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Expansion of the Antigenic Repertoire of a Single T Cell Receptor upon T Cell Activation

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Activated T cells and their naive precursors display different functional avidities for peptide/MHC, but are thought to have identical antigenic repertoires. We show that, following activation with a cognate mimotope (NRP), diabetogenic CD8+ T cells expressing a single TCR (8.3) respond vigorously to numerous peptide analogs of NRP that were unable to elicit any responses from naive 8.3-CD8+ T cells, even at high concentrations. The NRP-reactive, in vivo activated CD8+ cells arising in pancreatic islets of nonobese diabetic mice are similarly promiscuous for peptide/MHC, and paradoxically this promiscuity expands as the avidity of the T cell population for NRP/MHC increases with age. Thus, activation and avidity maturation of T lymphocyte populations can lead to dramatic expansions in the range of peptides that elicit functional T cell responses. The Journal of Immunology, 2001, 167: 655–666.

The poor shape complementarity of the TCR peptide/MHC interface allows certain TCRs to recognize different peptides in the context of a single MHC molecule, even peptides that do not show strong sequence homology (1–15). These peptide ligands induce T cell responses that range from partial or full activation (agonists) to antagonism (antagonists), owing to the ability of the peptides to elicit differential TCR signaling (16). The discovery that the TCR-peptide/MHC interaction has a high degree of degeneracy has provided a structural framework for the hypothesis that initiation of some autoimmune diseases may be triggered by foreign Ags capable of activating autoreactive T cells (molecular mimicry) (17, 18). It has been shown that, for at least some T cells, Ag recognition is highly degenerate, and that molecular mimicry at the CD4+ T cell level may play a role in autoimmune disorders like multiple sclerosis, myocarditis, rheumatoid arthritis, and herpes stromal keratitis (9, 12, 14, 15, 19–30).

The putative cross-reactive T cells capable of causing autoimmune disease via molecular mimicry must somehow evade normal immune tolerance mechanisms. Engagement of self-peptide/MHC complexes by autoreactive thymocytes on bone marrow-derived APCs usually leads to thymocyte death or to functional unresponsiveness to subsequent encounters with Ag (31). Autoreactive T cells, particularly those recognizing autoantigens that are not fer-

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3 Abbreviations used in this paper: NOD, nonobese diabetic; CM, complete medium; NAT, naturally occurring mimic of NRP; rhIL-2, recombinant human IL-2; mfl, mean fluorescence intensity.
mice expressing a transgenic, highly diabeticogenic, H-2Kd-restricted TCR (8.3-TCR) (8.3-NOD.RAG-2−/−) (41). This TCR recognizes the peptides NRP (KYNKANWF) and NRP-A7 (KYNKANFL) in the context of H-2Kd (42), and is representative of TCRs expressed by the CD8+ T cells that, upon avidity maturation, drive the progression of islet inflammation to diabetes in NOD mice (34). In this work we found that 1) in vitro activation of 8.3-TCR-transgenic (monoreceptor) T cells resulted in a dramatic expansion of the T cells’ antigenic repertoire; 2) the NRP-reactive T cell population arising spontaneously in the pancreatic islets of wild-type NOD mice displayed a similar degree of antigenic promiscuity as did in vitro activated 8.3-CD8+ T cells; and 3) the antigenic promiscuity of the wild-type, intraislet NRP-reactive CD8+ T cell population increased as the avidity of the population for NRP-A7/H-2Kd increased with age. These observations demonstrate that, upon activation and avidity maturation, T cell populations can mount functional responses against antigenic peptides that they previously ignored.

Materials and Methods

Mice, cell lines, and Abs

Mice (8.3-NOD.RAG-2−/−: 6- to 8-wk old) expressing the TCRαβ rearrangements of the H-2Kk-restricted β cell-reactive CD8+ T cell clone NY8.3 have been described (41). NOD mice were purchased from Taconic (Germantown, NY). RMA-SK3 cells were obtained from B. Wipke and M. Bevan (University of Washington, Seattle, WA). The GK1.5 (anti-CD4) hybridoma was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Anti-Lyt-2 (CD8α) (53-6.7), anti-L3T4 (IM7), anti-Vβ1/1.8/2 (MR5-5), anti-CD44-biotin (IM7), anti-CD69-biotin (H1.2F3), anti-H-2Kd (SF1-1.1), and anti-H-2Dd (KH95) mAbs were obtained from PharMingen (San Diego, CA).

Peptides/database searches

The peptide libraries were prepared using multipin synthesis technology and standard F-moc chemistry (Chiron Technologies, San Diego, CA). Specific single custom peptides were purified through reversed phase HPLC to >80% purity and sequenced by ion spray mass spectrometry. Peptides were resuspended in 0.1 M HEPES (Sigma, St. Louis, MO) in 40% acetonitrile (Fisher Scientific, Fair Lawn, NJ) at pH 7.4. The first screen was performed using the following NRP-based dipeptide libraries: DE1 (D or E/YNKANWF), NQ1, GS, FY1, VT1, AL1, WA1, DE1, KR3, GS3, FY3, VT3, AL3, W34, DE4, NQ4, GS4, FY4, VT4, AL4, WA4, DE5, KR5, NQ5, GS5, FY5, VT5, W56, DE6, KR6, GS6, FY6, VT6, AL6, W67, DE7, KR7, NQ7, GS7, FY7, VT7, AL7, DE8, KR8, NQ8, GS8, VT8, AL8, and W8. The single amino acid variants of NRP that were tested in subsequent experiments are listed in Fig. 2. Database searches were performed using the PIR database and a prosearch algorithm (version 1.3) using the search string (supertope): (KYNKANWF) and M. Bevan (University of Washington, Seattle, WA). The GK1.5 (anti-CD4) hybridoma was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Anti-Lyt-2 (CD8α) (53-6.7), anti-L3T4 (IM7), anti-Vβ1/1.8/2 (MR5-5), anti-CD44-biotin (IM7), anti-CD69-biotin (H1.2F3), anti-H-2Kd (SF1-1.1), and anti-H-2Dd (KH95) mAbs were obtained from PharMingen (San Diego, CA).

Proliferation assays

Naïve or NRP-differentiated spleen CD8+ T cells from 8.3-NOD.RAG-2−/− mice (2 × 105/well) were incubated in duplicate with peptide-pulsed (0.01, 0.1, and 1 μM), γ-irradiated (3000 rad) NOD splenocytes (105/well) for 3 days at 37°C in 5% CO2. Cytokines were pulsed with 1 μCi of [3H]thymidine during the last 18 h of culture and harvested.

Cytokine secretion

Naïve or NRP-differentiated splenic CD8+ T cells from 8.3-NOD.RAG-2−/− mice (2 × 105/well) were incubated with peptide-pulsed (0.001, 0.01, 0.1, 1, and/or 10 μM) γ-irradiated NOD splenocytes (105/well) in 96-well plates for 48 h at 37°C. Islet-derived T cell lines or FACS-sorted tetramer-positive and -negative CD8+ T cells (adjusted at 2 × 104 CD8+ T cells/well) were tested the same way, but using 1 μg of peptide. The supernatants (100 μl/well) were assayed for IL-2, IL-4, and/or IFN-γ content by ELISA using commercially available kits (Genzyme, Cambridge, MA).

Generation of islet-derived- and NRP-differentiated splenic CD8+ T cells

Naïve spleen cells from 8.3-NOD.RAG-2−/− mice were adjusted to 104 CD8+ T cells/100 μl of complete medium (CM; RPMI 1640 medium containing 10% FBS), stimulated with peptide-pulsed (1 μM) NOD splenocytes for 3 days, and expanded in the presence of rIL-2 for 4–6 days. Islet-derived CD8+ T cells from 8.3-NOD.RAG-2−/− and nontransgenic NOD mice were generated by culturing pancreatic islets in CM containing 0.5 U/ml Takeda recombinant human IL-2 (rhIL-2; 10–50 islets/well in 24-well plates). The in vivo activated, IL-2Rγ-lymphocytes migrating from islets into the culture medium were further expanded in rhIL-2-containing medium for an additional 3–6 days. T cells were used in functional assays within 6–9 days of islet isolation.

Tetramer staining and cell sorting

Tetramers were prepared as described by Altman et al. (43). Hand-picked pancreatic islets pooled from 15–40 mice/age group/experiment (9- or 20-wk-old NOD mice) were cultured in CM supplemented with 0.5 U/ml Takeda rhIL-2 for 7–9 days. T cells (105 per 20 μl) were then stained for 45 min at room temperature in 20 μl of wash medium (0.2% sodium bicarbonate, 0.1% sodium azide, and 2% FBS in RPMI 1640) containing anti-CD8-FITC (clone YTS169.4; 0.5 μg) and NRP-A7/Kd tetramer (88.5 nM). After washing, the cells were resuspended in wash medium and sorted with a FACStar flow cytometer (BD Biosciences, Mountain View, CA). Purity of sorting was 87–93% for tetramer-positive cells and 95–97% for tetramer-negative cells.

5Cr-release assays

RMA-SK5 cells (preincubated at 26°C overnight) were labeled with [51Cr]sodium chromate (DuPont-NEN, Boston, MA) for 2 h, washed, resuspended at 105 cells/ml in RPMI 1640 containing 0.25% BSA, seeded at 105 cells/100 μl/well, pulsed with peptides (0.1–1 μM) for 1 h at 37°C, and used as target cells in 5Cr-release assays. Effector cells (NRP-differen- tiated splenic CD8+ T cells; 100 μl) were added to each well in duplicate at a 1:10 target-effector ratio. Cultures of RMA-SK5 cells with peptides but no T cells were used as controls to confirm that the peptides were not cytotoxic. Plain medium or 1% Triton X-100 was added to sets of target cells for examination of spontaneous and total cell lysis, respectively. The plates were incubated at 37°C for 8 h, and the supernatants were collected for determination of specific 5Cr release: (% lysis = 100 × (test cpm – spontaneous cpm)/total cpm – spontaneous cpm)). Values >20% release were considered significant.

H-2Kd-stabilization assay and H-2Kd/peptide association rates

RMA-SK5 cells that had been cultured overnight at 26°C were seeded in quadruplicate, at 106 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, and stained with anti-H-2Kd-FITC or anti-H-2Dd-FITC, and the mean fluorescence intensity (mfi) for MHC class I expression was analyzed by flow cytometry (42). Controls used included: (H-2Kd-binding), lymphocytic choriomeningitis virus-GP33 (H-2Dd-binding), and no peptide. The Kd was measured by repeating the experiments described above except using different concentrations of peptides (10, 1, 0.1, 0.01, and 0.001 μM). The Kd values were calculated as the concentration of peptide required to rescue 50% of the H-2Kd molecules on RMA-SK5 cells (100% at 10 μM).

Statistical analyses

Data were compared by Mann-Whitney U test or χ2.

Results

Single amino acid substitution analysis of NRP

To determine which amino acid substitutions could be introduced at each position of the NRP sequence without inducing a major loss of functional activity, we first tested the agonistic activity of NRP-based combinatorial dipeptide libraries prepared in the positional scanning format. To generate the libraries, each of the seven nonanchor positions of NRP (P1 and P3–P8) was substituted with groups of two related amino acids (DE, KR, NQ, GS, FY, VT, AL, and WI). The libraries were tested for their ability to induce proliferation of, and IFN-γ, IL-2, and IL-4 secretion by, naïve splenic CD8+ T cells from 6- to 8-wk-old 8.3-NOD.RAG-2−/−, which
bear a monoclonal TCR repertoire (41). Negative and positive controls included the H-2K\(^{d}\)-binding peptide Tum (KYQAVTTTL) and NRP or NRP-A7, respectively. 8.3-NOD.RAG\(^{−/−}\) mice do not usually develop islet inflammation until after 10 wk of age, and most, if not all, of their peripheral CD8\(^{+}\) T cells have a naive phenotype (CD11\(\alpha\)low, CD44\(\text{low}^\), CD69\(^\text{mid}\), CD62L\(\text{high}^\)) (Ref. 41 and data not shown). In general, there was a good correlation between the magnitude of the proliferative and cytokine (IL-2 and IFN-\(\gamma\)) secretory responses induced by each library (Fig. 1; none of the peptide libraries induced IL-4 secretion; data not shown). Interestingly, naive 8.3-CD8\(^{+}\) T cells did not secrete IL-2 in response to 12 of the 24 libraries that elicited IFN-\(\gamma\) secretion (Fig. 1), and each of the remaining 12 libraries induced significantly more IFN-\(\gamma\) than IL-2 (Fig. 1). The sequences of dipeptide libraries that elicited functional responses from 8.3-CD8\(^{+}\) T cells \(\geq 10\%\) of the proliferation and/or cytokine secretion induced by NRP but not TUM in at least two of three experiments; see Fig. 1) were selected for deconvolution. This was performed by determining which of the 52 possible peptides deduced from the dipeptide library screen could induce the 8.3-CD8\(^{+}\) T cell responses mentioned above, over a range of concentrations (1, 0.1, and 0.01 \(\mu\)M) (Fig. 2 and data not shown). This set of experiments also included single amino acid variants of NRP that carried substitutions (other than cysteine and methionine) that were not tested in the dipeptide library screen (NRP-R1, NRP-H1, NRP-Q3, NRP-R4, NRP-H4, NRP-H5, NRP-A6, NRP-H6, NRP-H7, NRP-Y8, and NRP-H8) as well as two negative control peptides deduced from a negative dipeptide library (NRP-W8 and NRP-L8) (Fig. 2). Peptides NRP-L5, NRP-S5, NRP-Q6, and NRP-I7 were largely insoluble and could not be tested. As shown in Fig. 2, 42 of the 65 single amino acid variants of NRP that were tested in these experiments triggered proliferation of, and/or cytokine (IL-2 and/or IFN-\(\gamma\)) secretion by, naive 8.3-CD8\(^{+}\) T cells. As expected on the basis of previous studies in other systems (14), substitutions of the two primary TCR contact residues K4 and F8 for any other residue, whereas 11 peptides were unable to elicit proliferation of, or IL-2 and/or IFN-\(\gamma\) secretion by, naive 8.3-CD8\(^{+}\) T cells.
without the potentially confounding contribution of assay sensitivity to outcome. As shown in Fig. 3, in vitro differentiated 8.3-CD8 T cells secreted high levels of IFN-γ in response to 10 of the 20 nonagonistic peptides that were tested. These 10 peptides could also efficiently reconstitute H-2Kd cDNA-transfected RMA-S cells. These 10 peptides could not be reproduced in subsequent experiments. The Kd values were determined as described in Materials and Methods. Data are representative of two to three experiments. Shaded peptides are nonagonistic peptides chosen for further experimentation.

**FIGURE 2.** Single amino acid variants of NRP that were tested. Most correspond to NRP variants deduced from the dipeptide library screen shown in Fig. 1 (i.e., agonistic dipeptide libraries). The remaining variants (NRP-R1, NRP-Q3, NRP-R4, NRP-L5, NRP-Q6, NRP-I7, NRP-Y8, NRP-H1, NRP-H3, NRP-H4, NRP-H5, NRP-H6, NRP-H7, and NRP-H8) are peptides carrying amino acid substitutions not tested in the dipeptide library screen. Data are shown as the percentage of the responses obtained with each peptide at 1 and 0.1 μM (in brackets; values at 0.1 μM are shown only if >10% of the value induced by NRP at the same concentration) with respect to the corresponding responses induced by NRP at each concentration. The absolute values for the NRP-induced responses were 60,000 ± 3,075 cpm, 25 ± 11 pg/ml IFN-γ, and 28 ± 18 pg/ml IL-2 at 1 μM; and 1,705 ± 1,113 cpm, 9 ± 5 pg/ml IFN-γ, and 9 ± 9 pg/ml IL-2 at 0.1 μM. Similar differences among peptides were obtained at 0.01 μM (data not shown). The peptide-induced responses are color coded (low, blue; medium, yellow; high, green; and very high, red). Peptides are classified as full agonists (1/1), or partial agonists (0.1-0.9). NRP-H1, NRP-I6, and NRP-L8 were classified as nonagonists because the weak and partial responses observed in the experiment shown could not be reproduced in subsequent experiments. The Kd values were determined as described in Materials and Methods. Data are representative of two to three experiments.

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**Antigenic promiscuity upon T cell activation**

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To investigate whether the increased antigenic promiscuity of the 8.3-TCR on activated vs naive T cells also applied to NATs, we searched protein databases for NRP mimics. This was performed using a supertope that included residues that were tolerated by NRP (Fig. 1) plus M or C at any nonanchor position, F or Y at the first anchor position (P2), and L, I, or V at the second anchor position (P9). This analysis resulted in the identification of 92 potential mimics of NRP (data not shown). All these peptides were tested for their ability to bind to H-2Kd molecules on RMA-SK<sup>6</sup> cells, to elicit cytokine secretion by, and proliferation of, naive 8.3-CD8<sup>+</sup> T cells, and to enable the cytotoxic activity of NRP-differentiated 8.3-CD8<sup>+</sup> T cells. Thirty-two of these 92 peptides did not bind to H-2K<sup>d</sup> molecules and had no functional activity on 8.3-CD8<sup>+</sup> T cells. The remaining 60 peptides bound H-2K<sup>d</sup> molecules or were recognized by NRP-differentiated 8.3-CD8<sup>+</sup> T cells in cytotoxicity assays (>20% 51Cr release; data not shown), and a few of them elicited a functional response from naive 8.3-CD8<sup>+</sup> T cells (Fig. 4). These 60 peptides were classified into three broad groups based on their ability to elicit functional responses from naive 8.3-CD8<sup>+</sup> T cells: 1) peptides that did not elicit any responses (designated as “nonagonists”; n = 54); 2) peptides that elicited weak responses (designated as “partial agonists”; n = 3); and 3) peptides that elicited strong responses (designated as “full agonists”; n = 3) (labeled +, +/-, or +, respectively, in Fig. 4). It should be noted that the designations agonistic and nonagonistic are arbitrary and refer exclusively to the ability of the different peptides to induce functional T cell responses from naive 8.3-CD8<sup>+</sup> T cells; as shown below, some nonagonistic mimics can induce functional responses from differentiated 8.3-CD8<sup>+</sup> T cells. As expected on the basis of the data shown in Fig. 1, presence of the two TCR contact residues of NRP (K<sub>4</sub> and F<sub>8</sub>) was necessary for full agonism (see Fig. 4). However, their presence was insufficient because only 6 of 24 peptides carrying these two residues had agonistic activity on naive 8.3-CD8<sup>+</sup> T cells (Fig. 4). Interestingly, the number of agonistic residues carried by individual peptides was not a good marker of the degree of agonism because poorly agonistic substitutions (in NRP) were
present in mimics with full agonistic activity. However, the number of nonanchor residues within a peptide that were shared with NRP was a significant indicator of agonism ($1.9 \pm 0.1$ for nonagonistic peptides vs $3.5 \pm 0.3$ for agonistic peptides, $p < 0.0005$).

When taken together, these data indicated that 1) the agonistic activity of NATs was a function of their ability to contact the 8.3-TCR, rather than of their affinity for MHC; and 2) only a very small fraction of all the NATs carrying the two primary TCR contact residues of NRP could trigger naive 8.3-CD8$^+$ T cells.

**Responsiveness of ex vivo and in vivo differentiated 8.3-CD8$^+$ T cells against nonagonistic mimics of NRP**

We next compared the ability of NRP-A7 (an alanine mutant of NRP with superior agonistic properties), the six naturally occurring agonistic mimics, and the nonagonistic mimics described above, to trigger IFN-γ and IL-2 secretion by naive and NRP-differentiated 8.3-CD8$^+$ T cells. In general, agonistic mimics induced more IFN-γ and IL-2 secretion from differentiated 8.3-CD8$^+$ T cells than from naive 8.3-CD8$^+$ T cells (Fig. 5A). Nonagonistic mimics that lacked both K4 and F8 were unable to trigger IFN-γ and IL-2 secretion from NRP-differentiated 8.3-CD8$^+$ T cells (data not shown), as was the case for naive 8.3-CD8$^+$ T cells (see above). However, 12 of the 31 nonagonistic NRP mimics that carried TCR-contact residues K4 and F8 of NRP (comprising the 11 naturally occurring homologues and the 20 single amino acid variants that are highlighted in gray in Figs. 1 and 4) were able to trigger IFN-γ secretion by NRP-activated 8.3-CD8$^+$ T cells (Fig. 5A, upper panel). Nine of these 12 nonagonistic mimics were also able to elicit IL-2 secretion from differentiated 8.3-CD8$^+$ T cells (Fig. 5A, lower panel). Surprisingly, some of these peptides were at least as efficient as NRP-A7 or some of the six naturally occurring agonists at eliciting IFN-γ and/or IL-2 secretion by these T cells (Fig. 5A). Note that most of these peptides were unable to trigger IFN-γ secretion by naive 8.3-CD8$^+$ T cells, even at high concentrations (see below and Fig. 6). Kinetic studies indicated that, as seen with single amino acid variants of NRP, there was no correlation between the on-rates ($K_o$) of peptide binding to MHC (Fig. 4) and the degree or type of response elicited by the different peptides on differentiated 8.3-CD8$^+$ T cells (Fig. 5A). This indicated that the ability of nonagonistic NRP mimics to elicit cytokine secretion by differentiated CD8$^+$ T cells is stringently dependent on their ability to contact the TCR. Taken together, these results confirmed what the results with single amino acid variants of NRP had indicated, namely, that differentiated 8.3-CD8$^+$ T cells respond efficiently to many more peptides than their naive precursors.

To confirm that the promiscuity of differentiated 8.3-CD8$^+$ T cells was not an artifact of in vitro stimulation with NRP, we restested all the peptides on 8.3-CD8$^+$ T cells grown from pancreatic islets of 8.3-NOD.RAG-2$^{-/-}$ mice. Purified islets were cultured for 6–9 days in rIL-2-containing medium (in the absence of peptide) to selectively expand IL-2R-positive CD8$^+$ T cells. As shown in Fig. 5B, these in vivo activated CD8$^+$ T cells responded to even more nonagonistic mimics than NRP-differentiated 8.3-CD8$^+$ T cells, suggesting that the quality and/or degree of activation modulates the degree of antigenic promiscuity exhibited by an activated T cell. All the nonagonistic peptides capable of triggering IFN-γ secretion from differentiated 8.3-CD8$^+$ T cells could also efficiently elicit their cytotoxic activity (data not shown).

To ascertain whether the increased antigenic promiscuity of differentiated 8.3-CD8$^+$ T cells was the result of a reduction in the threshold of TCR triggering upon T cell activation, we performed titration experiments with different concentrations of peptides. Naive 8.3-CD8$^+$ T cells did not secrete, or secreted only very low levels of, IFN-γ when challenged with high concentrations of nonagonistic NRP mimics (Fig. 6, upper left panel). Surprisingly, differentiated 8.3-CD8$^+$ T cells secreted as much or more IFN-γ in response to low concentrations of some nonagonistic peptides (i.e., NRP-W3) than in response to similar concentrations of NRP-A7 or agonistic mimics (i.e., NAT-47) (Fig. 6). This indicated that the ability of nonagonistic peptides to trigger differentiated 8.3-CD8$^+$ T cells did not result from a concentration-dependent increase in the sensitivity of differentiated vs naive T cells to TCR ligation, but rather from an ability of differentiated T cells to respond efficiently to low concentrations of poor ligands of their TCRs.

**Responsiveness of NRP/NRP-A7-reactive CD8$^+$ T cells propagated from islets of prediabetic NOD mice**

It was possible that the reactivity of 8.3-CD8$^+$ T cells for nonagonistic mimics of NRP was a peculiarity of the 8.3-TCR. To rule this out, we investigated whether these peptides could also induce functional responses from the in vivo activated NRP/NRP-A7-reactive CD8$^+$ T cells that are spontaneously recruited into pancreatic islets in nontransgenic NOD mice (34). Islets from nine different nondiabetic 9-wk-old NOD mice were cultured in the presence of rIL-2 for 6–9 days to selectively expand IL-2R-positive CD8$^+$ T cells, which contain a significant percentage of NRP/NRP-A7-reactive CD8$^+$ T cells (34). These nine T cell lines were tested for their ability to secrete IFN-γ in response to 12 naturally occurring nonagonistic NRP mimics carrying K4 and F8 and the six naturally occurring agonistic NRP mimics (Fig. 7). Controls included Tum (negative control), NRP-A7 (positive control), and INS (an insulin-derived peptide recognized by islet-associated T cells from young NOD mice; Ref. 45). We used NRP-A7 instead of NRP as positive control peptide because NRP-reactive CD8$^+$ T cells from NOD mice bind NRP-A7/K4 tetramers with higher avid-
ity than NRP/K d tetramers (34). As shown in Fig. 7, there was a high degree of variability among lines in terms of the number of peptides they could respond to (positive responses highlighted). However, six of the nine lines responded efficiently to one or more nonagonists, and in some cases the response against nonagonistic mimics was more vigorous than the response against agonistic mimics. This demonstrated that, like in vitro activated 8.3-CD8 T cells, in vivo activated NRP-reactive CD8 T cells propagated from islets of nontransgenic NOD mice can also respond to non-agonistic mimics of NRP.

Increased antigenic promiscuity of a polyclonal NRP/NRP-A7-reactive CD8 T cell population upon avidity maturation

Progression of pancreatic islet inflammation to overt diabetes in the NOD mouse overlaps with the “avidity maturation” of the NRP/NRP-A7-reactive CD8 T cell population; as prediabetic NOD mice age (from 9 to 20 wk), their in vivo activated, islet-associated CD8 T cells contain increasing numbers of NRP-A7-reactive cells, and these cells bind NRP-A7/K d tetramers with increased avidity and longer half-lives (34). We reasoned that this increase in the avidity of in vivo activated NRP/NRP-A7-reactive CD8 T cells for peptide/MHC might allow the T cells to respond to lower affinity ligands of their TCR. If that were true, the avidity maturation of the NRP/NRP-A7-reactive CD8 T cell population would be paralleled by an increase in the antigenic promiscuity of the T cells. This hypothesis was tested by investigating whether activated CD8 T cells propagated from islets of 13 different 20-wk-old nondiabetic NOD mice could respond to more nonagonistic (but not agonistic) NRP mimics than the activated CD8 T cells propagated from islets of 9-wk-old NOD mice. Note that although lines derived from 20-wk-old mice have an overall higher avidity for NRP-K d than cells from younger animals, they contain percentages of NRP-A7/K d tetramer-positive T cells that are similar to those seen in lines derived from 9-wk-old mice (34). As shown in Fig. 7, CD8 T cells propagated from 20-wk-old mice responded to significantly more nonagonistic mimics of NRP (but not NRP-A7, as expected) than cells from 9-wk-old mice.
9-wk-old mice (Fig. 7). Staining of islet-derived T cells from 9- and 20-wk-old NOD mice with mAbs specific for activation markers confirmed that these differences in the ability of cells from 9- and 20-wk-old NOD mice to respond to nonagonistic mimics of NRP were not the result of differences in the percentage of tetramer-positive cells that had undergone activation in vivo. As shown in Fig. 8, the NRP-A7/Kk tetramer-positive CD8+ T cells that were contained within these lines expressed levels of CD44 and CD69 similar to those expressed by NRP-differentiated 8.3-CD8+ T cells, and significantly higher than those expressed by naive 8.3-CD8+ T cells.

Our next set of experiments aimed at confirming that these differences in the ability of islet-derived T cell lines from 9- vs 20-wk-old NOD mice to respond to NATs were not the result of differences in the numbers of NRP-A7-reactive cells that were contained within lines. This was performed by testing the ability of FACS-sorted, NRP-A7/Kk tetramer-negative, and tetramer-positive CD8+ T cells from 9- and 20-wk-old mice (pooled lines from

FIGURE 5. Responsiveness of naive and differentiated 8.3-CD8+ T cells to agonistic and nonagonistic NRP homologues. A, Secretion of IFN-γ and IL-2 by naive (filled columns) and NRP-differentiated (hatched columns) 8.3-CD8+ T cells in response to different peptides (at 1 μM). IFN-γ secreted in response to each peptide is shown as a percentage of the amount secreted by each responder in response to NRP-A7. The absolute levels of IFN-γ and IL-2 secreted by naive and differentiated 8.3-CD8+ T cells in response to NRP-A7 in the experiment shown were as follows: naive cells, 1110 ± 23 and 28 ± 5 pg/ml, respectively; differentiated cells, 1768 ± 78 and 338 ± 8 pg/ml, respectively. Data shown are representative of two to three different experiments. B, Secretion of IFN-γ by pancreatic islet-derived (in vivo activated) 8.3-CD8+ T cells vs splenic 8.3-CD8+ T cells (naive). Data shown are representative of experiments with five different islet-derived T cell lines. Assays were conducted as described in Fig. 3. Agonistic peptides are the six naturally occurring agonistic NRP mimics shown in Fig. 4. Nonagonistic peptides correspond to single amino acid variants and NATs (shaded in gray in Figs. 2 and 4).
15–40 mice per experiment) to secrete IFN-γ in response to these peptides. Unlike the tetramer-positive cells of 9- and 20-wk-old NOD mice (∼87–93% purity), the tetramer-negative cells from 20-wk-old NOD mice (∼97% purity) did not mount significant responses against NRP-A7, the 12 nonagonistic mimics (data not shown), or the six agonistic mimics (Fig. 9A). This indicated that nearly all the NRP-A7-reactive T cells of 20-wk-old NOD mice were contained within the NRP-A7/Kd tetramer-positive subpopulation. In contrast, the tetramer-negative cells from 9-wk-old NOD mice (∼96% purity) secreted significant levels of IFN-γ in response to NRP-A7 and three of the six agonistic mimics of NRP (Fig. 9A). This indicated that at least some of the NRP-A7-reactive cells of 9-wk-old (but not 20-wk-old) mice do not bind NRP-A7/Kd tetramers, or do so weakly (i.e., with low avidity), as we had previously shown (34). Finally, we compared the ability of NRP-A7/Kd tetramer-positive CD8+ T cells from 9- vs 20-wk-old NOD mice to secrete IFN-γ in response to NRP-A7 and the seven mimics of NRP that elicited quantitatively different responses from nonsorted lines (i.e., the peptides highlighted in dark in Fig. 7). As shown in Fig. 9B, the tetramer-positive CD8+ T cells of 20-wk-old NOD mice produced higher levels of IFN-γ than the tetramer-positive CD8+ T cells of 9-wk-old NOD mice upon stimulation with six of these mimics. When taken together, these results demonstrate that the ability of polyclonal, monospecific T cell populations to mount functional responses against nonagonistic mimics of a cognate autoantigen is a function not only of their activation state, but also of their overall avidity for peptide/MHC.

Discussion

The interactions between TCRs and peptide/MHC complexes are promiscuous, such that individual TCRs can recognize many different peptides in the context of a single MHC restricting element. Notwithstanding the observation that when naive T cells become activated they display higher functional avidities for agonistic ligands, it has been generally held that naive and activated T cells expressing identical TCRs have identical antigenic repertoires (i.e.,

**FIGURE 6.** Responsiveness of naive and differentiated 8.3-CD8+ T cells to agonistic and nonagonistic NRP homologues at different concentrations. Upper left panel. Amounts of IFN-γ secreted by naive 8.3-CD8+ T cells in response to high concentrations (10 μM) of agonistic and nonagonistic peptides (the same ones used in Fig. 5). Data correspond to one of two separate experiments. The other three panels show amounts of IFN-γ secreted by NRP-differentiated 8.3-CD8+ T cells in response to three different concentrations of agonistic and nonagonistic peptides. Data correspond to one of three separate experiments.
they respond to the same set of peptides). Here we provide experimental evidence indicating that this is not always the case. Our observations were made using CD8\(^{1}\) T cells from RAG-2\(^{2}\), TCR-transgenic NOD mice expressing a single, highly diabetogenic, H-2K\(d\)-restricted TCR (8.3-TCR), and numerous analogs of a target peptide (NRP). We have shown that differentiated 8.3-CD8\(^{1}\) T cells can respond efficiently to many mimics of NRP that have no activity on naive 8.3-CD8\(^{1}\) T cells, even at high concentrations. We have also shown that the antigenic promiscuity of the 8.3-TCR on activated cells is not a peculiarity of this TCR, but rather a phenomenon that is shared by polyclonal NRP-reactive CD8\(^{1}\) T cells propagated from islets of nontransgenic NOD mice. Furthermore, we have shown that the antigenic promiscuity of the 8.3-TCR on activated cells is not a peculiarity of this TCR, but rather a phenomenon that is shared by polyclonal NRP-reactive CD8\(^{1}\) T cells propagated from islets of nontransgenic NOD mice. Furthermore, we have shown that the antigenic promiscuity of the 8.3-TCR on activated cells is not a peculiarity of this TCR, but rather a phenomenon that is shared by polyclonal NRP-reactive CD8\(^{1}\) T cells propagated from islets of nontransgenic NOD mice. Furthermore, we have shown that the antigenic promiscuity of the 8.3-TCR on activated cells is not a peculiarity of this TCR, but rather a phenomenon that is shared by polyclonal NRP-reactive CD8\(^{1}\) T cells propagated from islets of nontransgenic NOD mice.

The numerical values correspond to picograms per milliliter of IFN-\(\gamma\) secreted by islet-derived T cells (adjusted at 2 \(\times\) 10\(^4\) CD8\(^{1}\) cells/well) from 9- and 20-wk-old NOD mice (low and high avidity, respectively (34), in response to peptide-pulsed (1 \(\mu\)M) NOD splenocytes. On average, lines derived from 9- and 20-wk-old mice contain similar percentages of NRP-A7/K\(d\)-tetramer-reactive CD8\(^{1}\) T cells; Ref. 34.). Values >100 pg/ml were considered significant and are shaded. Background responses (against the control peptide tum) were subtracted. Peptides that induced significantly different responses on T cells propagated from 9- vs 20-wk-old mice are darkly shaded. Values of \(p\) were calculated with the Mann-Whitney \(U\) test.

They respond to the same set of peptides. Here we provide experimental evidence indicating that this is not always the case. Our observations were made using CD8\(^{1}\) T cells from RAG-2\(^{2}/-\), TCR-transgenic NOD mice expressing a single, highly diabetogenic, H-2K\(d\)-restricted TCR (8.3-TCR), and numerous analogs of a target peptide (NRP). We have shown that differentiated 8.3-CD8\(^{1}\) T cells can respond efficiently to many mimics of NRP that have no activity on naive 8.3-CD8\(^{1}\) T cells, even at high concentrations. We have also shown that the antigenic promiscuity of the 8.3-TCR on activated cells is not a peculiarity of this TCR, but rather a phenomenon that is shared by polyclonal NRP-reactive CD8\(^{1}\) T cells propagated from islets of nontransgenic NOD mice. Furthermore, we have shown that the antigenic promiscuity of the NRP-reactive T cell population is not just a function of the activation state of the cells, but also a function of the overall avidity of the population for peptide/MHC. These results suggest the existence of two types of agonists: “primary agonists,” which can trigger naive T cells, and “secondary agonists,” which have no functional activity on naive T cells, but exhibit primary agonist-like activity on activated T cells. These results imply that following activation and avidity maturation, T cell populations can mount functional responses against antigenic peptides that they had previously ignored.

The systematic replacement of every residue of NRP by any other amino acid revealed that the TCR contact residues K4 and F8 could essentially not be replaced (with one exception) without abrogating recognition by 8.3-CD8\(^{1}\) T cells, as seen with other TCRs (14). The degree of tolerance for amino acid replacements was greatest at positions 1 and 7 and decreased toward the TCR contact residue K at position 4. Because amino acid substitutions at positions apparently not involved in MHC-binding and TCR contact can have dramatic consequences on T cell activation (5), we suspect that substitutions near K4 affect the ability of K4 to contact the 8.3-TCR. Also worth noting is the fact that replacement of the tryptophan (W) at position 7 by a number of small, neutral, or polar residues increased the agonistic properties of the resulting NRP analogs, suggesting enhanced availability of the phenylalanine at P8 for binding to the TCR.

A search of protein databases using a supertope resulted in the identification of 92 potentially cross-reactive peptides, but only six of these peptides were able to elicit significant proliferative and/or cytokine responses by naive 8.3-CD8\(^{1}\) T cells. This indicated that combinations of amino acids that were tolerated individually within the supertope did not have the same functional activity when combined in a single peptide, even when some of the individual residues had superagonistic activity in the context of the NRP sequence. This observation is at odds with the results of most, albeit not all, studies with CD4\(^{1}\) T cell clones or hybridomas, where recognition of hypermutated peptides could be predicted on the basis of the effects of single amino acid substitutions (12, 23, 46, 47). Surprisingly, studies with activated 8.3-CD8\(^{1}\) T cells revealed that the 8.3-TCR has a much more extensive degree of degeneracy in peptide recognition when expressed on activated cells than on their naive counterparts. Ex vivo and in vivo differentiated 8.3-CD8\(^{1}\) T cells mounted cytokine responses (IFN-\(\gamma\) and IL-2 secretion) against as many as 21 peptides that could not elicit any responses from unstimulated splenic 8.3-CD8\(^{1}\) T cells. Because the supertope that we used to search for naturally occurring

![FIGURE 7](http://www.jimmunol.org/)
9- and 20-wk-old NOD mice were stained with NRP-A7/Kd tetramer-PE, 1 CD8 Santamaria, unpublished observations). The ability of NOD islet-derived T cell lines to respond to secondary agonists vs 2.6 primary agonists shows that the stimulatory potency of peptides for a single TCR is 1 miscuity of activated 8.3-CD8 T cells of 9-wk-old (thin line) and 20-wk-old NOD mice (thick line).

Responsiveness of NRP-A7/Kd tetramer-negative and -positive CD8+ T cells from 9- and 20-wk-old NOD mice against agonistic and nonagonistic mimics of NRP. A, IFN-γ secretion by islet-derived, NRP-A7/Kd tetramer-positive, and tetramer-negative CD8+ T cells from 9- and 20-wk-old NOD mice (with low and high avidity for NRP-A7/MHC, respectively (34) in response to the six agonistic NATs and/or NRP-A7 (average of two experiments). B, IFN-γ secretion by islet-derived, NRP-A7/Kd tetramer-positive CD8+ T cells from 9- and 20-wk-old NOD mice in response to the seven NATs that were differentially recognized by nonagonistic mimics of NRP. A7/Kd tetramer-positive and -negative CD8+ T cells were tested against NRP-A7 (positive control), tum (negative control), and the different NRP mimics (at 5.10^4/well) were tested against NRP-A7 (positive control), tum (negative control), and the different NRP mimics (at 1 μM). Tum-induced (background) responses were subtracted from responses induced by all other peptides.

What could possibly be the mechanisms that underlie this phenomenon? The stimulatory potency of peptides for a single TCR is related to a number of factors, including their affinity for the MHC restricting element, the affinity of the corresponding peptide/MHC complexes for the cognate TCR, and the half lives (or K off) of the interactions (49). Our data has shown that primary and secondary agonists do not differ in their affinity for MHC binding and that the only mimics of NRP that can function as secondary agonists carry two TCR-contact residues of NRP (K4 and F8). In addition, NATs with primary and secondary agonistic activity differed in the number of nonanchor residues that they shared with NRP (3.5 ± 0.3 for primary agonists vs 2.6 ± 0.3 for secondary agonists). Because the ability of NOD islet-derived T cell lines to respond to secondary agonists increases with their avidity for NRP/MHC, these data suggest that the ability of activated T cells to respond vigorously to secondary agonists is related to the formation of the immunological synapse upon T cell activation. T cell activation induces the oligomerization of TCRs and associated coreceptors into supramolecular clusters by a serial triggering mechanism (50–52). It has
been shown that the number of peptide/MHC complexes in the clusters correlates with the half-life of the TCR-peptide/MHC interaction, such that low concentrations of agonists and low affinity ligands are inefficient at inducing cluster formation and stable signaling (51). The efficiency of TCR signaling in the clusters also depends on the ability of peptide/MHC complexes to ligate the TCR long enough to trigger the recruitment and activation of ZAP-70 by p56
<sup>1</sup>LA, the activation of the lipid raft-associated adaptor linker for activation of T cells, and downstream signaling (48). Because activated T cells express significantly higher levels of rafts and p56
<sup>1</sup>LA on the surface than naive T cells (53), the exquisite antigenic promiscuity of activated vs naive 8.3-CD8<sup>+</sup> T cells can be explained by a model in which short-lived interactions between TCRs and low affinity ligands (i.e., NRP mimics bearing the two major TCR contact residues of NRP but differing from NRP at minor TCR contact residues) would be able to elicit efficient signaling in activated T cells, but not in their naive progenitors.

The increase in the antigenic promiscuity of the NRP-A7-reactive CD8<sup>+</sup> T cell population with its avidity for peptide/MHC is likely due to preferential expansion of T cells bearing high affinity TCRs for NRP-A7 during the spontaneous anti-islet autoimmune response (34). Available structural data suggest that the Ag-binding site of TCR is flexible, that the low affinity and slow kinetics of TCR-peptide/MHC-binding is a consequence of this flexibility, and that this flexibility accounts for the cross-reactivity of TCRs for NRP-A7 reactive CD8<sup>+</sup> T cells can be explained by a model in which short-lived interactions between TCRs and low affinity ligands (i.e., NRP mimics bearing the two major TCR contact residues of NRP but differing from NRP at minor TCR contact residues) would be able to elicit efficient signaling in activated T cells, but not in their naive progenitors.

Whatever the mechanisms, this amplification of the antigenic repertoire of single TCRs upon T cell activation may have evolved as a means for the immune system to be able to fight mutants arising during microbial or viral infections (58–60). Some of these mutant viruses encode lower affinity ligands of reactive TCRs that compete with variants carrying high affinity epitopes for TCR recognition, or encode TCR antagonists that can assist in the survival of variants carrying wild-type epitopes (59, 60). Owing to their activation-induced promiscuity, activated and memory T cells would be able to mount immune responses against escape mutants that cannot elicit the activation of naive T cells bearing TCRs specific for the wild-type epitopes. This interpretation is consistent with the observation that viral infections expand polyclonal populations of T cells containing T cells capable of providing protection against a range of antigenic variants (61). However, this process is a double-edged sword that compromises the natural resistance of the hosts to autoimmunity afforded by the processes of immunological tolerance, which preferentially target TCRs with high affinity for self-peptide/MHC. Activation of a nonautoreactive T cell population by a foreign Ag during an infection would allow the differentiated T cell progeny to react against self-derived, low affinity ligands of their TCRs (autoantigens).

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