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Low-Avidity Self-Specific T Cells Display a Pronounced Expansion Defect That Can Be Overcome by Altered Peptide Ligands

Karin E. de Visser, Tanina A. Cordaro, Helmut W. H. G. Kessels, Felicia H. Tirion, Ton N. M. Schumacher, and Ada M. Kruisbeek

Thymic expression of self-Ags results in the deletion of high-avidity self-specific T cells, but, at least for certain Ags, a residual population of self-specific T cells with low-avidity TCRs remains after negative selection. Such self-specific T cells are thought to play a role in the induction of T cell-mediated autoimmunity, but may also be used for the induction of antitumor immunity against self-Ags. In this study, we examine the functional competence of a polyclonal population of self-specific CD8+ T cells. We show that low-avidity interactions between TCR and peptide are associated with selective loss of critical T cell functions. Triggering of low levels of IFN-γ production and cytolytic activity through low-avidity TCRs readily occurs provided high Ag doses are used, but IL-2 production and clonal expansion are severely reduced at all Ag doses. Remarkably, a single peptide variant can form an improved ligand for the highly diverse population of low-avidity self-specific T cells and can improve their proliferative capacity. These data provide insight into the inherent limitations of self-specific T cell responses through low-avidity TCR signals and the effect of modified peptide ligands on self-specific T cell immunity.


During T cell development, the fate of developing thymocytes depends on their avidity for thymically expressed Ag-MHC complexes. Developing T cells expressing TCRs with high affinity for peptide-MHC complexes will undergo cell death. This intrathymic negative selection process ensures the removal of high-avidity self-specific T cells from the T cell repertoire. However, low-avidity self-specific T cells may escape the clonal deletion process and reach the periphery (1–9). Under certain circumstances, this self-specific T cell repertoire can be triggered by antigenic challenges in the presence of a strong inflammatory response (4, 6, 8, 9).

A possible consequence of activation of self-specific T cells is the development of undesirable T cell autoimmunity. It has become clear that autoreactive T cells involved in autoimmune diseases can be triggered by exposure to viral and bacterial peptides that share homology with self-Ags (molecular mimicry) (10, 11). Yet, self-specific T cell responses may also be exploited for the induction of desirable responses against self-proteins overexpressed on tumors (6, 12–14). The same mechanism of molecular mimicry that has been shown to induce autoimmunity may thus be used to induce beneficial self-specific T cell responses.

It has become clear that the TCR is not an “on-off” switch, but instead a very versatile signaling complex. Depending on the nature of the interaction between Ag and TCR, a T cell can display a spectrum of cellular responses ranging from complete activation to inhibition (15–18). One way to manipulate the strength of the TCR-Ag interaction is to use altered peptide ligands (APL) that display different affinities for the TCR. A better understanding of the effect of APL on T cell activation and function could facilitate the rational design of strategies to enhance or inhibit self-specific T cell responses.

We studied a self-specific T cell population using a mouse model in which a fragment (aa 1, 2, 328–498) of the influenza nucleoprotein (NP) is expressed as a transgene under control of the H-2K promoter in C57BL/10 mice (B10NP mice) (19). We have previously reported that the ubiquitous expression of NP in these B10NP mice leads to thymic deletion of high-avidity NP-specific CD8 T cells (8). Here, we report that the residual population of low-avidity NP-specific CD8+ T cells displays a severely reduced capacity to expand and to produce IL-2. We show that a single amino acid substitution in the wild-type epitope generated a cross-reactive variant peptide displaying a higher equilibrium binding to and a lower off-rate from the complete polyclonal NP-specific T cell repertoire present in B10NP mice. Whereas vaccination of B10NP mice with the self-peptide does not result in measurable expansion of low-avidity self-specific T cells, vaccination with this higher affinity variant peptide resulted in a dramatic expansion of the self-specific T cell population with maintenance of effector functions. Thus, higher affinity ligands may be used to promote the expansion of self-specific T cells for Ags overexpressed on tumors without affecting their Ag specificity or sensitivity.

Materials and Methods

Mice

C57BL/10 (H-2b; B10) mice were obtained from the experimental animal department of The Netherlands Cancer Institute (Amsterdam, The Netherlands). B10 mice transgenic for a fragment of the influenza NP from which aa 3–327 are deleted under control of the widely expressed MHC-class I promoter H-2Kb (B10NP mice) were kindly provided by Dr. D. Kioussis (National Institute for Medical Research, London, U.K.) (19). All mice used in this paper: APL, altered peptide ligand; NP, nucleoprotein; HAU, hemagglutinin units.
were kept under specified pathogen-free conditions and used when 6–10 wk of age.

Viruses, peptides, and tetramers

Purified influenza A/NT/60/68 virus was kindly provided by Dr. R. Con sabate (National Institute for Medical Research). Virus was grown and titered in the Department of Virology, Erasmus University Rotterdam (Rotterdam, The Netherlands). Virus was stored at −70°C in 50% sucrose and thawed immediately before use. The NP366–374 Peptide (sequence AS NENMDAM) and variant peptides (sequences SSSNENMDAM, LSNEN MDAM, NSNENMDAM, AQNENMDAM, ALNENMD AM, ASNENMDTM, ASNENMEA, ASNENMDLM, and ASNENMDAM) were produced by Prome synthesis. All peptides were HPLC purified. Monomeric MHC-peptide complexes and tetrathers of soluble MHC class I molecules complexed with the NP366–374 Peptide or variant peptides were synthesized according to the original protocol developed by Altman et al. (20), modified as described elsewhere (21).

Cells and tissue culture conditions

All cell lines were cultured in IMDM (Life Technologies, Paisley, Scotland) supplemented with 5% FCS (PAA Laboratories, Linz, Austria), 100 IU/ml penicillin (Boehringer Mannheim, Mannheim, Germany), 100 μg/ml streptomycin (Boehringer Mannheim), and 5 × 10−8 M 2-ME (Merck, Hohenbrunn, Germany; complete medium) at 37°C in humidified air containing 5% CO2. Tumor cell lines used were the murine thymoma EL4 (H-2b) (22), EL4 tumor cells retrovirally transduced with a cDNA encoding for fragment 1, 2, 288–498 of the influenza A/NT/60/68 virus NP linked via an IRES sequence with enhanced green fluorescence protein (EL4NP cells) (23). The NK-sensitive murine T lymphoma cell line YAC (24) was used in some experiments. Low-avidity ASNENMDAM-specific hybridomas (independent clones 4F3, 1E2, and 5E5) were generated from 4-day bulk cultures of splenocytes from B10NP mice infected with the A/NT/60/68 virus.

Virus infection

For live virus infections, anesthetized mice were infected by intranasal administration of 50 μl of HBSS (Life Technologies) containing 25 hemagglutinin units (HAU) of A/NT/60/68 virus.

Flow cytometry

For evaluation of the percentage and phenotype of NP366–374-specific T cells, analysis by flow cytometry was performed on cells isolated from spleens and lungs. Single-cell suspensions of the different tissues were prepared by homogenizing the tissues over a nylon filter (NPBI, Emmen, The Netherlands). Erythrocytes were subsequently lysed by treatment with NH4Cl lysis buffer and the remaining cells were washed. Samples of 5 × 106 cells were washed twice with PBS containing 0.5% BSA and 0.02% NaN3 (PBS/BSA) and incubated for 20 min with 20 μl of the appropriate dilutions and combinations of allophycocyanin- or FITC-conjugated anti-CD8 (BD PharMingen, San Diego, CA) and PE- or allophycocyanin-conjugated tetrameric class I-peptide complexes at 4°C. Cells were washed twice and resuspended in PBS/BSA. Data acquisition and analysis were performed on a BD Biosciences FACSCalibur using CellQuest software (Mountain View, CA).

Generation of CTL bulk cultures

Spleens were isolated at indicated time points after infection and single-cell suspensions were obtained as described above. Splenocytes were seeded into 24-well culture plates at 5 × 106 cells/well in 2 ml of 10% FCS medium supplemented with or without 20 Cetus U of IL-2/ml (PerkinElmer/Cetus, Emeryville, CA) and indicated peptide concentrations. Cultures were tested at day 7 or restimulated with peptide and IL-2 and analyzed at day 14.

MHC-peptide binding assay

The relative ability of peptides to bind Dβ was determined by an RMA-S stabilization assay (25). RMA-S cells were incubated at 26°C overnight to acquire maximum MHC expression at the cell surface. Increasing concentrations of the tested peptides were added for 2 h at 37°C. Cells were washed and stained for Dβ expression with FITC-conjugated anti-Dβ Ab (BD PharMingen) at 4°C.

Peptide-TCR binding assays

To compare the equilibrium binding of wild-type peptide ASNENMDAM and cross-reactive variant peptides to self-specific TCRs, B10NP-derived hybridomas expressing low-avidity ASNENMDAM-specific TCRs were used. These hybridomas were stained with PE-conjugated variant peptide tetramers in the presence of increasing concentrations of unlabeled AS NENMDAM monomers or unlabeled variant peptide monomers for 20 min at 4°C. The mean fluorescence intensity of the tetramer signal was analyzed by flow cytometry.

The dissociation rates of MHC tetramers containing the wild-type peptide ASNENMDAM and the variant peptides from hybridomas expressing self-specific TCRs were determined by an off-rate assay. Briefly, 5 × 106 cells of each of the three hybridoma clones were stained with either ASN ENMDAM- or variant peptide tetramers for 20 min at 4°C. Cells were washed and resuspended in 400 μl of medium supplemented with 1% FCS. To determine the off-rate, 10 μM unlabeled variant peptide monomers were added and the decrease in tetramer signal was measured by flow cytometry at the indicated time points.

Peptide immunization protocol

Mice were injected s.c. at the tail base with 100 μg of ASNENMDAM peptide or variant peptides emulsified in CFA (Difco, Detroit, MI). In addition, at day 0, day 1, and day 2, mice were injected i.p. with 100 μg of anti-CD40 Abs (FGK.45) (26–28). After 10 days, isolated splenocytes were stimulated with increasing concentrations of the ASNENMDAM peptide or variant peptides and IL-2.

Intracellular cytokine staining

For determination of Ag-specific cytokine production, intracellular cytokine stainings were performed. Splenocytes were cultured as described above in bulk cultures. At day 7 or 14, cells were purified over a Lympholyte-M (CEDARLANE Laboratories, Hornsby, Ontario, Canada) gradient. Intracellular IL-2 and IFN-γ stainings were performed as described previously (29). Briefly, spleen cells were stimulated in 96-well flat-bottom tissue culture plates (Costar, Corning, NY) of a concentration of 1 × 106 cells/well in 200 μl of complete medium supplemented with 1 μM brefeldin A (GolgiPlug; BD PharMingen) and 50 μM human IFN-γ and stimulated for 5 h. Stimulation, cells were surface stained with allophycocyanin-conjugated anti-CD8 (BD PharMingen), washed, and stained intracellularly with FITC-conjugated antimonye IL-2 or IFN-γ and isotype controls with FITC-conjugated antimonye IgG2 or IgG1, respectively. Stainings were performed using the Cytofix/Cytoperm kit according to the manufacturer’s protocol (BD Pharmingen).

Cytotoxicity assay

For analysis of cytolytic activity, bulk cultures were prepared as described above. After 7 days of culture, cells were purified over a Lymphophyte-M (CEDARLANE Laboratories) gradient and tested in a chromium release assay performed according to standard protocols. Briefly, splenocytes were serially diluted in triplicate in U-bottom tissue culture plates (Costar). Target cells, 2 × 106, were incubated with 100 μCi of 51Cr (Amersham Pharmacia Biotec, Little Chalfont, U.K.) for 1 h at 37°C in a total volume of 100 μl of complete medium. In the case of peptide loading, indicated peptide concentrations were added during labeling. The labeled cells were washed three times with complete medium and 2 × 105 cells were added per well. To block NK cell activity, 50-fold excess of unlabeled YAC cells was added to the wells. Per target, six spontaneous release wells were obtained by incubating the labeled target cells in medium alone. Maximum release wells were prepared by incubating the labeled target cells in 2% Triton X-100. After 4–5-h incubation at 37°C, 25 μl of supernatant was harvested in Luma plates (Packard Instrument, Meriden, CT) and counted in a TopCount microplate scintillation counter (Packard Instrument). The percent specific 51Cr release was calculated as follows: percent specific release = 100 × (cpm experimental release – cpm spontaneous release)/ (cpm maximum release – cpm spontaneous release).

Results

Low-avidity self-specific T cells display an expansion defect

The ubiquitous expression of NP in B10NP mice influences the T cell repertoire in such a way that high-avidity NP366–374-specific CD8+ T cells (further referred to as NP-specific T cells) are deleted. The residual low-avidity NP-specific T cell population can be expanded in vivo by infection with the influenza A/NT/60/68 virus containing the same NP366–374 epitope as expressed in the
B10NP mice (Fig. 1A) (8). However, the in vivo expansion of the low-avidity T cell population during influenza infection is dramatically reduced as compared with the expansion of high-avidity T cells (8).

To study these low-avidity T cells in more detail and to define both their proliferative potential and their functional characteristics, we cultured splenocytes from influenza-infected B10NP and B10 mice with increasing concentrations of NP\textsubscript{366-374} peptide and determined the percentage of NP-specific T cells of the CD8\textsuperscript{+} T cell population by flow cytometry. Importantly, even at high peptide concentrations, the expansion of low-avidity self-specific T cells was dramatically impaired as compared with their high-avidity counterparts (Fig. 1B).

Because it has been suggested that exogenous IL-2 can increase CTL responsiveness (30), we determined whether the poor expansion of low-avidity self-specific T cells could be increased by the addition of IL-2. Indeed, addition of IL-2 during culture resulted in a low level of peptide concentration-dependent enrichment of NP-specific T cells in the B10NP cultures (Fig. 1C). Whereas optimal stimulation of high-avidity T cells was observed at peptide concentrations of 5 \times 10^{-2} \mu g/ml, optimal expansion of low-avidity self-specific T cells required a 100-fold higher peptide concentration. In parallel to the in vivo data (8), also during in vitro culture the frequency of the low-avidity T cells was reduced compared with the high-avidity T cells: the expansion of low-avidity T cells was at least 8-fold (in the presence of IL-2) to 30-fold (in the absence of IL-2) reduced compared with high-avidity T cells (Fig. 1, B and C). Furthermore, because the number of T cells retrieved from B10 mice was generally higher than the number of T cells from B10NP cultures, these values are an underestimate of the actual expansion defect. The addition of more IL-2 (100 U/ml) did not further improve the expansion of NP-specific CD8\textsuperscript{+} T cells from B10NP mice (data not shown). Similar expansion defects were observed in cultures started on days 23 and 33 after infection (Fig. 1D), excluding a refractory state of the low-avidity T cells due to delayed kinetics of in vivo priming (8). The observed poor expansion of the low-avidity T cells, even in the presence of IL-2, cannot be explained by defective expression of IL-2R α-, β-, or common γ-chains, since these IL-2R subunits are all expressed normally on cultured low-avidity T cells (data not shown).

Given that expansion of T cell populations with low Ag concentrations may result in selective expansion of high-avidity T cell clones, whereas activation with high Ag concentrations may result

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**FIGURE 1.** Ag and cytokine requirements for in vitro expansion of low- and high-avidity NP\textsubscript{366-374} specific T cells. B10NP and B10 mice were infected intranasally with 25 HAU of influenza A/NT/60/68 virus, and lungs and spleens were isolated 16 days after infection. A, Cells derived from lungs were stained with NP tetramers and anti-CD8 mAb. Tetramer staining of the gated CD8\textsuperscript{+} T cells of representative infected B10NP (black histogram) and B10 mice (gray histogram) is shown. As a control, tetramer staining of CD8\textsuperscript{+} T cells of a naive mouse is shown (black thin histogram). Splenocytes (5 \times 10^6/well) were cultured with increasing concentrations of the NP\textsubscript{366-374} peptide without (B) or with (C) 20 U of IL-2/ml. Seven days later, cells were harvested, stained with NP tetramers and anti-CD8 mAb, and analyzed by FACS. The percentages of NP-specific T cells are shown for the gated CD8\textsuperscript{+} T cell population. D, B10NP and B10 mice were infected with 25 HAU of influenza A/NT/60/68 virus, and spleens were isolated 16, 23, and 33 days after infection. Splenocytes were cultured in the presence of 5 \times 10^{-2} \mu g/ml (B10NP) or 5 \times 10^{-4} \mu g/ml (B10) NP peptide and 20 U of IL-2/ml, and analyzed by FACS 7 days later. E, The mean fluorescence intensity of the NP tetramer signal of the CD8\textsuperscript{+} T cells for C is shown as a measure for avidity.
in the preferential survival of low-avidity T cells (due to activation-induced cell death of high-avidity T cells) (31, 32), we examined whether the avidity profiles observed in vivo (Fig. 1A) were maintained in vitro. In vitro stimulation with titrated peptide doses resulted in an increase in the avidity of NP-specific T cells in B10 cultures (Fig. 1E). However, high-avidity T cells could not be generated from B10NP mice at any of the Ag concentrations tested. This inability to recover high-avidity NP-specific T cells from B10NP mice is in agreement with the notion of a "hole" in the T cell repertoire of B10NP mice: high-avidity T cells have been deleted, and only self-specific T cells with lower avidity remain.

**NP-specific intracellular cytokine production and cytolytic activity by low- and high-avidity T cells**

Since low-avidity interactions between TCR and ligand may result in partial activation of T cell effector functions (16, 33–37), we assessed whether the low-avidity self-specific T cells differ from high-avidity T cells with respect to their ability to perform effector functions.

First, we studied cytokine production by B10NP-derived low-avidity T cells and B10-derived high-avidity T cells upon peptide stimulation. Due to the low level of tetramer staining of the self-specific T cells, we were unable to isolate these cells by FACS sorting. Therefore, cytokine production by CD8+ T cells from B10NP and control B10 bulk cultures (stimulated under optimal conditions as defined above) were evaluated by intracellular cytokine stainings. As shown in Fig. 2A, low-avidity T cells produced IFN-γ upon stimulation with the NP366–374 peptide, but displayed at least a 100-fold reduced Ag sensitivity compared with high-avidity T cells. Fig. 2C shows the IFN-γ production corrected for the percentage of NP-specific T cells present in the analyzed B10 and B10NP populations. The low- and high-avidity T cell populations showed similar proportional responses in terms of the frequency of IFN-γ producers at Ag concentrations above 0.001 μg/ml. Despite this normal proportional response by the low-avidity T

**FIGURE 2.** NP-specific intracellular IL-2 and IFN-γ production by low- and high-avidity T cells. B10NP and B10 mice were infected intranasally with 25 HAU of influenza A/NT/60/68 virus, and spleens were isolated 16 days after infection. After 7 days of in vitro restimulation of the spleen cells (5 × 10⁶/well) with 5 × 10⁻² μg/ml NP366–374 peptide (B10NP mice) or 5 × 10⁻³ μg/ml NP366–374 peptide (B10 mice) and 20 U of IL-2/ml, NP-specific intracellular IFN-γ and IL-2 production was analyzed. Splenocytes were harvested and stimulated with the indicated concentrations of NP366–374 peptide for 5 h. Cells were stained with allophycocyanin-conjugated anti-CD8 and FITC-conjugated mAbs for IFN-γ (A and C) or IL-2 (B and D) and isotype control FITC-conjugated antimouse IgG1 or IgG2, respectively. Isotype control stainings did not exceed background levels. A and B, Percentages of cytokine-positive cells gated on the CD8+ T cells are depicted. C and D, The IFN-γ and IL-2 production is corrected for the percentage of NP-specific T cells present in the analyzed B10 and B10NP populations by dividing the percentage of IFN-γ+ cells (A) or IL-2+ cells (B) by the percentage of NP-specific T cells multiplied by 100. E, The mean fluorescence intensity of the IFN-γ signal (A) is plotted against the concentration NP peptide.
cells at high Ag concentrations, the amount of IFN-γ produced per cell is lower at each Ag concentration tested (Fig. 2E). Even more striking, we observed only very marginal levels of IL-2 production, even after stimulation with high Ag concentrations (Fig. 2, B and D). Both low- and high-avidity T cell populations did not produce detectable levels of IL-4 and IL-10 (data not shown).

In contrast to the severe impairment in IL-2 production, the low-avidity NP-specific T cells exhibited NP-specific cytolytic activity (Fig. 3A), not only for peptide-pulsed target cells but also for targets expressing endogenously processed NP. Given the differences in the frequency of tetramer-positive T cells derived from B10 and B10NP bulk cultures (Fig. 1B), we cannot draw any conclusions from the cytolytic activity per cell. However, we could at a fixed E:T ratio compare the Ag sensitivity of low- vs high-avidity T cells. To determine whether the Ag sensitivity for cytolytic activity of low-avidity T cells differed from that of high-avidity T cells, EL4 cells were pulsed with a wide range of NP<sub>366–374</sub> peptide concentrations. At least a 100-fold reduced sensitivity of low-avidity T cells was observed (Fig. 3B). However, at high Ag concentrations low-avidity T cells displayed a significant capacity to mediate target cell lysis.

Taken together, these data show that low-avidity T cells can perform Ag-specific effector functions; they can produce IFN-γ, although less efficiently at low concentrations of the Ag and lower amounts per cell. In addition, the low-avidity T cells do exhibit Ag-specific cytolytic activity, although high Ag concentrations on the target cells are required. In contrast, IL-2 production and the ability to expand is clearly reduced in the low-avidity T cell population.

**Bypassing the expansion defects of low-avidity T cells**

As outlined above, a major defect of the low-avidity T cells triggered by influenza infection is their poor ability to expand in vivo as well as in vitro. This expansion defect can even more dramatically be observed using an optimized peptide vaccination strategy. B10NP and B10 mice were vaccinated with the wild-type NP<sub>366–374</sub> peptide (ASNENMDAM) in combination with anti-CD40 treatment (26–28), and splenocytes were restimulated in vitro with ASNENMDAM. Using this strategy, we could activate an abundant NP-specific T cell response in normal B10 mice (Fig. 4A). However, this peptide vaccination regimen is completely unable to trigger expansion of low-avidity NP-specific T cells in B10NP mice (Fig. 4A).

We then asked whether this poor expansion is an intrinsic property of self-specific T cells that escaped tolerance or is directly related to the low affinity of the expressed TCR for the self-ligand. We searched for improved ligands that could trigger a more vigorous expansion of the low-avidity T cells. Variant peptides (see Materials and Methods) were generated by substitution of single amino acid residues at TCR contact residues of the wild-type NP epitope. To study the interaction between low-affinity TCRs with these modified ligands, we used the peptide vaccination strategy as described above. B10NP mice were vaccinated with the wild-type peptide or the variant peptides in combination with anti-CD40 treatment, and splenocytes were restimulated in vitro with the same peptide as used for immunization. The cross-reactive NP-specific T cell response was then visualized using wild-type AS NENMDAM tetramers (38).

One of 10 variants that were tested could overcome the expansion defect of the self-specific T cells: the introduction of an alanine into threonine replacement at position 8 of the wild-type NP<sub>366–374</sub> peptide resulted in a variant peptide (ASNENMDAM) that triggered a clear-cut expansion of T cells specific for the wild-type NP epitope in B10NP mice (Fig. 4B). The magnitude of this NP-specific T cell response reaches up to 60–80% of the response triggered in B10 mice (data not shown). Thus, replacement of a single amino acid at a TCR contact residue resulted in a variant peptide that could induce significant expansion of the T cell population with a low-avidity for the pertinent self-Ag. These data indicate that the poor expansion of self-specific T cells upon stimulation with the self-peptide is not an intrinsic defect of these cells, but can be corrected by variant peptides.

**FIGURE 3.** NP-specific cytolytic activity by low- and high-avidity T cells. A, B10NP mice were infected intranasally with 25 HAU of influenza A/NT/60/68 virus, and spleens were isolated 16 days after infection. After 7 days of in vitro restimulation of the spleen cells (5 × 10⁶/well) with 5 × 10⁻² μg/ml NP<sub>366–374</sub> peptide and 20 U of IL-2/ml, the cells were tested in a <sup>51</sup>Cr release assay against EL4 tumor cells, EL4 cells pulsed with 100 μg/ml NP<sub>366–374</sub> peptide, and EL4NP cells. The mean percentage of specific lysis is shown. The E:T ratio used is 80 (percentage of tetramer-positive cells of the CD8<sup>+</sup> population is 7%). B, Bulk culture cells of infected B10 and B10NP mice were tested in a <sup>51</sup>Cr release assay against EL4 cells pulsed with decreasing concentrations of the NP<sub>366–374</sub> peptide. The mean percentage of specific lysis per Ag concentration is shown. The E:T ratio used is 80 (percentage of tetramer-positive cells of the CD8<sup>+</sup> population is 43% (B10 cultures) and 7% (B10NP cultures).
on the CD8

the splenocytes were stimulated in vitro with ASNENMDTM or ASNEN

variant peptide ASNENMDTM or wild-type peptide ASNENMDAM and

binding capacities to H-2Db as the wild-type peptide (Fig. 5

stabilization assay. However, the variant peptide displayed similar

at day 0, day 1, and day 2, mice were injected i.p. with 100

Variant peptide ASNENMDTM binds with higher af

Bypassing the expansion defect of self-speci

FIGURE 4. Bypassing the expansion defect of self-specific T cells by a

variant peptide. A, B10 and B10NP mice were injected at day 0 s.c. at the
tail base with 100 µg of NP<sub>366–374</sub> peptide emulsified in CFA. In addition,
at day 0, day 1, and day 2, mice were injected i.p. with 100 µg of anti-

CD40 Ab. After 10 days, splenocytes were isolated and stimulated with the

peptide ASNENMDAM or wild-type peptide ASNENMDAM and the

splenocytes were stimulated in vitro with ASNENMDTM or ASNEN

MDAM, respectively, using the peptide vaccination protocol described in

A. After 14 days of culture, cells were harvested and stained with anti-CD8 mAb

and ASNENMDAM tetramers. Percentages of NP-specific T cells gated on

the CD8<sup>+</sup> T cell population are shown. B, B10NP mice were injected with

variant peptide ASNENMDTM or wild-type peptide ASNENMDAM and

the splenocytes were stimulated in vitro with ASNENMDTM or ASNEN

MDAM, and the splenocytes were isolated and stimulated with the indicated concentrations of the NP<sub>366–374</sub> peptide and 20 U/ml IL-2. After

14 days of culture, cells were harvested and stained with anti-CD8 mAb and

ASNENMDAM tetramers. Percentages of NP-specific T cells gated on

the CD8<sup>+</sup> T cell population are shown.

A

B

Figure 4

% ASNENMDAM-specific T cells of CD8 population

ASNENMDAM-Vaccination

0 5E-06 1E-05

Peptide (ASNENMDAM) concentration (µg/mL)

B10NP

B10

% ASNENMDAM-specific T cells of CD8 population

Peptide concentration (µg/mL)

5E-06 1E-05

Peptide vaccine:

ASNENMDAM

ASNENMDTM

Peptide vaccine:

ASNENMDAM

ASNENMDTM

B10NP mice

Variant peptide ASNENMDTM binds with higher affinity and slower off-rate to self-specific TCRs

Two possible explanations exist for the potential of the variant peptide ASNENMDTM to overcome the expansion defect of low-avidity T cells. First, the binding affinity of ASNENMDTM for H-2D<sup>b</sup> could be higher compared with ASNENMDAM. We therefore compared the capacity of the variant ASNENMDTM and wild-type ASNENMDAM peptides to bind to H-2D<sup>b</sup> in an RMA-S stabilization assay. However, the variant peptide displayed similar binding capacities to H-2D<sup>b</sup> as the wild-type peptide (Fig. 5A). A second possibility was that the variant peptide displayed a higher affinity for the self-specific TCRs. The binding properties of the ASNENMDTM epitope to the self-specific TCR were tested in both an equilibrium binding assay and in a dissociation rate assay. For these binding studies, three ASNENMDAM-specific hybridomas with low-affinity self-specific TCR were used. These hybridomas were independently generated from bulk cultures of influenza-infected B10NP mice. They all three displayed a cross-reactivity toward the ASNENMDTM epitope, as determined by staining with ASNENMDTM tetramers. ASNENMDTM tetramer binding of all three hybridomas could be inhibited more efficiently by ASNENMDTM monomers than by ASNENMDAM monomers (Fig. 5B), indicating that the interaction of the self-specific TCR with ASNENMDTM is of higher affinity than with ASNENMDAM. Consistent with this, ASNENMDTM tetramers displayed a decreased TCR off-rate compared with ASNENMDAM tetramers (Fig. 5C).

Thus, conservative replacement of one single amino acid residue in the wild-type self-peptide generated a variant peptide that increased the expansion of the residual low-avidity T cell repertoire in B10NP mice because of its more stable interaction with the TCR.

Functional capacities of ASNENMDTM-triggered ASNENMDAM-specific T cells

To study the functional capabilities of low-avidity T cells triggered by a variant peptide, B10NP mice were vaccinated with the ASNENMDTM variant as described above, and the splenocytes were expanded in vitro with the ASNENMDTM peptide in the presence of IL-2. As shown in Fig. 6A, B10NP cultures contained large cross-reactive populations of ASNENMDAM-specific T cells. The presence of these populations correlated well with production of IFN-γ upon stimulation with ASNENMDAM (Fig. 6B). In agreement with the observed defect in IL-2 production by influenza virus-triggered self-specific T cells (Fig. 2B), ASNENMDTM-triggered self-specific T cells produced only limited levels of IL-2 (Fig. 6B) upon stimulation with ASNENMDAM. In addition, the B10NP cultures displayed cytotoxicity toward target cells loaded with ASNENMDAM, provided that sufficient peptide is present (Fig. 6C). Thus, the low-avidity ASNENMDAM-specific T cell population triggered in B10NP mice by vaccination with the variant peptide ASNENMDTM maintains its effector functions upon recognition of the self-Ag.

Variant peptide ASNENMDTM forms a higher affinity ligand for the complete ASNENMDAM-specific T cell repertoire

During T cell development, a highly diverse T cell repertoire is formed (39). Even T cells which recognize the same peptide-MHC complex display a high degree of diversity (40, 41). This has also been shown for the NP-specific T cell population triggered in B10NP mice because of its more stable interaction with the TCR.

As a consequence, T cells that are specific for the same Ag generally respond differently to minor changes in the wild-type ligand (43–45). Specifically, in a nontolerized Ag-specific T cell population, peptide variants that form a higher affinity ligand for certain T cell clones generally form a lower affinity or null ligand for other clones (43, 44). To establish whether the same applies for self-specific T cell populations, we
studied which proportion of the polyclonal low-avidity T cell repertoire with specificity for ASNENMDAM cross-reacts with the higher affinity ligand ASNENMDTM.

The binding studies described in Fig. 5, B and C, show that each of the three independently generated low-avidity ASNENMDAM-specific hybridomas displayed a high-affinity cross-reactivity toward ASNENMDTM. This observation suggests that a large fraction of the polyclonal ASNENMDAM-specific T cell repertoire displays cross-reactivity toward ASNENMDTM. To study whether this hypothesis based on hybridomas can be extrapolated to polyclonal low-avidity ASNENMDAM-specific T cell populations, we determined which proportion of the influenza-triggered ASNENMDAM peptide under optimal stimulating conditions (as described above) and cultures were stained with ASNENMDAM and ASNENMDTM tetramers. Strikingly, a complete overlap was observed in percentages of ASNENMDAM- and ASNENMDTM-specific T cells in both B10 and B10NP cultures (Fig. 7A). In agreement with these data, addition of an excess of unlabeled ASNENMDTM monomers during staining could completely block the ASNENMDAM tetramer signal (data not shown). The mean fluorescence intensity of the ASNENMDAM tetramer signal in B10NP cultures is clearly lower (1.8-fold) than the signal in B10 cultures (Fig. 7A), whereas the difference in the ASNENMDTM tetramer signal between B10NP and B10 cultures is small at best. Moreover, the cultures of B10NP mice show at least a 105-fold higher sensitivity for variant ASNENMDTM compared with self-Ag ASNENMDA (Fig. 7B), confirming the observed differences in binding levels of ASNENMDTM and ASNENMDAM tetramers (Fig. 7A). In conclusion, a single variant peptide can form a higher affinity ligand for the complete polyclonal self-specific T cell repertoire.

Discussion

In this study, we investigated the impact of ubiquitous self-Ag expression on the polyclonal self-specific T cell response. We and others have demonstrated previously that the self-specific T cells...
that escaped negative selection do not display a broad avidity spectrum, but that they represent a residual population of T cells with low-avidity self-specific TCRs (3, 6, 8, 46, 47). Although several recent studies have demonstrated that such self-specific T cells can be activated in vivo through a number of interventions (1, 3–8, 47, 48), little is known about their functional behavior. In the present study, we address whether low-avidity self-specific T cells are functionally similar to normal T cells, provided sufficient Ag is present, or whether they display an intrinsically altered functional phenotype. We studied the activation, expansion, and differentiation of the endogenous polyclonal low-avidity CD8+ T cell population specific for a ubiquitously expressed model self-Ag in B10NP mice. Moreover, we studied the functional consequences of interactions between the low-affinity TCRs and variant peptides.

Here, we show that an important functional defect of the polyclonal low-avidity self-specific T cell population is its poor ability to expand in vivo and in vitro, even in the presence of high concentrations of the self-Ag. Whether this expansion defect is caused by an impaired ability to survive and/or by a poor proliferative potential remains to be investigated. Due to technical limitations in concurrent staining for tetramers and for hallmarks of apoptosis (such as TUNEL and annexin V) on the same cells, this issue cannot presently be resolved. We have previously shown that an NP-specific memory response can be generated in B10NP mice, suggesting that there is no major defect in the ability of self-specific T cells to survive (8). In addition, we show that there is a clear-cut difference in the quality of the Ag receptor repertoire, as high-avidity T cells cannot be generated from B10NP mice at any Ag concentration, while enrichment of B10-derived high-avidity NP-specific T cells readily occurs, especially at low Ag concentrations.

The main characteristics of T cell anergy as caused by deficient costimulation are defective clonal expansion and defective IL-2 production (49, 50). Importantly, stimulation of such anergic cells in the presence of exogenous IL-2 can overcome the block in proliferative potential (51). Consistent with this definition of anergy, we show that the ability to produce detectable levels of IL-2 is markedly reduced in low-avidity T cells. Furthermore, in contrast to high-avidity T cells, the expansion of low-avidity T cells greatly depends on exogenous sources of IL-2. However, contrary to what has been reported for T cells rendered anergic as a consequence of deficient costimulation, the expansion of low-avidity T cells remains very poor, even in the presence of high IL-2 concentrations.

In contrast to the pronounced expansion defect and the reduced IL-2 production, the production of IFN-γ and the ability to perform cytotoxic effector functions experience less drawbacks from a low-avidity TCR-ligand interaction. As expected, we find that high- and low-avidity T cells differ with respect to the Ag concentration needed for optimal cytolytic function and IFN-γ production, with low-avidity T cells being less sensitive. Additionally, the levels of IFN-γ produced per cell are consistently lower in the low-avidity T cell population. These differences in IL-2 and IFN-γ production are consistent with the hierarchical organization of TCR signaling thresholds for cytokine responses described by Itoh and Germain (52). They found that the ability to produce IL-2 requires stronger TCR signals than that for production of IFN-γ. In addition, a gradual increase in IFN-γ levels per cell at increased TCR signal strength was observed (52). Most likely, the self-specific TCRs in our model are of such a low affinity that the signaling thresholds required for IL-2 production cannot be reached, regardless of the Ag concentration. The low-avidity cells have the capacity to lyse tumor cells overexpressing the relevant Ag. Thus, a low-avidity interaction between TCR and ligand selectively impairs some but not other functions. Whether this differential response to low-avidity TCR interactions can be linked to specific signaling events is currently being investigated.

From studies using APL, it has become clear that Ag recognition is quite flexible and a given TCR can interact with a spectrum of
related ligands (15, 53). Only TCR-ligand interactions of appropriate longevity will result in activation of the complete intracellular signaling machinery, resulting in activation of a broad spectrum of cellular responses (17, 54, 55). Three types of such APL have been defined: fully activating ligands (agonists), partially activating ligands (partial agonists), and inhibitory ligands (antagonists) (15–18, 56–58). The question then arises whether the low-avidity self-specific T cells do display intrinsic defects, or whether the NP-derived ASNENMDAM epitope behaves as a partial agonist for low-avidity self-specific receptors and as a full agonist for high-avidity receptors. This justifies a search for APL with increased affinity for the self-TCR, which may induce the full range of T cell functions.

To provide better insight into the interaction of low-affinity TCRs with (modified) peptides, we have used a peptide vaccination strategy in combination with anti-CD40 treatment (26–28). We here show that the unresponsiveness of the low-avidity T cell repertoire toward vaccination with the ASNENMDAM peptide can be overcome by vaccination with the variant ASNENMDTM peptide. Two possible explanations exist for the increased immunogenicity of a given APL. First, an important determinant for T cell activation is the affinity of the peptide for MHC. APL with increased MHC affinity have been shown to exhibit increased immunogenicity. Not only replacement of MHC anchor residues (59–61), but also of nonanchor residues (13, 62, 63), as performed in this study, can influence the MHC affinity and thereby the immunogenicity. However, MHC-binding studies showed that the conservative change of A3T at position 8 in the ASNENMDAM peptide did not result in increased stabilization of H-2Db molecules, indicating that the increased immunogenicity of ASNENMDTM cannot be attributed to an increased affinity for MHC. The second explanation for increased immunogenicity may lie in the kinetics of the APL-TCR interaction (1, 7, 31). Substitution of a residue involved in the interaction between a peptide and the TCR can result in changes in TCR ligand on- and off-rates, and those in turn may lead to quantitatively and/or qualitatively different responses as a consequence of changes in the signaling pathways induced (14, 15, 57, 64, 65). TCR equilibrium-binding studies and TCR off-rate studies show that the conservative substitution of threonine for alanine at position 8 of the ASNENMDAM peptide significantly increased the affinity of the peptide for three of three different self-specific TCRs. In the x-ray structure of H-2Dd in complex with the NP epitope of influenza A/PR/8/34 (sequence ASNENMETM), the Thr8 side chain points outside of the peptide-binding groove and is considered a potential TCR-interacting

FIGURE 7. Variant peptide ASNENMDTM forms a higher affinity ligand for the complete ASNENMDAM-specific T cell repertoire. B10NP and B10 mice were infected intranasally with 25 HAU of influenza A/NT/60/68 virus, and spleens were isolated 16 days after infection. After 7 days of in vitro restimulation of the spleen cells (5 × 10^6/well) with 5 × 10^-2 μg of NP 366–374 peptide (B10NP mice) or 5 × 10^-3 μg of NP 366–374 peptide (B10 mice), cells were harvested. A. Cells were stained with anti-CD8 mAb and ASNENMDAM tetramers (left panels) or ASNENMDTM tetramers (right panels). Percentages of NP-specific cells gated on the CD8^+ population and the mean fluorescence intensity of the tetramer signals are shown. B. Cells were simulated with the indicated concentrations of ASNENMDAM (□, □) or ASNENMDTM (○, ●) for 5 h. Cells were stained with allophycocyanin-conjugated anti-CD8 and FITC-conjugated anti-IFN-γ. The IFN-γ production is corrected for the percentage of NP-specific T cells present in the cultures (see A) by dividing the percentage of IFN-γ^+ cells by the percentage of NP-specific T cells multiplied by 100.
residue (66). Thus, the functional consequences of the Ala-Thr mutation observed here are most likely due to a direct effect of this mutation on the interaction of the p8 side chain with the TCRs of self-specific T cells.

We here show that a single variant peptide can form a higher affinity ligand for a complete polyclonal low-avidity self-specific T cell population. Although it remains to be established whether this type of universal superligand can be identified for all self-specific T cell populations, it does suggest identification of APL as a feasible strategy to manipulate the self-specific T cell repertoire. In addition, the observation that mice in which the high-avidity AS NENMDAM-specific T cell population has been deleted harbor a fully functional ASNENMDTM-specific T cell repertoire suggests that self-Ag expression does not lead to the formation of substantial “holes” in the polyclonal T cell repertoire (K. E. de Visser, W. H. G. Kessels, A. M. Kruisbeek, and T. N. M. Schumacher, manuscript in preparation).

Summarizing, we show that even the immunogenecity of a self-Ag that is actively expressed in the thymus and on peripheral tissues (8, 19) can be enhanced by a single amino acid substitution, resulting in a variant peptide with increased TCR affinity. Triggering of self-specific T cells with this variant peptide results in a quantitatively enhanced T cell response with maintenance of effector function.

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