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The Impact of Self-Tolerance on the Polyclonal CD8$^+$ T Cell Repertoire[1]

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TCRs possess considerable cross-reactivity toward structurally related Ags. Because the signaling threshold for negative selection is lower than that required for activation of mature T cells, the question arises as to which extent thymic deletion of self-specific T cells affects T cell responsiveness toward foreign peptides. In this study we show, in three different mouse models systems, that the polyclonal CD8$^+$ T cell repertoire has a marked ability to react against the majority of Ags related to self despite self-tolerance, even in cases where self and foreign differ only marginally at a single TCR-contact residue. Thus, while individual T cells are markedly cross-reactive, the ability to distinguish between closely related Ags is introduced at the polyclonal T cell level. ***The Journal of Immunology*****, 2004, 172: 2324–2331.**

The polyclonal T cell repertoire is cleared of autoreactive T cells by the tightly controlled intrathymic process of negative selection (reviewed in Refs. 1 and 2). The critical parameter determining the outcome of this thymic selection process is the strength of the interaction between immature T cells and MHC/peptide (MHC/p)[6] complexes. T cells which express a TCR with a high affinity for any of the thousands of thymically expressed MHC/p complexes will undergo cell death, and as a consequence, an estimated 50–65% of positively selected thymocytes are eliminated by negative selection (3–5).

From studies using variant peptides, it has become clear that one single mature T cell identified by its potential to recognize a particular MHC/p combination can interact with many variants of this peptide (6–8); it has been estimated that a single TCR can recognize up to 10^6 different MHC/p complexes (8). This extensive cross-reactivity is suggested to be an intrinsic feature of TCR-MHC/p interactions that is due to flexibility of the complementarity determining region (CDR)3 TCR loops (9), and may be essential to allow the recognition of the $\sim 10^{12}$ possible foreign epitopes (8) by a T cell repertoire that has a diversity of $\sim 2.5 \times 10^7$ (10). However, this pronounced cross-reactive nature of T cell recognition is likely to also form a determining factor in the intrathymic molding of the T cell repertoire. Clonal deletion of T cells that can productively interact with a self peptide/MHC complex will at the same time ablate their ability to respond against related foreign Ags in the periphery. This effect may be further enhanced by the fact that the signal threshold for negative selection is lower than that required for activation of mature T cells (11–14). This higher sensitivity of immature T cells may be required to minimize the escape of potentially autoreactive T cells into the periphery. However, this "margin of safety" may likewise reduce T cell responsiveness toward peptides that structurally resemble self-Ags. The data on the effects of self-tolerance on the foreign Ag-specific T cell repertoire are conflicting. Early work from Matzinger and colleague (15) has suggested that tolerance to self may result in unresponsiveness to a foreign Ag. In line with this, Pircher et al. (11) have demonstrated that neonatal infection of mice with a lymphocytic choriomeningitis virus (LCMV)-strain encoding a variant T cell epitope resulted in clonal deletion of immature TCR-transgenic T cells specific for the wild-type LCMV epitope, whereas this variant LCMV virus was unable to trigger activation of the mature TCR-transgenic T cells. Likewise, Sandberg et al. (16) showed that T cells that recognized variants of a self-Ag were of lower avidity than T cells specific for a foreign Ag. In apparent contrast, anecdotal evidence that T cell immunity can be evoked toward foreign Ags that differ from a self-Ag by one or a few amino acids has been provided (17, 18).

Previous studies have defined the imprint of self-tolerance on TCR sequence diversity (4, 19, 20). Tolerance to self-Ags not only reduced the diversity of TCRα and TCRβ-chain sequences of self-specific T cells, but also of T cells specific for variants of these peptides (4, 19). However, whether and to what extent tolerance to self-Ags affects the ability of the remaining T cell repertoire to respond to foreign Ags remains largely unknown. It has previously been argued that in a polyclonal immune setting, the impact of self-Ag expression on the capacity to recognize foreign Ags may primarily depend on the nature of T cell cross-reactivity (8). Specifically, cross-reactivity may be 'focused' in that all T cells that react with a given self-peptide cross-react with a similar set of foreign peptides. Alternatively, T cells may display "unfocused" cross-reactive behavior, in which different T cells that react with a given self Ag cross-react with distinct sets of foreign peptides. Although in the former case self-tolerance will also affect T cell responsiveness toward related Ags, self-Ag expression may result

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[6] Abbreviations used in this paper: MHC/p, MHC/peptide; CDR, complementarity determining region; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; Tag, T Ag; APL, altered peptide ligand; TRAMP, transgenic adenocarcinoma mouse prostate; HAU, hemagglutinating unit.

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in much smaller defects in the functional T cell repertoire should cross-reactivity largely be unfocused (Fig. 1). In this study, we provide a comprehensive view of the effects of self-tolerance on the foreign Ag-specific T cell repertoire and demonstrate that the effects of self-tolerance on the functional T cell repertoire are remarkably small. The observation that the effects of self-tolerance in a polyclonal T cell repertoire are limited compared with those previously observed in a monoclonal repertoire (11) suggests that a major benefit of a diverse T cell repertoire may be to facilitate the distinction between self and foreign.

Materials and Methods

Mice

C57BL/10 (H-2^b) (B10) and C57BL/6 (H-2^b) (B6) mice were obtained from the Experimental Animal Department of The Netherlands Cancer Institute (Amsterdam, The Netherlands). B10 mice transgenic for a fragment of influenza nucleoprotein (NP) (aa 1,2, 328–498) under control of the MHC class I promotor (B10NP mice) were kindly provided by Dr. D. Kioussis (National Institute for Medical Research, London, U.K.) (21). B6 mice with transgenic expression of the SV40 large T Ag (Tag) under control of the prostate-specific rat probasin promotor, designated TRAMP (transgenic adenocarcinoma mouse prostate) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (22, 23). All mice were kept under specified pathogen-free conditions. B10, B6, and B10NP mice were used at 6–10 wk of age and TRAMP mice were used at 8–12 wk of age. Mice from the different groups were matched for gender in all experiments, except for the HY model, where T cell responsiveness of male and female mice was compared. All animal experiments were performed in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of The Netherlands Cancer Institute.

Peptides and MHC-tetramers

The H-2D^b binding NP$_{366–374}$ peptide (sequence: ASNENMDAM) and altered peptide ligands (APLs) (A1G, A1L, E4Q, M6I, D7E, D7N, A8L, and A8T) and the H-2D^b binding HY$_{738–746}$-peptide (sequence: KCSRNRQYL) and APLs (K1R, K1L, R6K, R6L, Q7E, Q7N, Y8F, and Y8L) and the H-2K^b binding SV40 large T$_{404–411}$ peptide (sequence: VVYDFLKC) (24) and APLs (V1A, V1L, and L6V) were synthesized by standard 9-fluorenylmethoxycarbonyl (FMOC) synthesis and purified by reversed-phase HPLC. Tetramers of soluble MHC class I molecules complexed with peptides were produced as described (25, 26). For increased stability, H-2K^b-tetramers of SV40 Tag were generated using peptides in which the C-terminal anchor residue cysteine was changed to leucine.

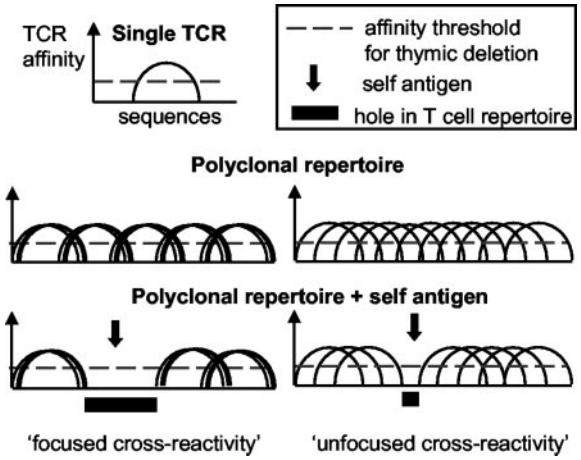

FIGURE 1. Schematic representation of the impact of self-Ag expression on the polyclonal T cell repertoire. In a situation in which individual Ag-specific T cells have similar cross-reactive behavior (focused cross-reactivity), deletion of immature thymocytes with the capacity to recognize thymically expressed self-Ags will result in the formation of significant holes in the repertoire of Ags that can be recognized. In contrast, in a situation in which individual Ag-specific T cells recognize different sets of peptides (unfocused cross-reactivity), thymic self-Ag expression will result in small holes in the T cell repertoire. In this situation, a maximally diverse T cell repertoire is generated while self-tolerance is maintained.

Virus infections and peptide vaccinations

For live virus infections, anesthetized mice were infected by intranasal administration of 50 μl of HBSS (Life Technologies, Grand Island, NY) containing 0.1 hemagglutinating unit (HAU) of A/HK/1/68 virus or 200 HAU of A/HKx31 virus. For peptide vaccinations, mice were injected s.c. at the tailbase with 100 μg of peptide emulsified in CFA (Difco, Detroit, MI). In addition, at days 0, 1, and 2, mice were injected i.p. with 100 μg of anti-CD40 Ab (FGK.45) (27, 28). After 10 days, splenocytes were isolated and used for the generation of splenocyte cultures.

Splenocyte cultures

Spleens were isolated and single cell suspensions were prepared by transferring the spleens through a nylon filter (NPBI, Emmer-Compascuum, The Netherlands). Erythrocytes were lysed by NH_4Cl treatment and the remaining cells were washed. Splenocytes were seeded into 24-well culture plates at 5×10^6 cells/well in 2 ml of IMDM (Life Technologies) containing 10% FCS (PAA Laboratories, Pasching, Austria), 100 IU/ml penicillin (Boehringer Mannheim, Germany), 100 μg/ml streptomycin (Boehringer Mannheim) and 5×10^{-5} M 2-ME (Merck, Darmstadt, Germany) supplemented with 20 Cetus U of IL-2/ml (Cetus, Emeryville, CA) and peptide at the indicated concentrations. Cultures were restimulated at day 7 of in vitro culture with peptide and IL-2, and analyzed at day 14.

Flow cytometry

Cells were harvested and samples of 5×10^5 cells were washed twice with PBS with 0.5% BSA and 0.02% NaN_3 (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 MHC tetramers (for the NP and HY models) or PE-conjugated anti-CD8 (Caltag Laboratories, Burlingame, CA) and allophycocyanin-conjugated MHC-tetramers (for the SV40 Tag model) 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). Log-transformed data of T cell responses in mice approximate a normal distribution, and T cell responses in different groups were therefore compared by an unpaired one-tailed Student's *t* test of log-transformed data.

Intracellular cytokine staining

Splenocytes were cultured as described above in bulk cultures. At day 14, cells were purified over a lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient. Intracellular IFN-γ stainings were performed as described (29).

Results

Negative selection of self-specific CD8$^+$ T cells does not affect responsiveness toward a structurally related viral epitope

To investigate the functional consequences of negative selection on the polyclonal T cell repertoire, we made use of B10NP mice that transgenically express a fragment (aa 1,2, 328–498) of the influenza A NP under control of the H-2K^b promotor (21). Evidence for tolerance toward the immunodominant H-2D^b binding NP$_{366–374}$-peptide (sequence: ASNENMDAM) has been provided by crossing B10NP mice with mice transgenic for the NP$_{366–374}$-specific F5 TCR (30). Infection of B10NP mice with an influenza A virus that contains the same NP$_{366–374}$ sequence (influenza A/HK/1/68) does not lead to a measurable expansion of NP$_{366–374}$-specific T cells either at day 8 (Fig. 2*A*), or at earlier time points (day 1, 2, 4 and 7) after viral infection (data not shown). In contrast, when B10NP mice are infected with a variant influenza virus (influenza A/HKx31) that encodes a mutant NP Ag (ASNENMETM) that differs from the A/HK/1/68-encoded epitope by two conservative substitutions, a strong Ag-specific T cell response ensues. This response in B10NP mice is indistinguishable from the response of wild-type animals both with respect to size and kinetics (Fig. 2*B* and data not shown). Thus, although the T

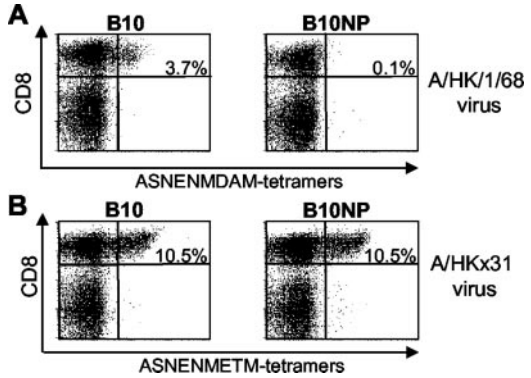

FIGURE 2. Tolerance toward influenza A/HK/1/68 $NP_{366–374}$ does not affect T cell responsiveness toward influenza A/HKx31 $NP_{366–374}$. B10 and B10NP mice were infected intranasally with 0.1 HAU of A/HK/1/68 virus (*A*) or with 200 HAU of A/HKx31 virus (*B*) and lungs were isolated 8 days after infection. Single cell suspensions were stained with anti-CD8 mAb and ASNENMDAM (*A*) or ASNENMETM (*B*) tetramers. Percentages of tetramer-binding cells of the total cell population are shown.

cell repertoire in B10NP mice is devoid of high-avidity ASNENMDAM-specific T cells and despite the fact that there is considerable structural similarity between A/HKx31 and A/HK/1/68 epitopes, ASNENMETM-specific T cell immunity is unaffected.

To study the responsiveness of self-tolerant mice toward structurally related foreign Ags in a more accessible system, a peptide immunization strategy was set up. Because for most Ags no expansion of Ag-specific T cells could be detected directly ex vivo, probably due to a low precursor frequency of Ag-specific T cells, splenocyte cultures were restimulated in vitro with the corresponding antigenic peptide. Following in vitro restimulation, Ag-specific T cell responses were monitored by MHC-tetramer staining. Because MHC-tetramers containing a particular ligand can bind to T cells that have a low functional avidity for that ligand (31), MHC-tetramer binding per se should not be taken as evidence for a high avidity interaction. In contrast, the ability of a particular ligand to induce the expansion of T cells has been shown a more reliable parameter for functional T cell avidity (32), and in the current experiments MHC-tetramers are simply used to monitor this expansion. In line with the viral infection experiments, vaccination of B10NP mice with the ASNENMDAM peptide did not result in detectable T cell responses, whereas vaccination with ASNENMETM did generate a sizable T cell response, as measured by MHC-tetramer staining (Fig. 3). The presence of expanded, tetramer-specific T cells in splenocyte cultures generated from vaccinated B10NP and B10 mice correlates well with the presence of IFN-γ-producing cells after stimulation with the corresponding peptide (Fig. 3), and ASNENMETM-specific cells from either B10 or B10NP mice displayed comparable Ag sensitivities (Fig. 3*E*). These data demonstrate in both a viral infection model and a peptide vaccination strategy that self-tolerance does not necessarily ablate reactivity toward a structurally related foreign Ag.

Impact of negative selection of NP-specific T cells on the functional T cell repertoire in B10NP mice

To investigate the impact of negative selection on the functional T cell repertoire specific for foreign Ags in more detail, we analyzed whether clonal deletion of ASNENMDAM-specific T cells in B10NP mice results in loss of T cell responsiveness to closely related peptide variants. A panel of variant peptides was generated by substituting single amino acids at $NP_{366–374}$-peptide N-terminal, central, or C-terminal positions. Conservative amino acid substitutions were tested first. Only in cases where no T cell responses were induced by these variants in NP-transgenic mice were additional peptide variants with nonconservative amino acid substitutions at the same position examined. Only variants of the self-peptide that did not affect the H-$2D^b$/peptide-complex stability were selected (33), and that did induce a T cell responses in wild-type mice. Both B10NP and control B10 mice were immunized with the variant peptides, and following in vitro restimulation, T cell responses were analyzed by flow cytometry using H-$2D^b$-tetramers containing the variant peptide (Table I). Despite tolerance to the NP-derived self-epitope, pronounced T cell responsiveness could be elicited in B10NP mice toward five of six variant peptides

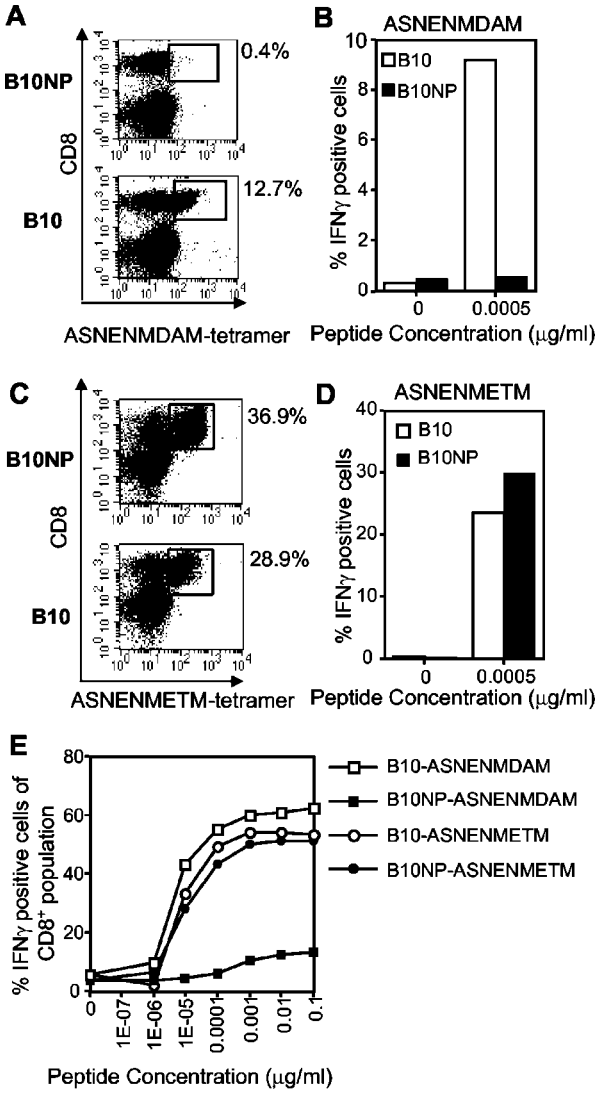

FIGURE 3. Unresponsiveness of B10NP mice upon ASNENMDAM but not ASNENMETM peptide vaccination. B10NP and B10 mice were vaccinated at day 0 with ASNENMDAM peptide (*A* and *B*) or ASNENMETM peptide (*C* and *D*). Splenocytes were stimulated with 5×10^{-4} μg/ml of the corresponding peptide and 20 U of IL-2/ml. After 14 days of culture, cells were stained with anti-CD8 mAb and H-$2D^b$-tetramers containing ASNENMDAM (*A*) or ASNENMETM peptide (*C*). Numbers represent the percentage of tetramer-binding cells of the total cell population. *B* and *D*, Cultured splenocytes from *A* and *C* were stimulated for 4 h with 5×10^{-4} μg/ml of the corresponding peptide or without peptide. Percentages of IFN-γ-producing cells of the total cell population are shown. *E*, Splenocytes from *A* and *D* were stimulated for 4 h with the indicated peptide concentrations. The mean percentages IFN-γ-producing cells of the $CD8^+$ T cell populations are shown ($n = 3$).

Table I. *Summary of tetramer reactivity of T cells elicited after immunization with the NP peptide or NP variants*[a]

Epitope	% Epitope-Specific T Cells of Total CD8$^+$ Cells			% NP-Specific T Cells of Total CD8$^+$ Cells	
	B10NP	B10	*p*	B10NP	B10
NP	0.4 ± 0.4	19.2 ± 22.0	**0.0027**		
NP A1G	0.2 ± 0.2	10.3 ± 14.9	**0.020**	0.1 ± 0.1	13.7 ± 21.9
NP A1L	1.3 ± 1.4	33.2 ± 13.3	**0.0051**	0.0 ± 0.0	10.7 ± 17.4
NP E4Q	24.4 ± 15.3	41.2 ± 24.5	0.16	0.4 ± 0.3	5.6 ± 5.2
NP M6I	4.8 ± 3.0	5.4 ± 1.9	0.34	0.0 ± 0.0	1.0 ± 1.6
NP D7E	1.0 ± 0.3	33.4 ± 26.1	**0.0009**	0.0 ± 0.0	4.9 ± 6.9
NP D7N	2.4 ± 2.2	1.6 ± 2.1	0.63	0.3 ± 0.4	0.6 ± 0.5
NP A8L	7.5 ± 5.9	25.2 ± 21.1	0.11	0.2 ± 0.1	4.4 ± 4.5
NP A8T	14.3 ± 8.1	14.7 ± 7.2	0.47	11.7 ± 8.0	15.2 ± 2.7

[a] The average percentages of tetramer-reactive T cells ±SD are shown for cells stained with MHC-tetramers containing the variant peptide used for immunization (left panels) and cells that were stained with MHC-tetramers containing the wild-type NP peptide (right panels). H2-D^b-tetramer stainings below 0.4% are background values. Statistical comparisons (log-transformed, unpaired, one-tailed Student's *t* test) of T cell responses in tolerant and nontolerant mice are shown (*p*). Values of $p < 0.05$ (indicating tolerance) are indicated in bold.

with a single conservative substitution at either a central or C-terminal position (P4, P6, P7, and P8) (Fig. 4). Furthermore, for two of these variant peptides (conservative substitutions E4Q and M6I) we verified that the presence of tetramer-positive cells coincided with T cell function, as determined by Ag-induced IFN-γ production (Fig. 5). Comparison of the magnitude of CD8$^+$ T cell responses in B10 and B10NP mice at days 7 and 14 demonstrated that T cell responses developed with identical kinetics in these mice, suggesting that the observed reactivity is not skewed during in vitro stimulation (data not shown). Importantly, the variant-specific T cell populations elicited in B10NP and B10 mice displayed comparable Ag sensitivities as determined both by the peptide concentration required for IFN-γ production and MHC-tetramer staining intensity (data not shown), indicating that for the majority of variants a functional T cell population can be activated in B10NP mice. Vaccination of B10NP mice with a peptide with a charge-conserving D7E substitution failed to induce a detectable T cell response in B10NP mice, although this Ag is highly immunogenic in nontransgenic B10 mice (Fig. 4). A peptide variant in which the aspartic acid at P7 is changed into asparagine (D7N) does form an effective immunogen in B10NP mice (Fig. 4), suggesting that TCR-MHC/p interaction is dependent on the charge at this position. Both a conservative (A1G) as well as a nonconservative (A1L) amino acid replacement at the N-terminal alanine residue of the $NP_{366–374}$-peptide results in a variant peptide that cannot be recognized by the T cell repertoire in B10NP mice (Fig. 4). This unresponsiveness of B10NP mice toward these variant peptides is entirely due to the endogenous expression of the $NP_{366–374}$-peptide, because T cell responses specific for these variant peptides can be generated in nontransgenic B10 mice.

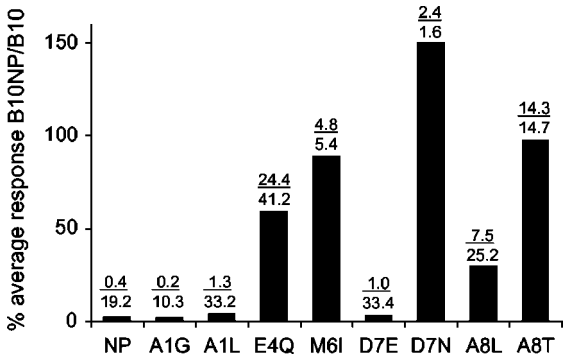

FIGURE 4. Impact of negative selection of ASNENMDAM-specific T cells on the functional T cell repertoire. B10NP and B10 mice were vaccinated with the indicated variant peptides and splenocytes were restimulated with the corresponding peptide at 5×10^{-4} μg/ml. After 14 days of culture, cells were harvested and stained with anti-CD8 mAb and H-2D^b-tetramers containing the corresponding peptide. The ratio plotted on the *y*-axis represents the average percentage of peptide-specific cells of the CD8$^+$ T cell population in B10NP mice ($n = 3$) divided by the average percentage of peptide-specific cells of the CD8$^+$ T cell population in B10 mice ($n = 3$) multiplied by 100.

In summary, although ubiquitous expression of NP results in the complete absence of functional high-avidity T cells specific for the ASNENMDAM-peptide, the polyclonal T cell repertoire is capable of inducing T cell responses against the majority of variants that contain single amino acid changes at P4, P6, P7, and P8. Self-tolerance to the NP Ag did extend to a variant peptide with a charge-conserving substitution at P7 and to variants with conservative or nonconservative substitutions at P1 (A1G and A1L).

Impact of negative selection of HY-specific T cells on the functional T cell repertoire in male mice

To examine whether the observed ability of the peripheral T cell repertoire to distinguish between self and foreign is epitope-specific, or a more general phenomenon, we studied the consequences of expression of the male-specific HY Ag on the capacity to respond to closely related peptide Ags. The immunodominant H-2D^b-binding $HY_{738–746}$-epitope (KCSRNRQYL) contains charged residues at both the central and at the N-terminal positions, allowing a further evaluation of the role of charge and position in self-nonself discrimination. Evidence for thymic deletion of $HY_{738–746}$-specific T cells in male mice has been provided by the analysis of mice transgenic for an $HY_{738–746}$-peptide-specific TCR by von Boehmer and colleagues (34). Consistent with this, vaccination of nontransgenic mice with the $HY_{738–746}$ peptide triggers a pronounced T cell response in female mice but not in male mice (Fig. 6). We subsequently compared T cell responsiveness in both male and female C57BL/6 mice against several HY-related peptide analogues. Immunization with two different Ags carrying conservative substitutions at P7 (Q7E and Q7N) resulted in comparable T cell responses in male and female mice (Table II, Fig. 6). Vaccination with a conservative single amino acid variant of $HY_{738–746}$ at position 8 (Y8F) did result in T cell responses in male mice. Vaccination with a second single amino acid variant at this position (Y8L) did not. Strikingly, replacement of the positively charged amino acid at either position 1 or 6 with similarly

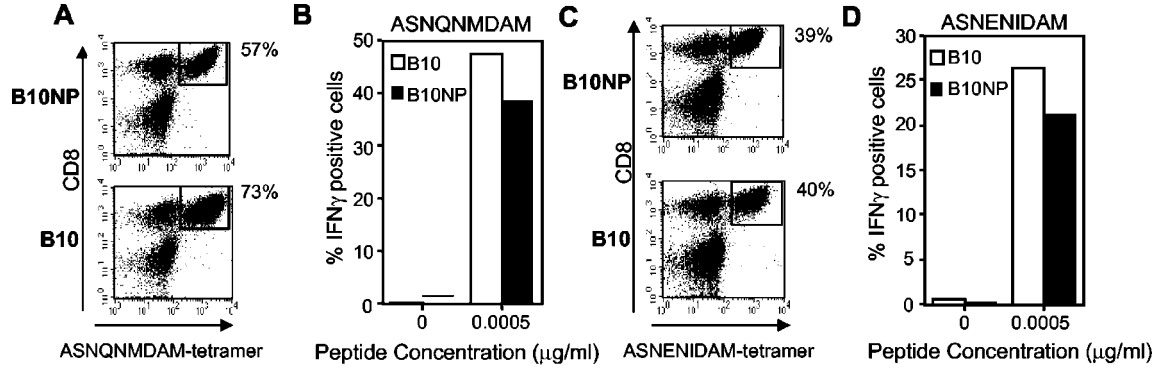

FIGURE 5. Negative selection of ASNENMDAM-specific T cells does not affect T cell activity against structurally related peptides. B10NP and B10 mice were vaccinated with the variant peptide ASNQNMDAM (E4Q) (*A* and *B*) or ASNENIDAM (M6I) (*C* and *D*) and splenocytes were stimulated with the corresponding peptide. After 14 days of culture, cells were stained with anti-CD8 mAb and H-2D^b-tetramers containing the ASNQNMDAM peptide (*A*) or the ASNENIDAM peptide (*C*). Percentages of tetramer-binding CD8$^+$ T cells of the total cell population are depicted. *B* and *D*, Cultured splenocytes from *A* and *C* were stimulated for 4 h with 5×10^{-4} μg/ml of the corresponding peptide or without peptide. Percentages of IFN-γ-producing cells of the total cell population are shown.

charged residues (K1R and R6K) results in peptide Ags that failed to induce any detectable T cell response in male mice, whereas substantial T cell responses were observed in female mice. Substitution of the arginine at P6 with an uncharged residue (R6L) results in a peptide variant that did evoke detectable T cell responses in both male and female mice. Combined with the data from the B10NP model (Fig. 4), these observations suggest that TCR recognition of charged contact residues depends primarily on charge interactions and may rely less on shape complementarity. Notably, substitution of the positively charged amino acid at P1 with an uncharged residue (K1L) resulted in a variant peptide that could not trigger a detectable T cell response in male mice (Fig. 6). These data are consistent with the data obtained with the B10NP mouse model (Fig. 4), and indicate that for these H-2D^b-bound peptides the self-tolerant T cell repertoire is unable to recognize homologues that solely differ at the N terminus.

The inability of the tolerized T cell repertoire to productively recognize peptide variants that diverge at P1 may be a particular feature of H-2D^b-restricted Ags. In contrast to other MHC molecules such as H-2K^b (35, 36), the peptide backbone in H-2D^b/peptide complexes is arched such that the N-terminal side chains may contribute less to TCR recognition (37). Alternatively, the inability of the polyclonal T cell repertoire to discriminate between self-peptides and variants that differ only at P1 may be explained by the fact that the orientation in which different TCRVα chains

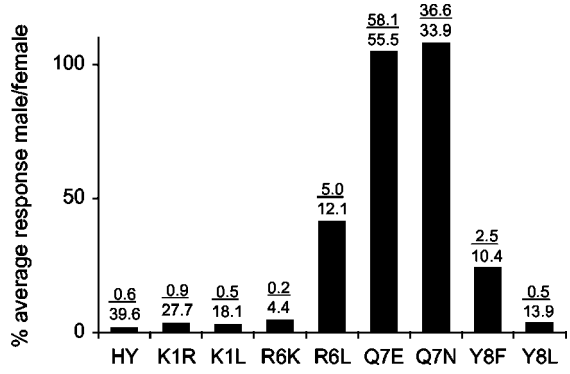

FIGURE 6. Impact of HY expression on the functional T cell repertoire. Female and male B6 mice were vaccinated with the $HY_{738-746}$-peptide (KCSRNRQYL), or with the indicated variant peptides, and splenocytes were stimulated with 5×10^{-2} μg/ml of the corresponding peptide. T cell responsiveness was determined as in Fig. 4 ($n = 3$ for each group).

interact with the N-terminal peptide residues of MHC/p complexes varies little (38). This conserved orientation of TCRα chains may result in a more focused cross-reactivity of different TCRs at this position. In addition, the N-terminal side chains of MHC-bound peptides are primarily contacted by the CDR1 loop of the TCRα-chain (38), and due to the limited structural variability within CDR1 structures, the formation of TCRs that can discriminate between the original epitope and N-terminal variants may be an infrequent event.

Impact of negative selection of SV40 Tag-specific T cells on the functional T cell repertoire in TRAMP mice

TRAMP mice are transgenic for SV40 Tag under control of the rat probasin regulatory elements (23). In both male and female TRAMP mice, T cells specific for SV40 Tag are tolerized by thymic deletion (39). Consistent with this, vaccination of TRAMP mice with the wild-type $Tag_{404-411}$ peptide (sequence: VVYDFLKC) does not result in $Tag_{404-411}$-specific T cell responses, whereas vaccination of control mice with the $Tag_{404-411}$-peptide does (Fig. 7 and Refs. 40 and 41).

To study the impact of Tag expression on the functional T cell repertoire, we generated variants of the Tag peptide by conservative replacement of single amino acid residues at the TCR contact residues P1 (V1A and V1L) or P6 (L6V). Although male TRAMP mice are completely unresponsive toward the wild-type Tag peptide, normal responsiveness could be elicited toward variant peptides with conservative changes either at central or N-terminal positions (Table III, Fig. 7). Thus, also for H-2K^b-presented peptides, foreign Ags that differ only minimally from a self-Ag can induce functional T cell responses. In contrast to the NP and HY models, variants with mutations at P1 are also efficiently recognized by the mature T cell repertoire. These data indicate that, at least in some cases, Ags that differ solely at P1 can also productively be recognized by the self-tolerant T cell repertoire and suggest that unresponsiveness may primarily correlate with the TCR accessibility of the mutant amino acid within the MHC/p complex.

Reactivity of T cells induced with APLs toward the self-epitope

All the T cell populations induced by vaccination of tolerant and nontolerant mice were also tested by staining with MHC-tetramers containing the unmodified epitope. MHC-tetramer staining with APLs can reveal both low and high avidity interactions, and it seemed useful to assess whether T cells with reactivity to self

Table II. *Summary of tetramer reactivity of T cells elicited after immunization with the HY peptide or HY variants*[a]

	% Epitope-Specific T Cells of Total CD8$^+$ Cells			% HY-Specific T Cells of Total CD8$^+$ Cells	
Epitope	Male	Female	*p*	Male	Female
HY	0.6 ± 0.4	39.6 ± 19.1	**<0.0001**		
HY K1R	0.9 ± 0.5	27.7 ± 25.0	**0.0033**	0.1 ± 0.1	28.7 ± 18.7
HY K1L	0.5 ± 0.3	18.1 ± 7.4	**0.0017**	0.9 ± 0.2	15.2 ± 7.2
HY R6K	0.2 ± 0.3	4.4 ± 1.7	**0.0005**	0.2 ± 0.1	2.2 ± 2.7
HY R6L	5.0 ± 3.1	12.1 ± 9.8	0.30	0.8 ± 0.3	1.6 ± 1.0
HY Q7E	58.1 ± 12.6	55.5 ± 24.4	0.63	16.5 ± 12.3	3.2 ± 2.1
HY Q7N	36.6 ± 21.6	33.9 ± 15.2	0.52	16.1 ± 11.1	20.3 ± 16.2
HY Y8F	2.5 ± 3.2	10.4 ± 12.8	0.28	4.3 ± 6.0	11.2 ± 13.3
HY Y8L	0.5 ± 0.2	13.9 ± 7.4	**0.0033**	0.4 ± 0.2	7.0 ± 7.6

[a] The average percentages of tetramer-reactive T cells ±SD are shown for cells stained with MHC-tetramers containing the variant peptide used for immunization (left panels) and cells that were stained with MHC-tetramers containing the wild-type HY peptide (right panels). H2-D^b-tetramer stainings below 0.4% are background values. Statistical comparisons (log-transformed, unpaired, one-tailed Student's *t* test) of T cell responses in tolerant and nontolerant mice are shown (*p*). Values of $p < 0.05$ (indicating tolerance) are indicated in bold.

could be induced by APL vaccination. In the NP model, the Ag-specific T cell populations induced by APL vaccination were not cross-reactive with tetramers containing the unmodified NP epitope for four of five variants tested (Table I), indicating that vaccination with these APLs does not lead to activation of T cells with any detectable self-reactivity. T cell populations reactive with wild-type NP-tetramers could efficiently be induced in B10NP mice by immunization with the A8T variant. However, while these cells have a high functional avidity for the A8T variant, they have a low functional avidity for the wild-type NP epitope (32). These data indicate that a substantial fraction of T cells that have a high functional avidity for this APL happen to display a low but detectable avidity for the self-Ag. However, a structural basis for this phenomenon is currently lacking. In the HY model, three variants (Q7E, Q7N, and Y8F) induced T cell populations in male mice that bind to wild-type HY-tetramers (Table II). In the SV40 model, vaccination of TRAMP mice with altered peptides with substitutions at position 1 (V1A and V1L) resulted in T cell pools that partly reacted with SV40-tetramers (Table III). Whether the self-specific T cell populations that can be induced through immunization of TRAMP mice with SV40 APLs or immunization of male mice with HY APLs are capable of recognizing endogenous levels of the self-epitope will require further evaluation. However, the fact that these T cell responses could not be induced by vaccination with the self-peptide, suggest that the capacity of these cells to recognize "self" may be limited. In line with this, analysis of the self-specific T cell repertoire induced by vaccination of B10NP mice with the A8T APL indicates that these cells are inefficient at recognizing endogenously produced levels of the NP-Ag (32).

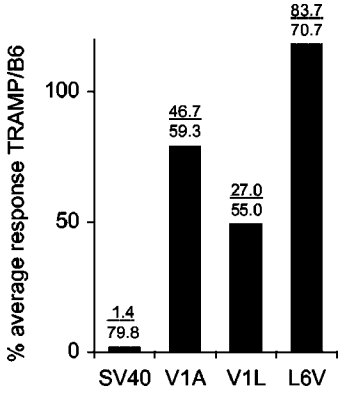

FIGURE 7. Consequences of expression of SV40 Tag in male TRAMP mice on the functional T cell repertoire. Male B6 and male TRAMP mice were vaccinated with the $Tag_{404-411}$-peptide (VVYDFLKC), or with the indicated variant peptides and T cell responsiveness was determined as in Fig. 4 ($n = 3$ for each group).

Discussion

Given the cross-reactive nature of T cell recognition, and given that the signal threshold for negative selection is lower than that required for activation of mature T cells, thymic deletion of self-specific T cells may at the same time destroy reactivity toward structurally related (foreign) Ags. Indeed, in a monoclonal T cell repertoire, establishment of self-tolerance has been shown to ablate reactivity toward a closely related variant peptide (11). However, it has previously been argued that in a polyclonal T cell repertoire the negative effect of T cell cross-reactivity on the postselection T cell repertoire may to some extent be compensated by the diversity of the T cell repertoire under the proviso that T cell cross-reactivity should in that case be unfocused (Fig. 1) (8), and in a number of cases reactivity to Ags that are similar to self could in fact be demonstrated (17, 18). In this study, we provide a systematic analysis of T cell responsiveness toward single amino acid variants of self-peptides in three antigenic systems in which a defined Ag is either self or nonself. We show that although a functional high avidity T cell repertoire is undetectable for all three self-Ags, deletion of self-specific T cells does not lead to the inability of the

Table III. *Summary of tetramer reactivity of T cells elicited after immunization with the SV40 peptide or SV40 variants*[a]

	% Epitope-Specific T Cells of Total CD8$^+$ Cells			% SV40-Specific T Cells of Total CD8$^+$ Cells	
Epitope	TRAMP	B6	*p*	TRAMP	B6
SV40	1.4 ± 0.9	79.8 ± 4.3	**0.0002**		
SV40 V1A	46.7 ± 44.3	59.3 ± 15.5	0.24	8.1 ± 4.4	51.3 ± 8.7
SV40 V1L	27.0 ± 17.4	55.0 ± 22.5	0.10	11.9 ± 6.3	41.6 ± 17.9
SV40 L6V	83.7 ± 1.8	70.7 ± 9.6	0.95	1.2 ± 1.2	27.4 ± 4.3

[a] The average percentages of tetramer-reactive T cells ±SD are shown for cells stained with MHC-tetramers containing the variant peptide used for immunization (left panels) and cells that were stained with MHC-tetramers containing the wild-type SV40 peptide (right panels). H2-K^b-tetramer stainings below 1.0% are background values. Statistical comparisons (log-transformed, unpaired, one-tailed Student's *t* test) of T cell responses in tolerant and nontolerant mice are shown (*p*). Values of $p < 0.05$ (indicating tolerance) are indicated in bold.

immune system to recognize a large fraction of structurally related Ags. This includes variants that differ from the self-epitope by only a single CH_2 bond (Q7N in HY-Ag, V1L and L6V in Tag-Ag) or hydroxyl group (Y8F in HY-Ag). Although in some cases small changes in the peptide sequences may result in T cell recognition of minor conformational adjustments in MHC structure (42), this is unlikely to account for all the observed reactivities (18, 43).

Substitutions at P1 in two H2-D^b-restricted epitopes and charge-conserving substitutions cannot be distinguished from self by the self-tolerant T cell repertoire. The former may be explained by the limited accessibility of the P1 side chain in H2D^b/peptide complexes. The latter suggests that recognition of charged peptide residues by the TCR is relatively independent of shape complementarity, consistent with the proposed role of charge-charge interactions during TCR-MHC/p association (44) possibly through long-range electrostatic steering (45).

The current data demonstrate that T cell immunity can be induced against the majority of Ags (12 of 19 tested) that differ from self by only a single amino acid substitution. Although in this study we have only demonstrated T cell responsiveness toward a set of foreign Ags that most closely resemble self, it stands to reason that the vast collection of more distantly related foreign Ags will be similarly unaffected. In support of this, the lack of T cell tolerance of B10NP mice to the A/HKx31 epitope that diverges from self at both P7 and P8 can be mapped to the alanine to threonine mutation at the penultimate position (Fig. 4). Thus, while the T cell repertoire is deprived of self-specific T cells, it still provides a nearly maximal Ag recognition capacity for foreign Ags that differ from the self-Ag at TCR contact residues.

This marked ability of the polyclonal T cell repertoire to react against the majority of Ags that are closely related to self fits well with the finding that small structural differences in minor histocompatibility Ags between donor and recipient suffice to trigger T cell responsiveness upon allogeneic transplantation. For example, for human minor histocompatibility Ags such as the *HB-1*-derived HLA-B44 binding epitope (H8Y) (46), and the DFRY-derived HLA-A1 binding epitope (C4S) (47), single amino acid differences in TCR-contact residues do result in alloresponses. Likewise, in mice, a conservative substitution in the H13 minor H-2D^b epitope (I4V) explains the strong T cell response upon H13 mismatched transplantation (48).

Based on these data, we conclude that despite the substantial cross-reactive behavior of T cells, and the lower Ag sensitivity of immature T cells, deletion of self-specific T cells does not greatly affect T cell responsiveness toward foreign Ags. This ability to respond to self-like Ags suggests that T cell cross-reactivity is primarily unfocused (Fig. 1). As a consequence, polyclonal T cell populations can readily distinguish self from nonself Ags where monoclonal T cell populations can fail (11).

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