

Selective Expansion of Cross-reactive CD8⁺ Memory T Cells by Viral Variants

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Summary

The role of memory T cells during the immune response against random antigenic variants has not been resolved. Here, we show by simultaneous staining with two tetrameric major histocompatibility complex (MHC)–peptide molecules, that the polyclonal CD8⁺ T cell response against a series of natural variants of the influenza A nucleoprotein epitope is completely dominated by infrequent cross-reactive T cells that expand from an original memory population. Based on both biochemical and functional criteria, these cross-reactive cytotoxic T cells productively recognize both the parental and the mutant epitope *in vitro* and *in vivo*. These results provide direct evidence that the repertoire of antigen-specific T cells used during an infection critically depends on prior antigen encounters, and indicate that polyclonal memory T cell populations can provide protection against a range of antigenic variants.

Key words: influenza virus • major histocompatibility complex tetramers • peptides • C57BL mice • *in vivo*

The small size of the contact surface between the TCR and MHC-bound peptide suggests that the peptide specificity in TCR–MHC interactions is limited (1). Indeed, a large number of studies have demonstrated that the sequence requirements for ligand recognition by T cell clones *in vitro* are quite minimal (2–5). However, in certain situations, variations in CTL epitopes may lead to a total or partial loss of functional recognition by cytotoxic T cells, due to qualitatively different TCR signaling upon interaction with these altered peptide ligand–MHC complexes (for a review, see reference 6). Indeed, altered peptide ligands have been shown to antagonize antigen-specific T cell responses both *in vitro* and *in vivo* (7–14). These experiments have firmly established that selected mutations in T cell epitopes can abolish productive T cell recognition. However, it is unresolved whether such abortive T cell responses are common upon *in vivo* encounter of antigen variants. In an MHC outbred population, T cell epitope mutations encountered during transient infections are likely to be random, and we therefore set out to examine how a polyclonal T cell population would react to such random antigenic variants in an *in vivo* model.

After infection with influenza A viruses, large numbers of influenza A-specific cytotoxic T cells can be recovered from pulmonary tissue, lymphoid organs, and peripheral blood in mice and humans (15–17). In C57BL/10 mice, the immunodominant CTL epitope of influenza A viruses is located in the

viral nucleoprotein (NP),¹ amino acids 366–374. Within the TCR-exposed side chains of the COOH-terminal region of this peptide (positions 6, 7, and 8), significant variation exists among naturally occurring influenza A strains. Early work from Townsend and Skehel (18) showed that certain influenza A virus NP-specific T cell lines can recognize viral variants in *in vitro* assays, but the extent and *in vivo* relevance of such cross-reactivity have remained elusive. To assess the consequences of exposure to naturally occurring variants on T cell reactivity in an *in vivo* setting, we have analyzed the effects of polyclonal T cell memory, formed during a primary influenza A virus infection, on the subsequent response against a series of influenza A virus variants. To directly visualize T cells displaying antigen receptors that are monospecific for a certain viral variant or that cross-react between different variants, we used differentially labeled oligomeric peptide–MHC class I complexes (19). Contrary to conventional functional assays, such as ⁵¹Cr release, this strategy allows for a direct assessment of the potential for cross-recognition of individual cells in mixed T cell populations. The results thus obtained show that cross-reactive memory T cells generated during a primary infection dominate the T cell response during a secondary infection with a variant virus even when such cross-reactive cells are rare

¹Abbreviations used in this paper: APC, allophycocyanin; HAU, hemagglutinin unit(s); NP, nucleoprotein; PI, propidium iodide.

in the original memory T cell pool. The implications of these findings for peripheral T cell repertoire selection and viral variation are discussed.

Materials and Methods

Animals. C57BL/10 mice at 5–6 wk of age were obtained from the animal department of the Netherlands Cancer Institute. Mice were handled at all times in accordance with institutional guidelines.

MHC Tetramers and Peptides. Peptides were produced using standard g-fluorenylmethoxycarbonyl (Fmoc) chemistry. Soluble fluorochrome (PE or allophycocyanin [APC])-labeled MHC tetramers were produced as described previously (17, 19) and stored frozen in Tris-buffered saline/16% glycerol/0.5% BSA.

Viruses and Cells. Influenza A viruses A/NT/60/68 and A/HKx31 were provided by Dr. R. Gonsalves, National Institute for Medical Research, London, UK. Influenza virus B/Lee/40 was obtained from the American Type Culture Collection. Mice were killed at indicated time points after infection, and organs were removed for further analysis. Inflamed lung tissue and spleens were minced in single chamber mesh filters. The single cell suspensions obtained were treated with NH_4Cl solution to get rid of contaminating erythrocytes, before staining for flow cytometry purposes.

The influenza A virus (A/NT/60/68) NP-derived H-2D^b-restricted CTL epitope, ASNENMDAM, was introduced into EL4 tumor cells by retroviral insertion as a COOH-terminal fusion with the enhanced green fluorescent protein (eGFP) gene product (Wolkers, M.C., manuscript in preparation).

Flow Cytometry. In all instances, mononuclear cells were stained with directly labeled mAbs or MHC tetramers. Analysis was performed on a FACSCalibur™ (Becton Dickinson) using CELLQuest™ software (Becton Dickinson). Before staining, propidium iodide (PI) was added to gate for PI-negative (living) lymphocytes.

Cytotoxicity Assay and MHC Monomer Competition Assay. Cytolytic activity of sorted CD8⁺ T cells derived from inflamed pulmonary tissue was determined in a standard 5-h ⁵¹Cr-release assay. EL4 target cells were preincubated with peptides for 1 h at 37°C. Percent specific lysis was calculated from the equation: [(experimental ⁵¹Cr release – spontaneous ⁵¹Cr release)/maximal ⁵¹Cr release – spontaneous ⁵¹Cr release] × 100%.

Spleen-derived mononuclear cells were stained with anti-CD8 mAb in the presence of PE-labeled MHC tetramers containing increasing concentrations of MHC monomers. Cells were subsequently analyzed by flow cytometry.

Intracellular IFN- γ Staining. Intracellular cytokine staining was performed as described (20). In brief, spleen cells were incubated with peptide (0.5 μM) for 5–6.5 h at 37°C in the presence of recombinant human (rh)IL-2 (50 U/ml) and Brefeldin A (0.1 $\mu\text{l/ml}$). After incubation, cells were surface stained with anti-CD8a-APC mAb (PharMingen), incubated in Cytofix/Cytoperm solution (PharMingen) for 20 min on ice, washed, and stained for intracellular cytokine with anti-IFN- γ -FITC (PharMingen) or FITC-labeled isotype control antibody (PharMingen). Analyses were performed on a FACSCalibur™ (Becton Dickinson) using CELLQuest™ software. Isotype control antibodies resulted only in background staining (data not shown).

Peptide Immunization. Mice were injected subcutaneously with 100 μg peptide in IFA, 4–6 wk after a primary influenza A virus infection. On days 0, 1, and 2, 100 μg anti-CD40 mAb (FGK.45) was injected intravenously (21). On day 10 after peptide immunization, spleen cells were used for flow cytometry analysis.

Results

Selective Expansion of Cross-reactive Influenza A Virus-specific T Cells. To assess the effect of random antigen variation on the dynamics of T cell responses in vivo, we infected mice with pairs of influenza A viruses. These viruses expressed either the same NP_{366–374} epitope or epitope variants. The specificity of the resulting T cell repertoire was assessed by two-parameter MHC tetramer staining, and association of MHC tetramers to NK receptors was ruled out through analysis of CD8b-expressing cells only. When mice are infected once or twice with either influenza virus strain A/NT/60/68 or A/PR/8/34, which differ in the sequence of the immunodominant NP CTL epitope at positions 7 and 8 (ASNENMDAM vs. ASNENMETM), the vast majority of the resulting NP-specific T cells selectively recognize the epitope of the strain encountered and not that of the opposite strain (Fig. 1 A, panels 1 and 3). This dominant role of the peptide side chains at positions 7 and 8 in ligand recognition by the majority of T cells is in accord with the prominent contribution of p8 and especially p7 to the TCR-exposed surface of this peptide–MHC complex (22).

This virus strain specificity of the NP-reactive CTLs contrasts sharply with the apparent lack of strain specificity during secondary responses against a variant strain. When mice that had recovered from a previous infection with influenza virus strain A/NT/60/68 are challenged with viral strain A/PR/8/34, most if not all NP^{PR}-reactive T cells are fully cross-reactive between the two NP variants (Fig. 1 A, panel 4). This phenomenon is reciprocal: in mice that have previously experienced infection with strain A/HKx31 (a reassortant strain with the NP gene from A/PR/8/34), the NP-reactive T cell population that emerges upon infection with A/NT/60/68 is fully cross-reactive between the two viral strains (Fig. 1 A, panel 2).

Although somewhat variable between individual mice, the extent of binding of the two MHC tetramers appears independent of the order in which the epitopes were encountered over a large series of experiments. To compare the affinity for primary and secondary antigen in a more direct manner, competition studies were performed. These experiments demonstrate that the binding of fluorochrome-labeled MHC tetramers can be inhibited by similar concentrations of ASNENMDAM- and ASNENMETM-containing monomers, indicating equal affinity of the cross-reactive TCRs for either antigen (Fig. 1 B). In addition, these data rule out the possibility of dual TCR expression (23, 24) by the cross-reactive T cells, since both MHC monomers compete for binding of ASNENMDAM-containing MHC tetramers.

Cross-reactivity Correlates with Cross-recognition. The above results indicate that the subsequent encounter of variants of a T cell epitope results in the expansion of a cross-reactive T cell population, as established by biochemical assays. In fact, this expansion inhibits the expansion of the largely strain-specific population observed during a regular primary response. However, T cell recognition of ligands with similar affinities can have drastically different functional outcomes, due to differences in off rates of TCR–ligand interactions

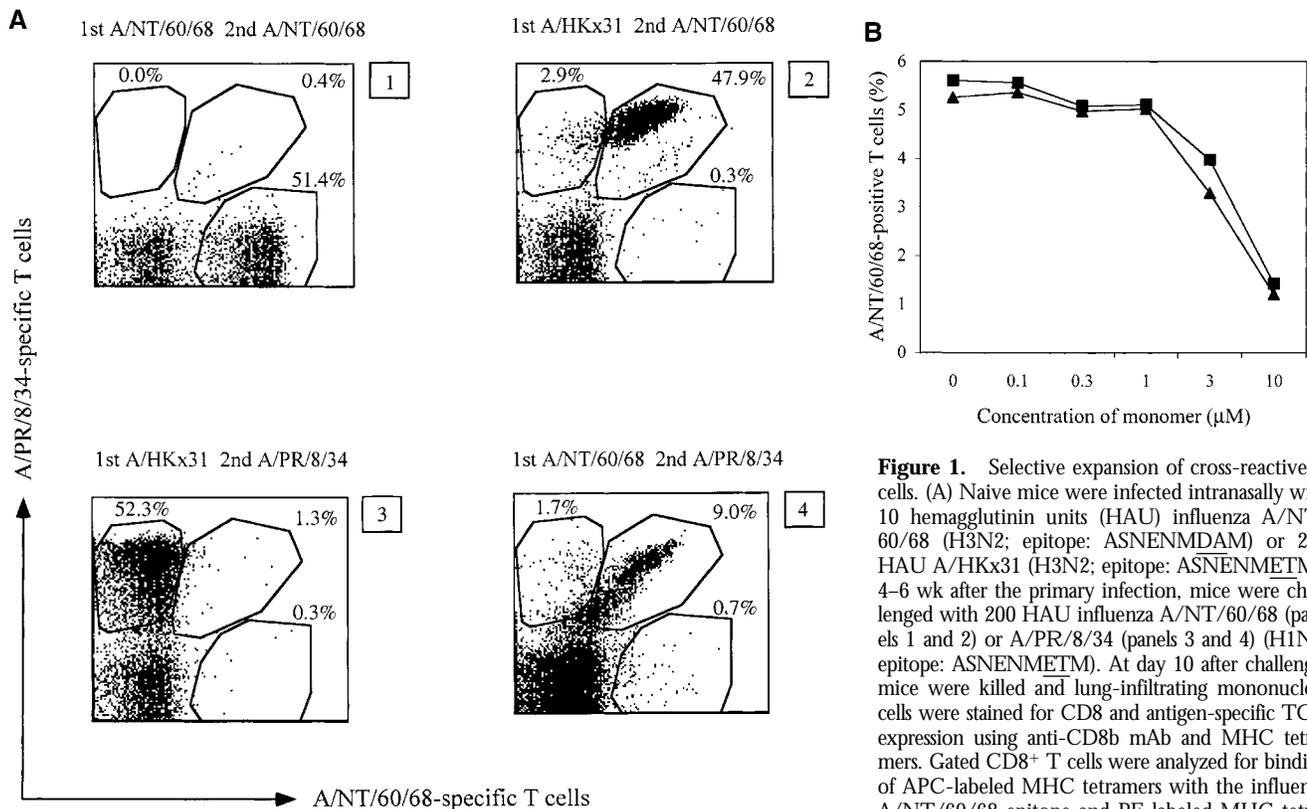


Figure 1. Selective expansion of cross-reactive T cells. (A) Naive mice were infected intranasally with 10 hemagglutinin units (HAU) influenza A/NT/60/68 (H3N2; epitope: ASNENMDAM) or 200 HAU A/HKx31 (H3N2; epitope: ASNENMETM). 4–6 wk after the primary infection, mice were challenged with 200 HAU influenza A/NT/60/68 (panels 1 and 2) or A/PR/8/34 (panels 3 and 4) (H1N1; epitope: ASNENMETM). At day 10 after challenge, mice were killed and lung-infiltrating mononuclear cells were stained for CD8 and antigen-specific TCR expression using anti-CD8b mAb and MHC tetramers. Gated CD8⁺ T cells were analyzed for binding of APC-labeled MHC tetramers with the influenza A/NT/60/68 epitope and PE-labeled MHC tetramers with the influenza A/PR/8/34 epitope. Identical results are obtained when challenge is performed with influenza A 81/HO (H7N2; NP_{366–374} sequence ASNENMDAM) instead of A/NT/60/68, and is therefore independent of HN subtype (not shown). Results represent the outcome of more than five experiments performed. (B) Mice were sequentially infected with influenza virus A/HKx31 and A/NT/60/68. On day 7 after the second infection, spleen cells were incubated with MHC tetramers containing the NP_{366–374} epitope (ASNENMDAM) of A/NT/60/68. H-2D^b monomers complexed with ASNENMDAM (■) or ASNENMETM (▲) were added in increasing amounts, and inhibition of the binding of MHC tetramers to CD8⁺ T cells was determined.

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(25–29). Therefore, we examined the functional behavior of this biochemically cross-reactive T cell population towards both the primary and the mutant epitope. The cross-reactive T cells that are observed during a recall influenza A virus infection lyse target cells pulsed with both the primary and the recall antigen directly ex vivo to the same extent over a wide range of E/T ratios, and over a range of peptide concentrations (Fig. 2 A).

As an independent test of functional cross-recognition, we measured the ability of these cells to initiate IFN- γ synthesis upon stimulation with the original or variant epitope. Since simultaneous staining of cells with MHC tetramers and intracellular IFN- γ staining is technically difficult, cells were stimulated in the presence of the original and variant peptide separately or simultaneously (Fig. 2 B). The proportion of IFN- γ -positive CD8⁺ T cells is similar in all three cases, indicating that the T cell populations that recognize the two epitopes are largely overlapping and therefore cross-reactive.

We conclude that the T cell population that emerges upon encounter of an antigenic variant is biochemically cross-reactive, and this cross-reactivity is fully reflected in their functional behavior.

Cross-reactive T Cells Are Selectively Expanded Memory T Cells. To better understand the ontogeny of the cross-

reactive T cell population, we examined the kinetics and composition of this T cell pool. The cross-reactive cytotoxic T cell population appears 2–3 d earlier at the site of infection (i.e., pulmonary tissue; data not shown) than the antigen-specific T cells during infection of naive mice (15–17), suggesting that these cells originate from a pre-existing memory T cell population. Several studies have shown a narrowing of the antigen-specific polyclonal TCR repertoire during recall infection, due to the preferential outgrowth of a subpopulation of memory cells (30–33). In naive mice that are infected with influenza virus A/PR/8/34, the repertoire of NP-specific T cells involves a variety of BV elements (Fig. 3 A). The slight preferential usage of the BV8.3 element reported previously for C57BL animals infected with influenza virus A/PR8/34 was not observed in these experiments (34). In contrast, in A/NT/60/68-primed mice that are infected with influenza virus A/PR/8/34, the repertoire of A/PR/8/34-specific T cells is highly restricted (Fig. 3 A). This narrow T cell repertoire is likely to reflect the affinity maturation observed previously (33) compounded by the low number of cross-reactive T cells within the original memory population. In certain animals, the oligoclonal nature of the cross-reactive T cell population appears to be directly visible from double-tetramer analyses of the influenza-reactive CD8⁺ T cell population. In these mice,

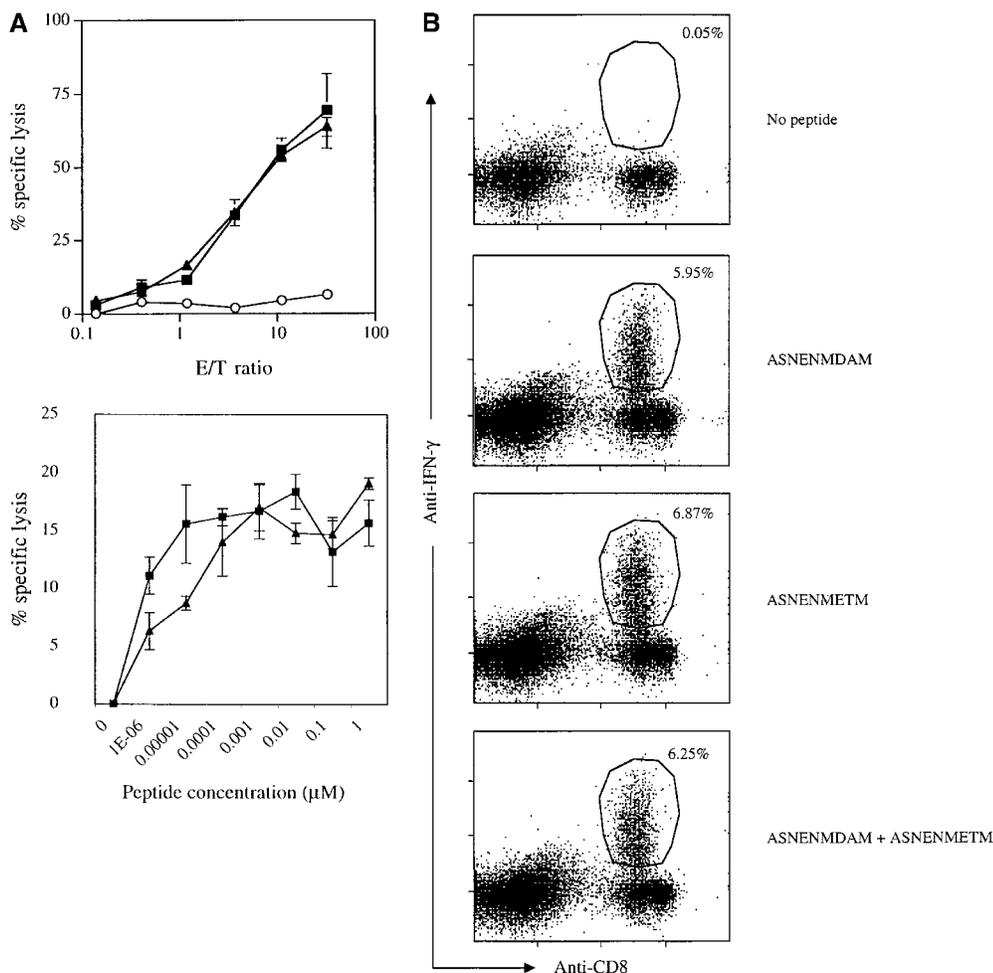


Figure 2. Ex vivo function of cross-reactive T cells. (A) Cytotoxic activity of cross-reactive T cells. Mice were sequentially infected with A/NT/60/68 (10 HAU) and influenza A/PR/8/34 (200 HAU) and at day 7 after infection, lung-infiltrating CD8⁺ T cells were purified by flow cytometry and directly used as effector cells in a 5-h ⁵¹Cr-release assay at the indicated E/T ratios. Top panel: targets were EL-4 cells (○), EL4 cells incubated with 100 μM of the A/NT/60/68 NP₃₆₆₋₃₇₄ epitope ASNENMDAM (■), or the A/PR/8/34 NP₃₆₆₋₃₇₄ epitope ASNENMETM (▲). Bottom panel: purified CD8⁺ T cells were used as effector cells in a 5-h ⁵¹Cr-release assay at an E/T ratio of 7:1. Target cells were EL4 cells preincubated with indicated concentrations of either ASNENMDAM (■) or ASNENMETM (▲). Titration of the peptide concentration to the picomolar range also results in similar lysis of both targets. (B) Intracellular IFN-γ staining of spleen cells. Mice were sequentially infected with A/NT/60/68 and A/PR/8/34, and on day 7 after the second infection, spleen cells were stimulated with peptides corresponding to the CTL epitopes of A/NT/60/68 and A/PR/8/34 separately or combined for 6.5 h. Subsequently, cells were stained for surface CD8 and intracellular IFN-γ. Numbers represent the percentage of total lymphocytes that are IFN-γ positive.

the expanded cross-reactive T cell population appears as two to three separate populations (an example is shown as Fig. 3 B), which may reflect slightly distinct affinities for the primary and secondary antigen.

Specificity of Cross-reactive T Cells. Several reports have indicated that the requirements for activating memory T cells differ from those for activating naive T cells, making them more susceptible to low-affinity TCR triggering or cytokine-mediated stimulation (35–39). To address the possible contribution of aspecific or more broadly reactive T cells in the formation of the cross-reactive T cell population, influenza A/NT/60/68-primed mice were infected with the antigenically unrelated influenza B virus (B/Lee/40). Both at day 4 (data not shown) and day 8 after infection (Fig. 4, panel 1), no expansion of the influenza A/NT/60/68-specific memory T cell population is observed. This is in agreement with results by others showing that bystander activation during an acute viral infection is minimal (20, 40, 41). Furthermore, the influenza A virus-specific T cells in mice that underwent sequential infections with A/NT/60/68 and A/PR/8/34 do not bind peptide-

MHC tetramers that contain an adenovirus E1A-derived CTL epitope (Fig. 4, panel 2). Thus, the cross-reactive T cell population that expands upon infection of primed mice does require a significant structural homology between two antigens that are encountered sequentially, and is specific for these two peptide-MHC complexes. Finally, selective outgrowth of cross-reactive T cells does not take place to an appreciable extent when the two related antigens are introduced simultaneously (Fig. 4, panel 3). This suggests that the selective advantage of cross-reactive cells relies on quantitative or qualitative traits of the T memory population, such as homing properties or the requirements for co-stimulatory signals.

Structural Requirements for Cross-reactivity. To provide a first estimate of the extent of structural homology between two antigens that is required for the selective expansion of cross-reactive T cells, we searched the National Center for Biotechnology Information (NCBI) GenBank database for other natural variants of the H-2D^b-restricted influenza NP epitope. Thus far, 10 variants of this epitope have been identified, 7 of which contain mutations that affect TCR-

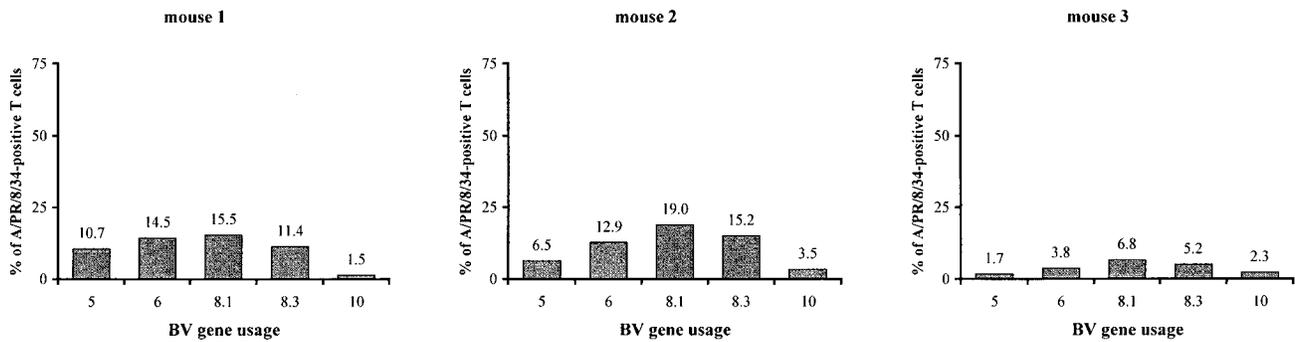
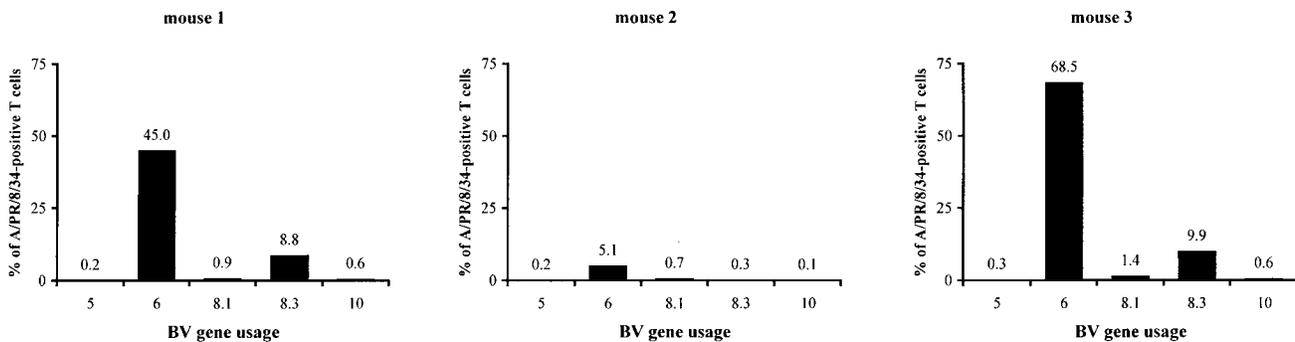
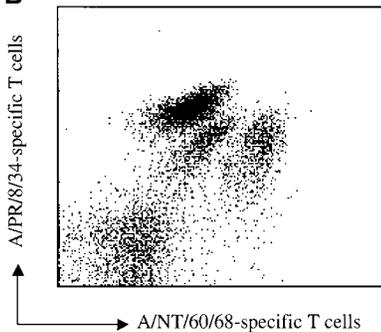
A**1st A/PR/8/34****1st A/NT/60/68, 2nd A/PR/8/34****B**

Figure 3. Cross-reactive cells are oligoclonal T memory cell expansions. (A) Cross-reactive CD8⁺ T cells display a restricted BV repertoire. Naive (top) or A/NT/60/68-primed mice (bottom) were infected with 10 and 200 HAU of influenza A/PR/8/34, respectively. At 7 d after infection, lung-infiltrating A/PR/8/34-specific CD8⁺ T cells were analyzed for expression of a series of BV elements by using a panel of fluorochrome-labeled anti-BV antibodies (PharMingen). Values represent the percentage of A/PR/8/34-specific CD8⁺ T cells that stain with a particular anti-BV antibody. (B) Mice were sequentially infected with influenza virus A/HKx31 and A/NT/60/68. 7 d after the second infection, mononuclear cells recovered from pulmonary tissue were stained with anti-CD8 and APC-labeled MHC tetramers with the influenza A/NT/60/68 epitope and PE-labeled MHC tetramers with the influenza A/PR/8/34 epitope, and analyzed by FACS[®] analysis. The dot plot reveals three distinct populations of selectively expanded cross-reactive CD8⁺ T cells.

exposed side chains (positions 6, 7, and 8) (reference 22, and Table I). Because influenza A virus is not a common mouse pathogen, this set of mutants should be random with respect to T cell recognition. Synthetic peptides corresponding to these mutants were generated, and mice that had previously been exposed to influenza A/NT/60/68 or A/HKx31 were challenged by subcutaneous immunization. To circumvent the need of helper T cells for the induction of an effective CTL response, mice were treated with anti-CD40 mAb (FGK.45) after vaccination (21, 42–44). These experiments establish that in most if not all cases where influenza A virus-primed mice are confronted with antigens that contain a single mutation within the T cell epitope, an influenza-specific T cell population emerges that is fully cross-reactive be-

tween the primary and secondary antigen (Table I). This phenomenon is not only observed for conservative substitutions, but also for more drastic amino acid changes (e.g., Ala8→Asn). For variants that contain multiple alterations in TCR-exposed residues, cross-reactivity is observed for some sequences (e.g., ASNENMDAM→ASNENMETM), but not for others (e.g., ASNENMDAM→ASNENVEAM). Both the type of mutation and the contribution of the mutated residues to the TCR-exposed surface of the peptide are likely to be determining factors in this regard. For one of the mutant epitopes (ASNENVETM), the functional behavior of the cross-reactive T cells was also tested. In line with the biochemical data, intracellular IFN- γ staining of spleen cells that were stimulated with the primary antigen

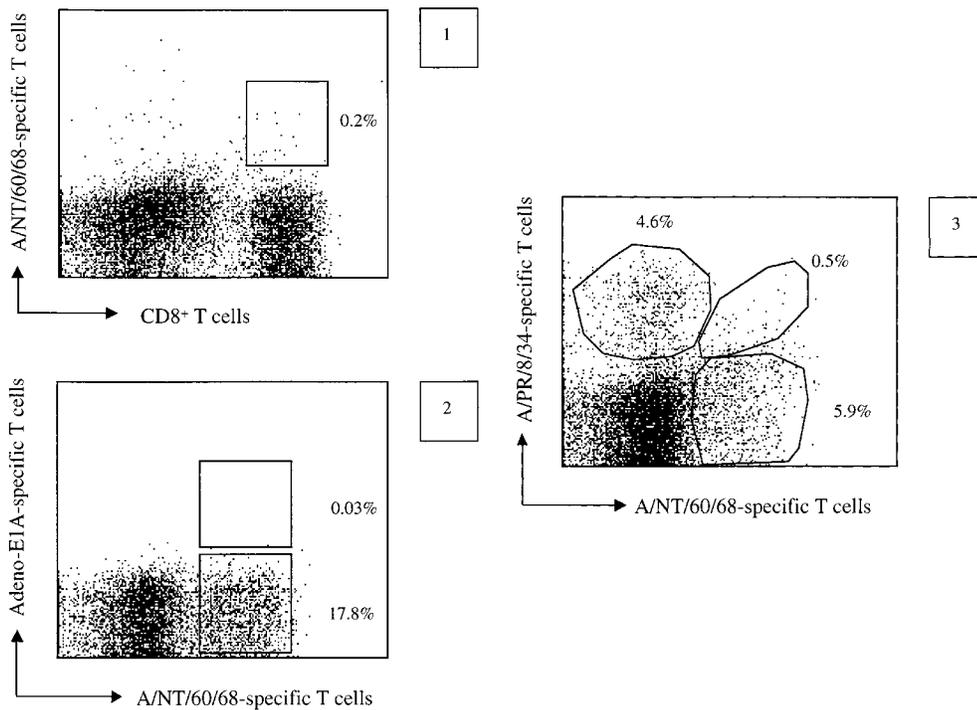


Figure 4. Specificity of cross-reactive T cells. Panel 1: mice were sequentially infected with A/NT/60/68 (10 HAU) and influenza B/Lee/40 (125 HAU) and at day 8 after infection, lung-infiltrating lymphocytes were analyzed for binding of MHC tetramers expressing the A/NT/60/68-derived epitope, ASNENMDAM, and CD8 expression. Panel 2: mice were sequentially infected with A/NT/60/68 (10 HAU) and A/PR/8/34 (200 HAU) and at day 7 after infection, lung-infiltrating CD8⁺ T cells were analyzed for binding of APC-labeled MHC tetramers containing the ASNENMDAM peptide and PE-labeled MHC tetramers with the adenovirus E1A-derived epitope, SGPSNT-PPEI. Panel 3: mice were infected with A/NT/60/68 (10 HAU) and A/HKx31 (200 HAU) simultaneously and at day 7 after infection, lung-infiltrating CD8⁺ T cells were analyzed for the presence of A/NT/60/68- and A/PR/8/34-specific T cells. In all panels, the numbers given represent the percentage of CD8⁺ T lymphocytes that stain with the indicated MHC tetramer(s).

(ASNENMETM) or the peptide variant used for vaccination revealed an identical percentage of responding cells (data not shown).

In Vivo Function of Cross-reactive T Cells. Cross-protection or the lack thereof by an epitope-specific T cell population cannot readily be tested through the use of mutant or recombinant viral strains. Even in settings where B cell immunity is absent, such as in μ -MT mice, it is difficult to exclude a possible contribution of T cell responses against subdominant epitopes that are conserved between the primary and the recall strain. To circumvent these difficulties, we developed a model system in which only the T cell epitope under study is shared between the primary and secondary antigen encounter. The NP₃₆₆₋₃₇₄ CTL epitope of influenza virus strain A/NT/60/68 was introduced into the EL4 tumor cell line as a COOH-terminal fusion with the eGFP gene product. Although the parental cell line grows progressively when injected subcutaneously in C57BL/10 mice, the NP epitope-expressing tumor is rejected over a 2–3-wk time period. Furthermore, tumor rejection is paralleled by the appearance of an NP₃₆₆₋₃₇₄-specific T cell population and is markedly enhanced by simultaneous infection with influenza virus A/NT/60/68 (Wolkers, M.C., manuscript in preparation). Thus, the introduced NP epitope appears to function as a bona fide (neo)tumor antigen in this setting, as recognition of this antigen is correlated with tumor rejection.

To examine the in vivo effects of cross-reactive T cell populations, mice that had previously been exposed to in-

fluenza virus A/NT/60/68 (carrying the homologous NP epitope), A/HKx31 (reassortant of A/PR/8/34; carrying a variant NP epitope), or an unrelated influenza B virus were challenged with EL4-NP₃₆₆₋₃₇₄ tumor cells. After tumor cell injection, blood samples were taken from individual mice and the frequency of virus strain-specific and cross-reactive CD8⁺ T cells was measured. In mice that had been infected with the unrelated influenza B virus (Fig. 5, panel 1), tumor growth is comparable to that in uninfected mice (data not shown). As expected, mice that were previously exposed to influenza virus A/NT/60/68, which shares the CTL epitope with the EL4-NP₃₆₆₋₃₇₄ tumor, showed a strong reduction in tumor growth (Fig. 5, panel 1). This protection is accompanied by a massive and rapid increase in the number of A/NT/60/68-mono-specific T cells, which were apparently reactivated from the memory T cell pool by the NP epitope-expressing tumor (Fig. 5, panel 2). Importantly, intermediate tumor growth was seen in mice that had previously been infected with variant virus A/HKx31 (Fig. 5, panel 1). In addition, the reduced tumor outgrowth in these mice is accompanied by the expansion of a large population of CD8⁺ T cells, which cross-react between the NP₃₆₆₋₃₇₄-expressing EL4 tumor and the viral strain used for priming (Fig. 5, panel 3). These results illustrate that the presence of a structurally related T cell antigen promotes the expansion of infrequent cross-reactive T cells even in the absence of any further noticeable homology between primary and secondary challenge, and this expansion is accompanied by a significant reduction in tumor outgrowth.

Table I. Sequence Requirements for Cross-reactive T Cells

Secondary vaccination	Mutation(s)	No. of CD8 ⁺ T cells (×1,000)/spleen	
		Cross-reactive	Variant-specific
Primary infection A/NT/60/68 (ASNENMDAM)			
ASNENMDTM	A→T	1,600	22
ASNENMDTM	A→T	650	≤2
ASNENMDNM	A→N	260	≤5
ASNENMDNM	A→N	110	≤2
ASNENMEAM	D→E	160	≤2
ASNENMEAM	D→E	12	≤5
ASNENMETM	DA→ET	220	12
ASNENMETM	DA→ET	41	7
ASNENVEAM	MD→VE	20	≤1
ASNENVEAM	MD→VE	≤3	≤2
ASNENVETM	MDA→VET	≤3	≤1
ASNENVETM	MDA→VET	≤2	≤5
Primary infection A/HKx31 (ASNENMETM)			
ASNENMEAM	T→A	100	≤4
ASNENMDTM	E→D	200	≤3
ASNENVETM	M→V	73	≤4
ASNENMDAM	ET→DA	10	6
ASNENMDNM	ET→DN	≤3	≤2
ASNENVEAM	M.T→V.A	8	≤1

Mice were infected with influenza A/NT/60/68 (10 HAU) (top) or A/HKx31 (200 HAU) (bottom). 4–6 wk after the primary infection, mice were challenged with synthetic peptides corresponding to the various naturally occurring influenza A NP_{366–374} variants, in combination with anti-CD40 mAb, FGK.45. At day 10 after challenge, spleen CD8⁺ T cells were analyzed by flow cytometry. Data represent the number of CD8⁺ T cells per spleen that stain with MHC tetramers of both the primary and secondary NP_{366–374} epitope or that are specific for the variant antigen. Each data point represents a single mouse; two mice per peptide challenge with A/NT/60/68, one mouse per peptide challenge with A/HKx31. Variants are depicted in order of descending homology with the primary antigen, based on the contribution of the mutated residue to the TCR-exposed surface (reference 22) and the nature of the amino acid substitution. Background values are $\leq 0.5 \times 10^4$ MHC tetramer-positive CD8⁺ T cells/spleen. For the NP_{366–374} epitope, no detectable antigen-specific T cell population can be induced in naive mice by this vaccination strategy, suggesting that this type of vaccination may not be as effective as a viral infection in inducing a CD8⁺ T cell response (not shown).

Discussion

The ability of cytotoxic T cell populations to cross-react between different viral strains was first appreciated by Townsend and Skehel in 1984 (18). They showed that repeated *in vitro* restimulation protocols of splenic lymphocytes derived from influenza A virus-primed mice could lead to the selection of NP-specific T cell lines that cross-reacted between different influenza A viral strains. At that time, it was unknown whether these T cell lines cross-reacted at the level of the (variable) immunodominant NP_{366–374} CTL epitope, or whether shared subdominant epitopes were recognized. However, with the benefit of hindsight, these experiments can be said to have revealed for the first time the capacity of cytotoxic T cells to productively recognize viral variants.

In spite of the early recognition of T cell cross-reactivity in *in vitro* assays, the biological *in vivo* significance of this cross-reactivity for the immune system to cope with viral

variants has since remained unclear. Our findings now document that prior antigen exposure dramatically affects the repertoire of T cells used in a subsequent response to antigenic variants *in vivo*. The propensity for cross-reactivity between two T cell antigens appears roughly proportional to the sequence similarity between the epitopes tested, and seems to be a common event for antigens that are closely related. These cells that expand *in vivo* are phenotypically indistinguishable from conventional effector T cell populations (CD44^{high}, CD62L^{low}; data not shown) and are functional *ex vivo* and *in vivo*. This process appears to be due to the selective expansion of cross-reactive T cells present in the memory T cell pool, and is not dependent on shared B or T helper epitopes between primary and recall antigen.

In recent years, several groups have studied the impact of epitope variants on antigen-specific T cell responses (for reviews, see references 6, 45, and 46). These variant epitopes

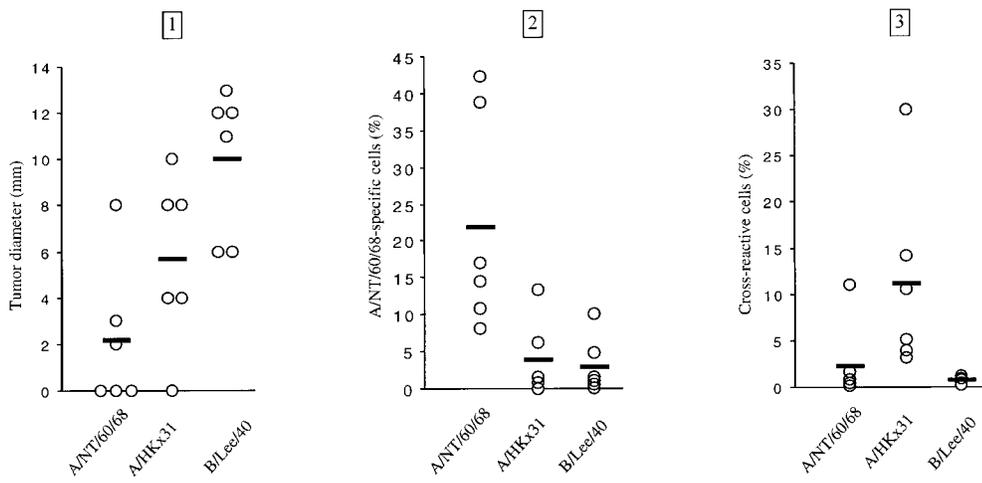


Figure 5. In vivo function of cross-reactive T cells. C57BL/10 mice (six mice per group) were infected with either A/NT/60/68 (10 HAU), A/HKx31 (100 HAU), or B/Lee/40 (125 HAU). 3 mo after the infection, all mice were injected subcutaneously with 3×10^6 live ELA-NP₃₆₆₋₃₇₄ tumor cells. 7 d after tumor challenge, tumor size was measured and the mean diameter per mouse is plotted (panel 1). At the same time point, peripheral blood samples were taken and surface stained with anti-CD8 mAb (PharMingen) and differentially labeled MHC tetramers containing the ASNENMDAM and ASNENMETM epitopes. The percentages of A/NT/60/68-mono-specific CD8⁺ T cells (panel 2) and cross-reactive CD8⁺ T cells (panel 3) are plotted for individual mice.

were generally isolated as immune escape variants, or were identified in *in vitro* assays by their aberrant recognition by T cell clones. These selected antigen variants function as either partial agonists or antagonists of antigen-specific T cell responses not only *in vitro* but also *in vivo*. We sought to examine whether this type of T cell antagonism or partial agonism is a common phenomenon when a polyclonal T cell repertoire is confronted with antigenic variation. To this purpose, we studied the development of antigen-specific T cell repertoires after encounter of random natural variants of influenza A viruses. We conclude that during such encounters, the T cell repertoire generally reacts with the outgrowth of a T cell population for which the variant epitope is a full agonist. The TCRs encoded by these T cells bind with equal affinity to MHC molecules complexed with wild-type and variant epitopes. Furthermore, the functional capacity of this T cell population towards target cells expressing the original or the variant antigen is indistinguishable both *in vitro* and *in vivo*.

How do these data fit in with previous observations that mutations in T cell epitopes can lead to CTL escape by the virus? During chronic HIV and HBV infections (10, 11), and also in a murine lymphocytic choriomeningitis virus (LCMV) model (12), the mechanism that we have identified apparently does not operate efficiently. In theory, this could be due to the type of amino acid changes in the T cell epitopes involved. However, examination of the type of mutations in the T cell epitopes in those cases and the mutations studied here does not point towards obvious differences (not shown). Alternatively, the impaired ability to react to emerging antigenic variants in those settings may be due to alterations at the T cell level. Specifically, repetitive antigen-specific T cell stimulation results in a narrowing of the reactive T cell repertoire (33). Indeed, the antigen-specific T cell expansions observed during chronic HIV infection are oligoclonal (47). It may be hypothesized that for such restricted antigen-specific

T cell populations, a single antigenic variant could antagonize a substantial part of the antigen-specific T cell response, and it will be a challenge to test this notion in a direct manner.

Why do cross-reactive T cells dominate the response against antigenic variants over the largely strain-specific response observed normally during a primary T cell response? The cross-reactive T cells that we have identified appear to originate from the preexisting memory T cell pool, and naive and memory T cells differ both in quantitative and qualitative terms. Specifically, even though cross-reactive cells are infrequent in the original memory pool, these cells may still outnumber the strain-specific T cells that—due to their specificity—failed to get activated in the primary response. In addition, the activation requirements of memory T cells are less stringent than those of naive T cells (38, 48, 49). This is reflected in a lessened requirement both for costimulatory signals and for prolonged antigenic stimulation. Finally, the ability of memory T cells to enter peripheral tissues may promote the early encounter of viral antigens (50). Indirect evidence for functional T cell cross-reactivity during subsequent viral infections has been obtained previously. Experiments by Welsh and colleagues (51) have suggested that a measure of heterologous immunity may occur even for apparently unrelated viruses. Although in that case the antigenic determinants involved were not identified and cross-reactive cells were of low avidity, these results do suggest that the selective expansion of cross-reactive memory T cells could be a more general phenomenon.

We conclude that a polyclonal T cell repertoire responds to the encounter of random variants by the expansion of memory T cells that are best fit for the recognition of the variant antigen. These findings suggest that random mutations in T cell epitopes are unlikely to result in CTL escape, and that a polyclonal T cell memory population can provide protection not only against the index sequence, but also against a swarm of related sequences.

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