mice were followed for diabetes (**Fig. 3a**) and killed either at onset of diabetes or at the end of the follow-up period, to investigate the effects of treatment on the size and avidity of the islet-associated IGRP$_{206–214}$-reactive CD8$^+$ T-cell subpopulation (**Fig. 3a–e**).

NRP-I4 was antidiabetogenic in a dose-dependent manner: it was not protective at all when given at ≤25 μg/injection, but was highly antidiabetogenic when given at 100 μg/injection (**Fig. 3a**). Notably, the protective effect of NRP-I4 (at 100 μg/injection) was associated with the presence of predominantly low-avidity IGRP$_{206–214}$-reactive CD8$^+$ cells in islets, rather than with massive deletion of the entire IGRP$_{206–214}$-reactive CD8$^+$ cell subset. Whereas the percentages (**Fig. 3a**) and absolute numbers (**Fig. 3b**) of NRP-V7–reactive CD8$^+$ cells contained in the islets of these mice were similar to those seen in TUM-treated controls, the islet-associated CD8$^+$ cells of NRP-I4–treated mice bound NRP-V7 tetramers with significantly lower avidity (higher K_d) than those derived from TUM-treated mice ($P < 0.05$; **Fig. 3a**,**e**). Given that NRP-I4 induces partial deletion of 8.3-CD8$^+$ T cells (**Fig. 2b**), which recognize NRP-V7 with intermediate-to-high avidity, the most logical interpretation of these results was that the protective effect of high-dose NRP-I4 treatment resulted from selective deletion of high-avidity IGRP$_{206–214}$-reactive CD8$^+$ cells. In agreement with this, NRP-A7, a higher-affinity ligand of these cells, protected mice from diabetes at a lower dose than NRP-I4 (50 μg/injection), and this, too, coincided with accumulation of low-avidity IGRP$_{206–214}$-reactive CD8$^+$ T cells within islets (**Fig. 3a**). The absolute number of NRP-V7–reactive T cells in the islets of these mice was reduced, but not significantly compared to TUM-treated mice ($0.6 \times 10^5 \pm 0.3$ versus $1 \times 10^5 \pm 0.3$, respectively). Cytotoxicity assays confirmed that the CD8$^+$ T cells that accumulated in islets of NRP-I4–treated mice were significantly less cytotoxic than those derived from TUM-treated animals, despite containing more tetramer-positive cells ($P < 0.05$; **Fig. 3c**). NRP-I4 also fostered the accumulation of low-avidity CD8$^+$ T cells in islets and had antidiabetogenic activity when administered intravenously (**Fig. 3d**,**e**). Notably, initiation of treatment at 10 weeks (an age when virtually all mice display severe insulitis) delayed the onset of diabetes without reducing its incidence (**Fig. 3d**). Altogether, these results indicated that repeated triggering of high-avidity IGRP$_{206–214}$-reactive CD8$^+$ T cells by NRP-I4 in the periphery before development of full-blown insulitis induces tolerance and facilitates the occupation of the intraislet T-cell niche by noncytolytic low-avidity clonotypes.

The idea that protection by high doses of NRP-I4 and intermediate doses of NRP-A7 resulted solely from deletion of high-avidity clonotypes was at odds with two unexpected observations. First, high doses of NRP-A7 (100 μg) were ineffective. It should be noted that here we prepared the inoculum by diluting concentrated stocks of peptide, as opposed to dissolving material dried from diluted stocks[13]. As the latter strategy results in peptide loss, it should not be unexpected that in a previous study NRP-A7 was protective when given at the 100 μg dose[13]. The second unexpected observation was that NRP-V7 was not protective at any dose (**Fig. 3a**). This was unexpected because the islets of NRP-A7– (100 μg/dose) and NRP-V7–treated mice (over a range of doses) contained very significantly reduced numbers of NRP-V7 tetramer-reactive CD8$^+$ cells ($P < 0.02$; **Fig. 3a**,**b**), and lacked NRP-V7–reactive cytotoxic T lymphocytes (CTLs; **Fig. 3c**). Thus, the antidiabetogenic activity of NRP-I4 (at 100 μg) and NRP-A7 (at 50 μg) could not be attributed to deletion of (high-avidity) IGRP$_{206–214}$-reactive CD8$^+$ cells alone. This suggested that enhanced recruitment of noncytolytic, low-avidity IGRP$_{206–214}$-reactive CD8$^+$ clonotypes to islets had an active role in the resistance of NRP-I4– and NRP-A7–treated mice against diabetes.

Islet-derived CD8$^+$ cells target many IGRP epitopes

Treatment of NOD mice with high doses of IGRP$_{206–214}$ (75–100 μg) yielded results very similar to those obtained with NRP-V7 (**Fig. 4a**). The ineffectiveness of IGRP$_{206–214}$ treatment was also associated with near complete deletion of the intraislet IGRP$_{206–214}$-reactive CD8$^+$ T-cell subset (**Fig. 4b**,**c**).

These results prompted us to consider the possibility that near complete deletion of the IGRP$_{206–214}$-reactive CD8$^+$ subset might somehow foster the creation of a 'niche' for subdominant specificities. Conceivably, enhanced recruitment or accumulation of these clonotypes could have counteracted any protection afforded by depletion of the dominant IGRP$_{206–214}$-reactive pool. Because staining of islet-derived T cells of TUM- versus NRP-V7– or IGRP$_{206–214}$-treated mice with insulin 15-23L/K^d tetramers (the only other known target of beta cell–autoreactive CD8$^+$ cells[29]) did not show significant differences (data not shown), we wondered whether the response of CD8$^+$ T cells against IGRP in diabetes involved multiple epitopes.

To investigate this, we designed an IGRP-based peptide library comprised of 33 K^d- and 43 D^b-binding nonamers (**Supplementary Table 1** online). Only one of these peptides (IGRP$_{207–215}$; peptide 6) was cross-reactive with IGRP$_{206–214}$ (peptide IG) as determined by its ability to elicit 8.3-CD8$^+$ responses (data not shown). We tested the ability of each of these IGRP peptides to elicit interferon (IFN)-γ secretion by CD8$^+$ cells propagated from islets of prediabetic (21 ± 1 weeks) or acutely diabetic NOD mice (18 ± 1 weeks). Islets of most mice, regardless of diabetes status, contained IGRP$_{206–214}$- and IGRP$_{207–215}$-reactive CD8$^+$ cells (**Fig. 5a** and **Supplementary Table 2** online). Experiments using IGRP$_{206–214}$ tetramers confirmed the existence of a correlation between the magnitude of IFN-γ secretion by islet-associated CD8$^+$ cells and the percentage of tetramer-positive cells ($P < 0.001$), suggesting that differences in IFN-γ secretion reflect differences in cell numbers (data not shown). Notably, a substantial number of mice also mounted responses against other IGRP epitopes, particularly peptides 72, 7, 8 and 39 (**Fig. 5a** and **Supplementary Table 2** online). Limited studies with tetramers confirmed the presence of peptide 72– and peptide 39–reactive CD8$^+$ T cells in at least some animals (**Fig. 5b**). Notably, the combined response of islet-associated T cells against all the tested IGRP epitopes was significantly higher in diabetic animals than in prediabetic animals, as measured by comparing the average number of responses against IGRP per group ($P < 0.0001$; **Fig. 5a**) or the average amount of IFN-γ secreted by T cells of individual mice against all peptides ($P < 0.03$; **Fig. 5c**). Thus, diabetic and, to a lesser extent, nondiabetic NOD mice mount dominant CD8$^+$ responses against IGRP$_{206–214}$ and subdominant responses against numerous other IGRP epitopes.

Increased responses against IGRP in IGRP$_{206–214}$-treated mice

We next investigated whether depletion of the IGRP$_{206–214}$-reactive CD8$^+$ subset by IGRP$_{206–214}$ might have failed to protect mice from diabetes because it fostered the expansion of the smaller pool of intraislet T cells recognizing subdominant epitopes of IGRP. To that end, we evaluated the presence of anti-IGRP CD8$^+$ T cells in untreated, TUM-treated and IGRP$_{206–214}$-treated NOD mice. TUM treatment did not significantly increase the frequency or magnitude of responses against IGRP (**Fig. 6a**,**b** and **Supplementary Table 2** online). In contrast, whereas IGRP$_{206–214}$ treatment significantly reduced both the frequency and magnitude of the response against

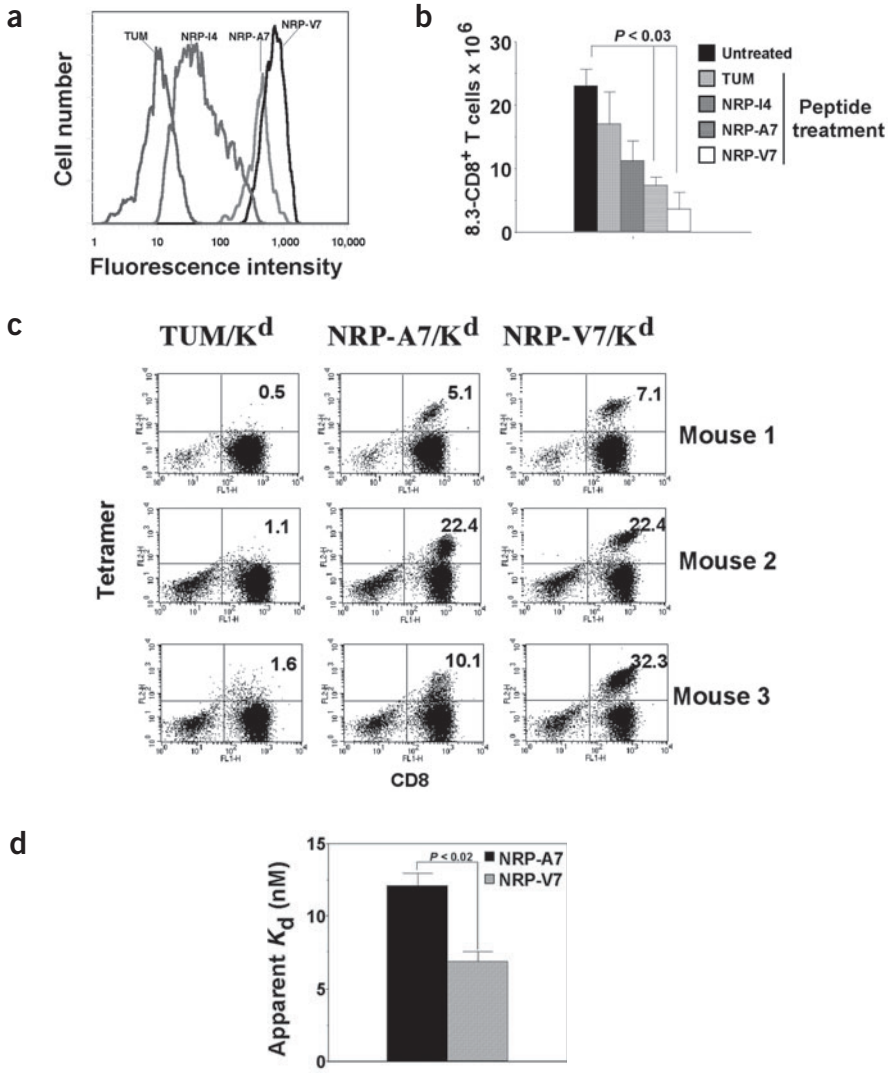

Figure 2 Differences in the functional avidity of APLs correlate with differences in peptide-MHC–binding avidity and tolerogenic activity. (**a**) Fluorescence intensity of tetramer staining on naive splenic 8.3-CD8$^+$ T cells. (**b**) Tolerogenic activity of APLs against 8.3-CD8$^+$ T cells *in vivo*. Comparison of total numbers of CD8$^+$ splenocytes in untreated (10 ± 1 weeks old; $n = 9$) or APL-treated 8.3-NOD mice. Mice received one intraperitoneal injection of 100 μg of peptide in PBS 7 d before analysis. The age and number of mice studied were: TUM-treated (11 ± 1 weeks; $n = 5$); NRP-I4–treated (9 ± 1 weeks; $n = 3$); NRP-A7–treated (11 ± 1 weeks; $n = 7$); and NRP-V7–treated (14 ± 2 weeks; $n = 3$). (**c**) Tetramer-binding ability of islet-associated CD8$^+$ T cells from 20-week-old nontransgenic NOD mice. Vertical panels correspond to three individual mice. Numbers in the upper right quadrants of each panel correspond to percentage of tetramer-positive CD8$^+$ cells. (**d**) CD8$^+$ T cells propagated from islets of 9-week-old female NOD mice bind NRP-V7/K^d tetramers ($n = 6$ mice) with higher avidity (lower K_d) than NRP-A7/K^d tetramers ($n = 6$ mice).

IGRP$_{206–214}$ ($P < 0.0001$), it significantly increased the frequency of responses against other IGRP epitopes ($P < 0.003$; **Figs. 4c** and **6a** and **Supplementary Table 2** online). As a result, the magnitude of the total intraislet response against IGRP in IGRP$_{206–214}$-treated mice was similar to that in untreated or TUM-treated animals (**Fig. 6b**). Notably, the frequency (**Fig. 6c**) and magnitude (**Fig. 6d**) of subdominant IGRP epitope-specific CD8$^+$ responses in islets of IGRP$_{206–214}$-treated mice were significantly higher in mice that had progressed to diabetes, implying that these T-cell specificities were involved in progression of diabetes ($P < 0.0001$ and $P = 0.05$, respectively). Because these differences were not seen in NRP-I4- versus TUM-treated mice (**Fig. 6e**,**f** and **Supplementary Table 3** online), we propose that expansion of the subdominant IGRP epitope-specific T-cell pool accounts for the paradoxical ineffectiveness of IGRP$_{206-214}$ (and NRP-V7) peptide treatment for prevention of diabetes. The data also support the view that, in the presence of significant numbers of low-avidity IGRP$_{206–214}$-reactive clonotypes, subdominant IGRP epitope-specific clonotypes cannot effectively foster progression of diabetes.

DISCUSSION

Recent years have witnessed the emergence of CD8$^+$ cells as major effectors of tissue damage in organ-specific autoimmunity[15]. We have previously shown that a significant fraction of islet-associated CD8$^+$ cells in NOD mice recognize a peptide from the islet-specific protein IGRP (IGRP$_{206–214}$)[20]. We have also shown that this subset of T cells undergoes a process of avidity maturation that results from the competitive outgrowth of a small pool of high-avidity clonotypes at the expense of a larger pool of nondiabetogenic, low-avidity clonotypes[13]. Here we investigated the conditions under which manipulation of the IGRP$_{206–214}$-reactive CD8$^+$ T-cell pool with APLs had therapeutic importance. Our data show that chronic treatment of mice with inter-

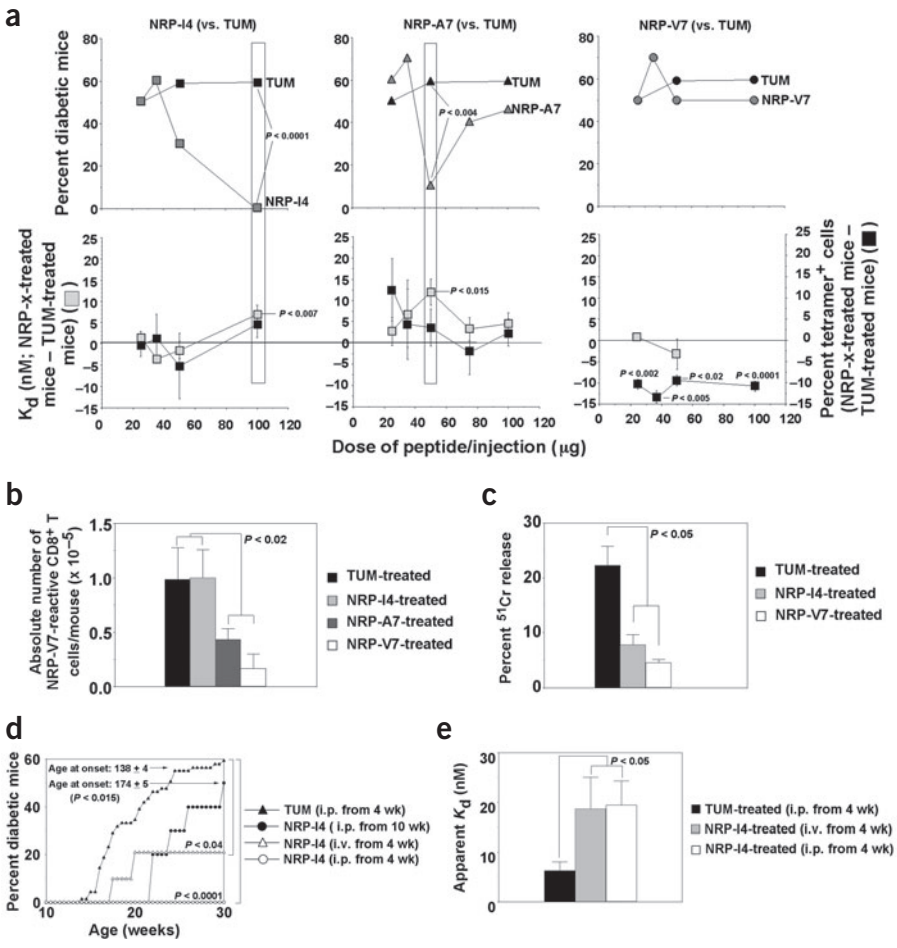

Figure 3 Antidiabetogenic activity of APLs in wild-type NOD mice. (**a**) Incidence of diabetes in TUM versus APL-treated female NOD mice. TUM: ≤25 μg ($n = 29$), 50 μg ($n = 10$), 100 μg ($n = 69$); NRP-I4: ≤25 μg ($n = 30$), 35 μg ($n = 10$), 50 μg ($n = 10$), 100 μg ($n = 10$); NRP-A7: ≤25 μg ($n = 9$), 35 μg ($n = 10$), 50 μg ($n = 10$), 75 μg ($n = 10$), 100 μg ($n = 50$); NRP-V7: ≤25 μg ($n = 30$), 35 μg ($n = 10$), 50 μg ($n = 10$), 100 μg ($n = 59$) (upper panels). Effects of APL versus TUM treatment on the percentages and avidity of NRP-V7/K^d tetramer–binding cells within islet-derived CD8$^+$ cells (lower panels). Mice were killed at onset of diabetes or at the end of the study (32 weeks) to isolate islet-associated CD8$^+$ T cells. Data are presented as differences in values obtained in APL- versus TUM-treated mice (± s.e.). Values above or below zero indicate that APL treatment was associated with recruitment of lower (high K_d) or higher (low K_d) avidity T cells, and/or in recruitment of higher or lower percentages of tetramer-reactive CD8$^+$ T cells, respectively, as compared to TUM treatment. NRP-I4: ≤25 μg ($n = 15$, 7 T1D), 35 μg ($n = 4$, 3 T1D), 50 μg ($n = 3$, 1 T1D), 100 μg ($n = 7$, 0 T1D); NRP-A7: ≤25 μg ($n = 9$, 5 T1D), 35 μg ($n = 4$, 4 T1D), 50 μg ($n = 5$, 1 T1D), 75 μg ($n = 3$, 0 T1D), 100 μg ($n = 10$); NRP-V7: ≤25 μg ($n = 10$, 7 T1D), 35 μg ($n = 6$, 3 T1D), 50 μg ($n = 10$, 5 T1D), 100 μg ($n = 47$, 31 T1D). Measurements of avidity were only possible in mice containing tetramer-positive cells in islets. NRP-I4: ≤25 μg ($n = 15$), 35 μg ($n = 4$), 50 μg ($n = 2$), 100 μg ($n = 7$); NRP-A7: ≤25 μg ($n = 6$), 35 μg ($n = 4$), 50 μg ($n = 5$), 75 μg ($n = 2$), 100 μg ($n = 10$); NRP-V7: ≤25 μg ($n = 7$), 50 μg ($n = 2$). Except where indicated in the graph, values obtained in APL-treated mice were statistically similar to those seen in TUM-treated animals. (**b**) Absolute number of NRP-V7/K^d tetramer–binding CD8$^+$ T cells in mice treated with different peptides at 100 μg/injection. (**c**) Cytotoxicity of islet-derived T cells from APL-versus TUM-treated mice (at 100 μg/injection) against NRP-A7–pulsed RMA-SK^d cells at a 1:10 target/effector ratio. Background responses against the control peptide TUM were subtracted. Data are shown as mean ± s.e.m. ^{51}Cr-release values correspond to $n = 18$ (control group; 13 T1D), $n = 4$ (NRP-I4) and $n = 45$ (NRP-V7; 30 T1D) mice. No significant differences were noted between samples from diabetic versus nondiabetic mice and thus were pooled. The percentages of tetramer-positive cells in these samples were: 9 ± 1 (TUM), 20 ± 4 (NRP-I4) and 1 ± 1 (NRP-V7). (**d**) Cumulative incidence of T1D in mice treated with TUM intraperitoneally ($n = 69$; from 3–4 weeks of age), NRP-I4 intraperitoneally ($n = 10$; from 3–4 weeks), NRP-I4 intravenously (from 3–4 weeks; $n = 9$) and NRP-I4 intraperitoneally ($n = 10$; from 10 weeks). All mice received 100 μg peptide/injection. (**e**) K_d values of NRP-V7 tetramer–binding to CD8$^+$ T cells derived from islets of additional cohorts of mice treated with TUM intraperitoneally ($n = 5$; 1 T1D), NRP-I4 intraperitoneally ($n = 12$; 0 T1D) or NRP-I4 intravenously ($n = 4$; 0 T1D). Samples were collected at 18–22 weeks for intraperitoneally treated mice, or at 30 weeks for intravenously treated mice. Please note that K_d values corresponding to non-peptide-treated 20-week-old mice (data not shown) are similar to those seen in TUM-treated animals (6 ± 1 nM). i.p., intraperitoneal; i.v., intravenous; wk, weeks.

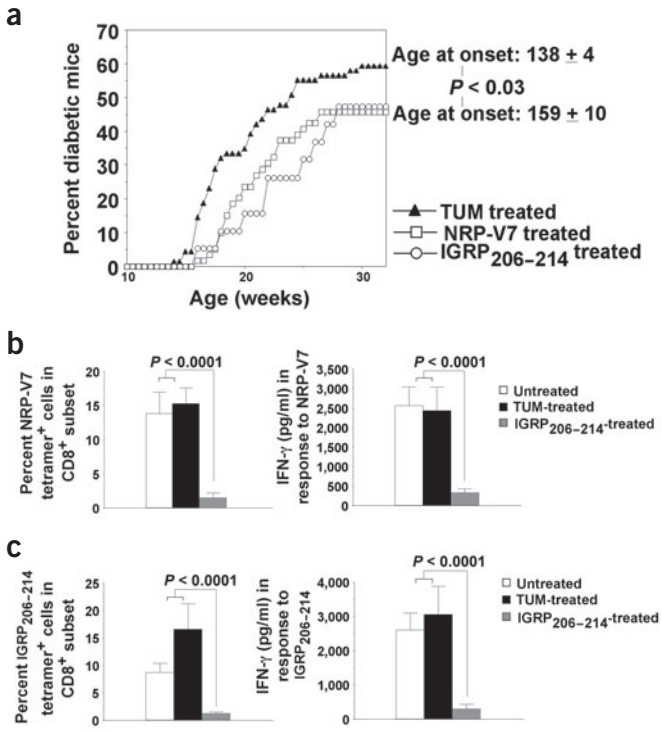

Figure 4 NRP-V7 and $IGRP_{206-214}$ cannot blunt progression of diabetes, despite depleting the $IGRP_{206–214}$-reactive $CD8^+$ T-cell pool. (**a**) Cumulative incidence of T1D in TUM ($n = 69$), NRP-V7 ($n = 59$) and $IGRP_{206–214}$-treated NOD mice ($n = 19$). All mice received 100 μg (TUM, NRP-V7 and $IGRP_{206–214}$) or 75 μg of peptide/injection ($IGRP_{206–214}$; $n = 9$). No differences were noted between groups of mice receiving 75 or 100 μg of $IGRP_{206–214}$, hence the data were pooled. *P* value refers to TUM- versus NRP-V7– and $IGRP_{206–214}$-treated mice. (**b**) Percentage of NRP-V7/K^d tetramer–reactive cells (left) and IFN-γ secretion (right) by islet-associated $CD8^+$ T cells from untreated, or TUM- and $IGRP_{206–214}$-treated NOD mice. Responses to the negative control peptide TUM were subtracted. No significant differences were noted between diabetic and nondiabetic mice within individual treatment groups. *N* values for tetramer staining were: untreated group, $n = 11$ (2 T1D); TUM-treated group, $n = 20$ (9 T1D); NRP-V7–treated group, $n = 52$ (32 T1D); $IGRP_{206–214}$-treated group, $n = 17$ (8 T1D). For IFN-γ secretion: untreated group, $n = 28$ (19 T1D); TUM-treated group, $n = 11$ (7 T1D); and $IGRP_{206–214}$-treated group, $n = 12$ (6 T1D). (**c**) As in **b**, but using $IGRP_{206–214}/K^d$ tetramers or $IGRP_{206–214}$ peptide. For tetramer staining: untreated group, $n = 28$ (19 T1D); TUM-treated group, $n = 3$ (0 T1D); $IGRP_{206–214}$-treated group, $n = 14$ (6 T1D). For IFN-γ secretion: untreated group, $n = 29$ (9 T1D); TUM-treated group, $n = 7$ (4 T1D); and $IGRP_{206–214}$-treated group, $n = 11$ (5 T1D).

mediate doses of an intermediate-affinity APL (NRP-A7) or high doses of a low-affinity APL (NRP-I4) afforded near complete protection from diabetes. Disease protection was associated with local accumulation of low-avidity $IGRP_{206-214}$-reactive $CD8^+$ T cells at the expense of their high-avidity counterparts, which were deleted. Unexpectedly, repeated treatment of mice with high doses of a very high–affinity APL (NRP-V7) or the natural ligand ($IGRP_{206–214}$) only afforded marginal protection. Notably, our detailed systematic analyses showed that the islets of these mice contained very few $IGRP_{206-214}$-reactive $CD8^+$ T cells, but increased populations of $CD8^+$ T cells recognizing several other IGRP epitopes. These results strongly argue against the usefulness of 'high-avidity' (high-affinity and high-dose) peptide therapy for the prevention of autoimmunity by eliminating prevalent subsets of autoreactive lymphocytes.

Because soluble peptides are usually cleared within 2 d, particularly when given intravenously[30], they induce a weak and short-lived activation state that, in the absence of costimulatory signals, leads to anergy and deletion[27,28]. Because this tolerogenic stimulus must reach an undefined threshold of TCR occupancy, the effectiveness of soluble peptides for induction of tolerance should be a function of dose as well as affinity for TCR and MHC. Accordingly, the observation that the effectiveness of NRP-I4 and NRP-A7 therapy increased with dose was expected, as was the fact that NRP-A7 reached maximum protective activity at a lower dose than NRP-I4 did. Notably, the islets of mice treated with protective doses of NRP-A7 and NRP-I4 contained normal or only slightly reduced numbers of $IGRP_{206–214}$-reactive $CD8^+$ T cells. These cells bound NRP-V7 tetramers with considerably lower avidity and were less cytotoxic against peptide-pulsed target cells than those isolated from TUM-treated controls, suggesting that NRP-I4 treatment

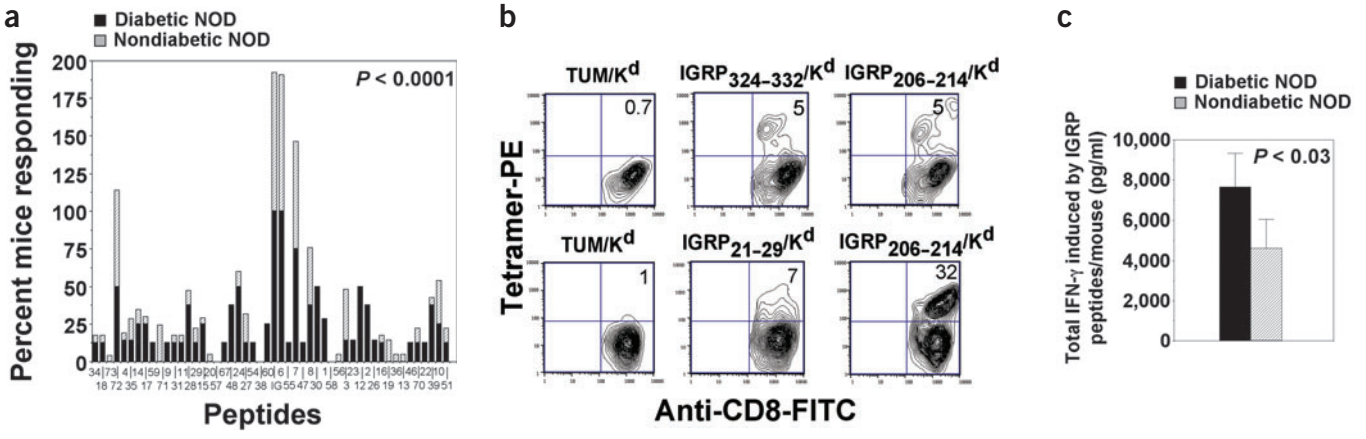

Figure 5 NOD mice spontaneously mount intraislet $CD8^+$ T-cell responses against multiple IGRP epitopes. (**a**) Percentage of diabetic and nondiabetic NOD mice that contain intraislet $CD8^+$ T cells recognizing epitopes of IGRP. Data are from **Supplementary Table 2** online. Numbers on *x*-axis refer to the different peptides tested, ordered from the amino to the carboxy terminus of IGRP (**Supplementary Table 1** online). (**b**) Examples of mice containing $IGRP_{324–332}$- or $IGRP_{21–29}$-reactive $CD8^+$ T cells within islets, as determined with tetramers. (**c**) Cumulative amounts of IFN-γ secreted by islet-derived $CD8^+$ T cells in response to IGRP peptides. All values correspond to responses above background (in response to TUM).

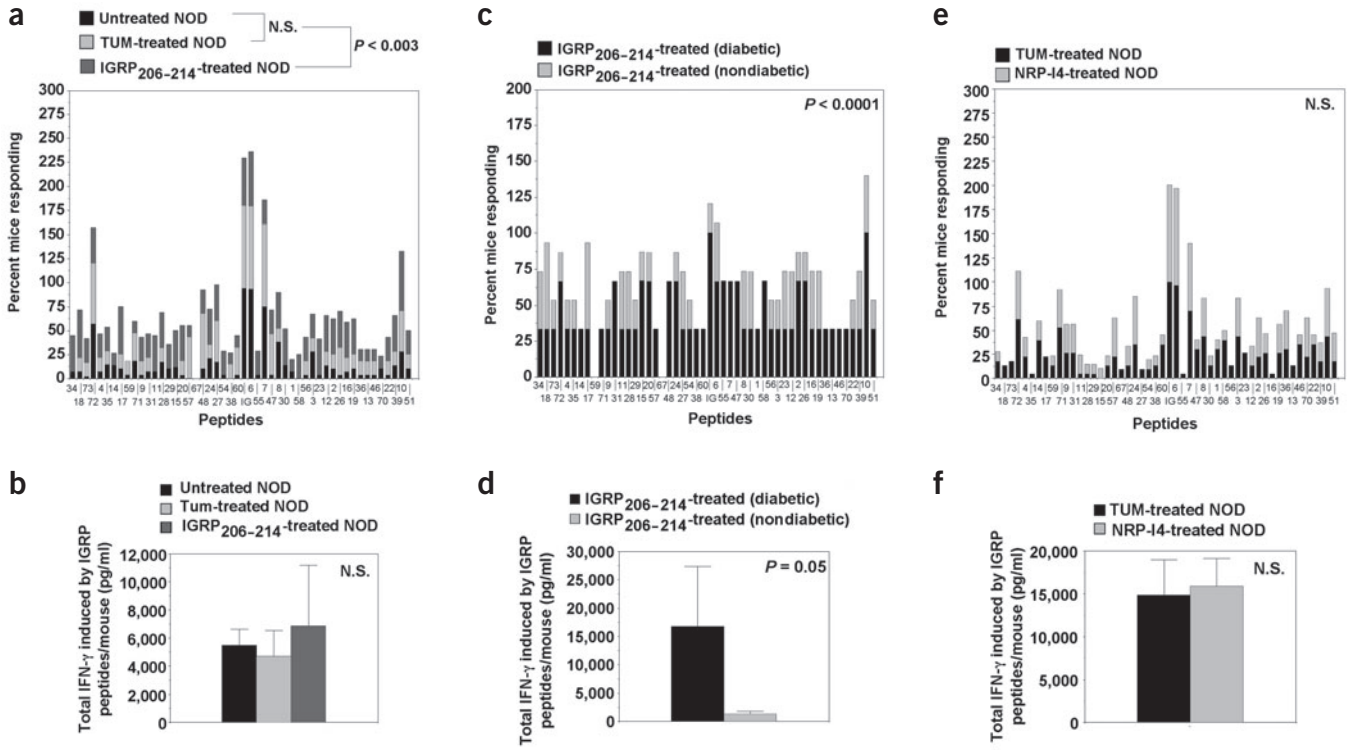

Figure 6 Unlike NRP-I4-treatment, treatment with $IGRP_{206–214}$ induces increased responses against other IGRP epitopes. (**a**) Percentage of untreated and TUM- or $IGRP_{206–214}$-treated NOD mice that contain intraislet $CD8^+$ T cells recognizing epitopes of IGRP. *P* values reflect differences in the total number of positive responses (>50 pg/ml) in the different groups. Data are from **Supplementary Table 2** online. (**b**) Cumulative amounts of IFN-γ secreted by islet-derived $CD8^+$ T cells of individual mice in response to IGRP peptides. (**c**) As in **a**, except **c** compares $IGRP_{206–214}$-treated NOD mice that developed diabetes and those that did not. (**d**) Cumulative amounts of IFN-γ secreted by islet-derived $CD8^+$ T cells of individual diabetic and nondiabetic $IGRP_{206–214}$-treated NOD mice in response to IGRP peptides. Data are from **Supplementary Table 2** online. (**e**) As in **a**, except that **e** compares NRP-I4–treated mice (intraperitoneally starting at 3–4 weeks) and a new cohort of TUM-treated (control) mice. Data are from **Supplementary Table 3** online. (**f**) Cumulative amounts of IFN-γ secreted by islet-derived $CD8^+$ T cells of individual NRP-I4– or TUM-treated NOD mice (intraperitoneally starting at 3–4 weeks) in response to IGRP peptides. Although the total amounts of IFN-γ secreted by the islet-associated T cells of these cohorts of mice were higher than those corresponding to the cohorts studied in **a**–**d**, no differences were seen for NRP-I4– versus TUM-treated mice. Data are from **Supplementary Table 3** online. All values correspond to responses above background (against TUM). N.S., not significant. Numbers on *x*-axis in **a**,**c** and **e** refer to the different peptides tested, ordered from the amino to the carboxy terminus of IGRP (**Supplementary Table 1** online).

preferentially targeted high-avidity clonotypes. In support of this interpretation, NRP-V7 and NRP-I4 were equally tolerogenic in 8.3-NOD mice, expressing an intermediate-to-high avidity $IGRP_{206–214}$-reactive TCR, but showed substantially different tolerogenic activities in NOD mice expressing a low avidity $IGRP_{206–214}$-reactive TCR (P. Serra & P. Santamaria, unpublished data). Altogether, these observations suggest that administration of protective doses of NRP-A7 and NRP-I4 results in the selective deletion of pathogenic, high-avidity clonotypes, and that sustained deletion of high-avidity $IGRP_{206–214}$-reactive $CD8^+$ clonotypes is accompanied by progressive occupation of the corresponding intraislet space by their low-avidity counterparts.

Given that the efficacy of vaccination protocols for induction of antitumor immunity requires the recruitment of high-avidity CTLs[31–33], it is safe to assume that deletion of prevalent high-avidity clonotypes by NRP-I4 and NRP-A7 does protect mice from T1D. The loss of NRP-A7's antidiabetogenic potential with increased doses of peptide despite substantial depletion of the total $IGRP_{206–214}$-reactive T-cell pool implied that the protective effect of NRP-I4 and NRP-A7 must have also required the recruitment of low avidity clonotypes, a concept supported by mathematical modeling of the data (A.F.M.M., P.S. & L.E.K., unpublished data). Whether these cells afford diabetes protection by secreting immunoregulatory factors or other mechanisms remains to be determined. Autoreactive T cells with immunoregulatory properties have been found in normal individuals[34], and APLs are known to be able to induce specific immunoregulatory T cells[11,35].

Another fundamental observation of this study is that near-complete deletion of the $IGRP_{206–214}$-reactive $CD8^+$ subset was associated with markedly increased responses against subdominant epitopes of IGRP, especially in mice that had become diabetic. This suggests that depletion of the $IGRP_{206-214}$-reactive $CD8^+$ T-cell niche created a 'vacuum' that somehow promoted the expansion of diabetogenic, subdominant epitope-specific clonotypes. These subdominant epitope-specific (and potentially high-avidity) CTLs may be highly effective at destroying their cellular targets because they readily evade mechanisms of tolerance, as proposed recently[36]. Because NRP-I4 treatment afforded diabetes protection without inhibiting (or enhancing) the recruitment of these subdominant IGRP epitope-specific clonotypes, it is reasonable to suspect that enhanced recruitment of low-avidity $IGRP_{206–214}$-reactive clonotypes is both necessary and sufficient for the ability of NRP-I4 to blunt the progression of diabetes. Conceivably, these two opposing phenomena (tolerance of dominant epitope-specific T cells over a large avidity spectrum and recruitment of subdominant epitope-specific T cells) might account for the ineffectiveness of human trials using full-length protein autoantigens[6–12]. It is important to note, however, that

these data do not imply that deletional strategies will be inappropriate for the prevention of autoimmunity. Rather, our observations suggest that for these strategies to work, they will have to target multiple epitope specificities, rather than only dominant ones.

In sum, our findings suggest that complete elimination of a dominant T-cell subpopulation by using high doses of high-affinity APLs is an inefficient way to halt the progression of cellularly complex, polyclonal autoimmune responses. Rather, we argue that effective prevention of such diseases with APLs requires the selective elimination of high-avidity clonotypes and the unopposed recruitment of their low-avidity, nonpathogenic counterparts. The fact that this outcome occurs only within a narrow range of dose and functional avidity bears an important lesson that may aid in the design of APL- or self-antigen–based vaccines as 'tolerogens' in autoimmunity. Careful examination of peptide affinity for MHC and TCR and dose are therefore warranted in the design of clinical trials.

METHODS

Mice, cell lines and antibodies. 8.3-NOD mice, expressing the TCRαβ rearrangements of the $IGRP_{206–214}$-reactive $CD8^+$ clone NY8.3, have been described[19]. We purchased NOD mice from Taconic Farms and Lyt-2 (CD8α)-, L3T4-, Vβ8.1/8.2-, $H\text{-}2K^d$- and $H\text{-}2D^b$-specific monoclonal antibodies from PharMingen. All experiments were approved by the University of Calgary Animal Care Committee and were performed in compliance with guidelines from the Canadian Council of Animal Care.

Peptides and peptide libraries. We prepared the peptide libraries used to identify APLs utilizing multipin synthesis technology and standard Fmoc chemistry (Chiron Technologies)[22]. Representative APLs (**Fig. 1**) were chosen for *in vivo* experimentation. We prepared specific single custom peptides at >80% purity and sequenced them by ion-spray mass spectrometry (Chiron Technologies). Peptides were resuspended at 10 mg/ml in 0.1% acetic acid, separated into aliquots at –80 °C and resuspended in PBS before use. We designed $H\text{-}2K^d$- or $H\text{-}2D^b$-binding IGRP peptide libraries by screening the IGRP amino acid sequence with RANKPEP and SYFPEITHI. We synthesized peptide sets composed of predicted MHC binders with scores >39 (Rankpep) or >25 (Syfpeithi) and used them at 10 μM.

Generation of NOD islet-derived $CD8^+$ T-cell lines. We generated islet-derived $CD8^+$ cells by culturing 10–50 islets/well in 24-well plates in RPMI-1640 media containing 10% FBS and 0.5 U/ml Takeda recombinant human interleukin (IL)-2, for 6–10 d.

Proliferation assays. Naive or NRP-A7–differentiated splenic $CD8^+$ cells from 8.3-NOD mice (2×10^4/well) were incubated, in duplicate, with peptide-pulsed (0.01, 0.1 and 1 μM), γ-irradiated (3,000 rad) NOD splenocytes (10^5/well) for 3 d at 37 °C in 5% CO_2. We pulsed cultures with 1 μCi of [^{3}H]-thymidine during the last 18 h of culture and harvested them.

Cytokine secretion. We incubated naive splenic $CD8^+$ cells from 8.3-NOD mice (2×10^4/well) with peptide-pulsed (0.001–10 μM) γ-irradiated NOD splenocytes (10^5/well) in 96-well plates for 48 h at 37 °C. We tested short-term islet-derived T-cell lines (at 2×10^4 $CD8^+$ cells/well) the same way, but used 10 μM of peptide. We assayed the supernatants (100 μl) in duplicate for IL-2, IL-4 and/or IFN-γ content by ELISA using commercially available kits (R&D Systems).

Cytotoxicity assays. We performed cytotoxicity assays using peptide-pulsed (1 μg/ml) ^{51}Cr-sodium chromate-labeled RMA-S/K^d cells as targets (1×10^4) and islet-derived $CD8^+$ T cells (1×10^5) at a 1:10 target/effector ratio, as described elsewhere[21]. Values obtained with the negative control peptide TUM were subtracted.

Tetramer staining. We prepared tetramers and used them as described previously[13]. Islet-derived T cells (~0.5×10^6/20 μl) were stained for 45 min at 25 °C in 20 μl of wash media (0.2% sodium bicarbonate, 0.05% sodium azide and 2% FBS in RPMI-1640) containing a FITC-conjugated $CD8_β$-specific monoclonal antibody and tetramer (85.5 nM). For the analysis of tetramer staining at equilibrium, we stained T cells with different concentrations of tetramers (8.55, 17.1, 42.75 and 85.5 nM). After washing, we resuspended cells in 100 μl of wash media, fixed them in 1% paraformaldehyde and analyzed them with a flow cytometer. We determined the apparent K_d values by plotting the negative reciprocal of the slope of the line fit to Scatchard plots of fluorescence units (median of $CD8^+$ population tetramer staining)/nM versus fluorescence units.

$H\text{-}2K^d$-stabilization assay. RMA-SK^d cells that had been cultured overnight at 26 °C were seeded at 10^4 cells/well in 96-well plates, pulsed with peptides in RPMI-1640, 0.25% BSA for 1 h at 26°C, incubated at 37°C for 3 h, washed, stained with FITC-conjugated $H\text{-}2K^d$- or $H\text{-}2D^b$-specific monoclonal antibodies and analyzed for MHC class I expression by cytometry[21]. Controls used included TUM (K^d-binder), LCMV-GP33 (D^b-binder) and no peptide. We measured the dissociation constant (K_d) by using different concentrations of peptides (10, 1, 0.1, 0.01, 0.001 μM). We calculated the K_d values as the concentration of peptide required to rescue 50% of K^d molecules on RMA-SK^d cells (100% at 10 μM).

Peptide treatment. We injected cohorts of 3–4-week-old female NOD mice with 1–100 μg of peptide in PBS intraperitoneally or intravenously. We repeated this every 2 weeks until the third injection, and every 3 weeks thereafter. We treated a cohort of 10-week-old NOD females with 100 μg of NRP-I4 intraperitoneally to ascertain the ability of this peptide to blunt progression of diabetes in mice with full-blown insulitis. Mice were killed at onset of diabetes or between 18–22 weeks of age to characterize the specificity and avidity of their islet-associated $CD8^+$ T cells, or monitored for development of T1D until at least 28 weeks of age. We determined the 8.3-$CD8^+$ tolerogenic activity of APLs by treating 8.3-NOD mice with one intraperitoneal injection of 100 μg of peptide. Mice were killed 1 week after treatment, and their spleens analyzed for presence of 8.3-$CD8^+$ T cells by flow cytometry.

Statistical analyses. We compared data using linear regression and variance analysis, Mann-Whitney U test or χ^2.

URLs. Rankpep, http://immunax.dfci.harvard.edu/Tools/; SYFPEITHI, http://www.syfpeithi.de/

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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