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MHC Class I/Peptide Stability: Implications for Immunodominance, In Vitro Proliferation, and Diversity of Responding CTL

Dirk H. Busch and Eric G. Pamer

Infection of BALB/c mice with *Listeria monocytogenes* primes CD8⁺ cytotoxic T cells specific for four different H2-K¹-restricted peptides. In vitro restimulation of *L. monocytogenes* immune splenocytes with each of these peptides resulted in larger T cell responses to p60 217–225 and mpl 84–92 than to LLO 91–99 and p60 449–457. Direct frequency analyses of immune splenocytes, however, revealed that LLO 91–99 and p60 217–225 elicit dominant T cell responses, while p60 449–457 and mpl 84–92 elicit minor, subdominant responses. Restimulation of immune splenocytes with a range of peptide concentrations revealed that T cells with dominant specificities respond optimally to low peptide concentrations, while T cells specific for subdominant epitopes expand maximally to high peptide concentrations. This disparity correlates with the stability of H2-K¹/epitope complexes: the two dominant epitopes form stable complexes, while the subdominant epitopes form less stable complexes with H2-K¹. Interestingly, T cells specific for LLO 91–99 and p60 217–225 express more complex TCR-Vβ repertoires than p60 449–457- and mpl 84–92-specific T cells. Thus, in our system, dominant T cell responses have relatively diverse TCR repertoires and are specific for peptides that form stable complexes with MHC class I molecules. Determining the precise roles of epitope/MHC class I stability and TCR repertoire in the generation of dominant T cell responses will require further investigation. *The Journal of Immunology*, 1998, 160: 4441–4448.

Infection with complex pathogens elicits T cell responses that are specific for a limited number of pathogen-derived peptides. The magnitude of T cell responses to different peptide epitopes is variable, with robust (dominant) responses to some epitopes and minor (subdominant) responses to other peptides (1). The mechanisms that underlie dominance and subdominance are complex, since virtually every step of Ag processing, T cell priming, and T cell expansion can have implications for T cell responses. In the MHC class I Ag processing pathway, it has been suggested that proteasome specificity (2), transporters associated with Ag processing (TAP)³ selectivity (3), MHC class I affinity for different epitopes (2–7), and epitope dissociation from MHC class I molecules (5, 8) contribute to the outcome of a T cell response. The distinction between immunodominance and subdominance is less clear during chronic infections (6, 9), suggesting that prolonged in vivo Ag production modulates T cell response magnitudes. The dynamics of T cell responses are further complicated during persistent infections with pathogens that undergo antigenic variation. For example, point mutations in HIV can eliminate dominant T cell epitopes, changing the hierarchy of T cells specific for the pathogenic virus (10).

The role of the TCR repertoire in determining the magnitude of a T cell response is unknown. Approaching this issue has been difficult because of the complexity of Ag-specific T cell populations and the difficulty of identifying, isolating, and characterizing Ag-specific T cells ex vivo (11). TCR analyses of T cell clones specific for the same epitope demonstrated highly diverse repertoires in some cases (12–14) and restriction to specific TCR-α- and β-chains in others (15–19). While it is clear that positive and negative selection determine the ultimate number of naive peripheral T cell specific for a particular Ag, the relationship between naive precursor frequencies and immunodominance has not been established. The role of T cell avidities for different MHC/peptide complexes is also unknown.

CD8⁺ cytolytic T cells (CTL) play an important role in the host immune defense against *Listeria monocytogenes*, a Gram-positive intracellular bacterium that enters the cytoplasm of infected cells (20, 21). Intravenous infection of mice with a subethal dose of *L. monocytogenes* primes specific CTL, which participate in clearing the infection and mediate long-lasting, protective immunity (22). Four different *L. monocytogenes* epitopes are presented by MHC class I H2-K¹ molecules to CTL (23–26). These epitopes derive from bacterial virulence factors that are expressed and degraded in the cytosol of infected cells. Remarkably, there is no correlation between the amount of epitope that is presented by infected cells and the magnitude of the CTL response. Thus, LLO 91–99, a relatively sparsely presented epitope, elicits a quantitatively large T cell response, while p60 449–457, which is presented in large quantities, elicits a small response (27).

In this report, we investigate differences between two dominant and two subdominant T cell responses following *L. monocytogenes* infection. We find that short term, in vitro T cell expansion with peptide-coated stimulator cells provides misleading results when
Materials and Methods

**Mice, bacteria, and cell lines**

CB6 (C57BL/6 x BALB/c)F1; H2k+) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). M. monocyogenes strain 10403S was purchased from the American Type Culture Collection (ATCC, Rockville, MD) and grown in brain-heart infusion broth.

Mastocytoma cell line P815 (H2b) was obtained from ATCC, and H2-Kb-transfected RMS-S cells were kindly provided by M. Bevan (University of Washington, Seattle, WA). Cells were cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 1-glutamine, HEPES (pH 7.5), β-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml).

**Immunization with Listeria and harvesting spleen cells**

Mice were immunized by injecting 2 x 10^6 L. monocytogenes 10403S into the tail vein. Spleens were removed 7 days after immunization, and splenocytes were harvested by dissociation through a wire mesh and lysis of erythrocytes with ammonium chloride, and subsequently resuspended in RPMI 10^3.

**Peptides**

Synthetic peptides LLO 91–99 (GYKDVQDI), p60 217–225 (KYGVSS-VQDI), p60 449–457 (IVYGNGQMI), and mpl 84–92 (GTYLDNTDEI) were obtained from Research Genetics (Huntsville, AL). Peptide stock solutions (1 mg/ml dissolved in PBS) were stored at -20°C until use.

**IFN-γ ELISPOT assays**

ELISPOT assays were performed as described previously (27). Briefly, 96-well nitrocellulose plates (Millititer HA, Millipore, Bedford, MA) were coated with anti-mouse IFN-γ mAb (PharMingen, San Diego, CA) and then blocked with 1% FCS. To determine in vivo frequencies for epitope-specific CD8+ T-cells, 1 x 10^5 spleen cells from an immunized mouse and 1 x 10^5 peptide-coated irradiated P815 cells were added per well in a total volume of 200 μl RPMI containing 30 U/ml IL-2. For each peptide, measurements were performed at least in triplicates, and for no-peptide controls uncoated P815 were used. Cells were incubated for 27 h at 37°C; thereafter, nitrocellulose membranes were washed with PBS/0.05% Tween-20, incubated with biotinylated anti-mouse IFN-γ mAb (PharMingen), and developed using peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and DAB substrate (Bio-Rad, Melville, NY). The areas where a single cell was stimulated were analyzed with CELLQuest software (Becton Dickinson, Mountain View, CA).

**Results**

In vitro restimulation of peptide-specific T cells suggests a hierarchical CTL response

A conventional method for characterizing CTL following infection is to restimulate primed splenocytes in vitro with peptide-coated, irradiated, naive splenocytes and to assay for peptide-specific cytotoxic activity 5 days later in a standard chromium release assay. We restimulated L. monocytogenes-immune splenocytes with irradiated, naive spleen cells coated with 10^-6 M LLO 91–99, p60 217–225, p60 449–457, and mpl 84–92 and tested responders for specific lysis using peptide-coated P815 (H2b) cells. When equivalent proportions of different responders from the original cell cultures were tested, we obtained different degrees of % specific lysis for the various epitopes. Responders restimulated with mpl 84–92 and p60 217–225 showed a high percentage of specific lysis, while LLO 91–99- and p60 449–457-restimulated responders gave a lower specific lysis (Fig. 1, a and c). Although it is well known that the amount of specific lysis correlates with the ratio of effector (CTL) to target cells, we wanted to be certain that the different degrees of lysis obtained with CTL stimulated with the four H2-Kb-restricted epitopes could be attributed to different numbers of responding CTL and not to differences in the lytic capacity of the T cells.
different responder populations. Because Ag-specific T cells constitute only a small fraction of the total cellular content of short term, in vitro restimulations, we quantified Ag-specific T cells in responder flasks by ELISPOT. The number of IFN-\(\gamma\)-secreting, peptide-specific T cells in flasks restimulated with each of the four H2-K\(d\) -restricted epitopes correlates precisely with the specific lysis obtained in CTL assays (Fig. 1b). Thus, the differences in specific lysis that we observe can be attributed to the number of CTL following in vitro peptide restimulation.

In vivo frequencies of T cells specific for the four H2-K\(d\) restricted epitopes

The frequency of T cells specific for LLO 91–99, p60 217–225, and p60 449–457 1 wk following \(L. \)monocytogenes infection was previously found by ELISPOT to be roughly 80, 40, and 4 per 100,000 immune splenocytes (27). Because the frequency of T lymphocytes specific for the recently described mpl 84–92 epitope was unknown, we performed ELISPOT frequency analysis on immune splenocytes for this epitope and the previously characterized H2-K\(d\) -restricted epitopes. Surprisingly, the frequency of mpl 84–92-specific T cells is very low and is comparable to p60 449–457-specific responses (Fig. 2). As shown previously, the level of responses to LLO 91–99 and p60 217–225 is high. Thus, as determined by direct frequency analysis, the relative magnitude of the T lymphocyte responses can be ranked as follows: LLO 91–99 > p60 217–225 >> p60 449–457 = mpl 84–92. These frequencies contrast dramatically with the results obtained by in vitro peptide restimulation (Fig. 1, a and b), indicating that LLO 91–99-specific T cells proliferate poorly in vitro, while at the other extreme, mpl 84–92-specific T cells undergo far greater in vitro expansion.

FIGURE 1. Short-term, in vitro peptide restimulation of \(Listeria\)-specific CTL. \(L.\) monocytogenes-immune splenocytes were restimulated with irradiated, naive spleen cells coated with \(10^{-6}\) M LLO 91–99, p60 217–225, p60 449–457, and mpl 84–92, respectively. a. Five days later, the degree of peptide-specific lysis was determined in standard \(^{51}\)Cr release assays, using equivalent volumes from responder cultures, and tested at serial 1:3 dilutions (A–D). b. The number of peptide-specific T cells in responder cultures was determined by IFN-\(\gamma\) ELISPOT assays. Equivalent volumes from T cell cultures were tested at serial 1:5 dilutions (a–d) for peptide-dependent IFN-\(\gamma\) secretion. c. Maximal degree of % specific lysis of CTL cultures generated by short term peptide restimulation; data averaged from five independent experiments and the SD are shown.

FIGURE 2. In vivo frequencies of T cells specific for 4 H2-K\(d\)-restricted \(Listeria\) epitopes. Numbers of epitope-specific CD8\(^{+}\) T cells were determined using IFN-\(\gamma\) ELISPOT assays. Seven days after immunization with \(L.\) monocytogenes, immune splenocytes were incubated in the presence of peptide-coated and uncoated P815 cells, as described in Materials and Methods. Peptide-induced IFN-\(\gamma\) secretion by a single cell was visualized and quantified in triplicates and expressed as the number of IFN-\(\gamma\)-secreting cells per 100,000 immune splenocytes. The SD is shown.
Mitogenic stimulation conserves in vivo hierarchy

Different in vitro expansion rates may result from intrinsic differences between responding T cells with differing peptide specificities. Perhaps vigorous in vivo activation and expansion of LLO 91–99-specific CTL interferes with further in vitro expansion following peptide restimulation. It is possible that the majority of these cells are programmed to undergo cell death, since it is well known that most of the primary effector cells rapidly disappear as a result of apoptosis or other mechanisms (30). To determine whether this is the case, we stimulated immune splenocytes non-specifically with the mitogen Con A (37) and tested responders 5 days later with CTL and ELISPOT assays for their peptide specificity. As shown in Figure 3, mitogenic stimulation of immune splenocytes expands epitope-specific T cell populations in proportions that reflect the immunodominance hierarchy observed in vivo. Even small differences, such as the lower number of p60 217–225-specific CTL as compared with LLO 91–99, are conserved. Thus, CTL specific for immunodominant epitopes do not have a diminished ability to expand in vitro. This suggests that some aspect of in vitro peptide restimulation causes disparate expansion of CTL specific for different epitopes.

Dominant T cell responses are inhibited by high in vitro peptide concentrations

Recent studies have demonstrated that in vitro expansion of some T cells is inhibited when they are restimulated with high peptide concentrations (31, 32). To determine whether the peptide concentration that we used to stimulate T cells inhibited their in vitro expansion, we coated naive splenocytes with a range of peptide concentrations before in vitro restimulation of immune splenocytes. We found significant differences in the optimal peptide concentrations required for maximal in vitro CTL expansion (Fig. 4). T cells specific for immunodominant epitopes expanded most vigorously with lower peptide coating concentrations (10^-9 M for LLO 91–99 and 10^-8 M for p60 217–225), while CTL specific for subdominant epitopes responded best when stimulators were coated with a 100- to 1,000-fold higher peptide concentration (10^-6 M for p60 449–457 and mpl 84–92). Notably, T cells specific for subdominant epitopes did not undergo in vitro expansion in response to peptide concentrations of 10^-8 or 10^-9 M. Thus, when immune splenocytes are stimulated with high concentrations of dominant and subdominant peptides, T cells responding to subdominant peptides have a selective advantage and, following short term restimulation, can appear to be more prevalent than dominant T cell populations.

In vitro T cell expansion and immunodominance correlates with H2-Kd/peptide stability

The observation that T cells specific for immunodominant epitopes expand most vigorously when lower peptide concentrations are used for stimulation prompted us to determine the stability of MHC/peptide complexes with each of the four H2-Kd-restricted epitopes. RMA-S-Kd cells, which lack functional TAP2 molecules, express negligible levels of surface H2-Kd in the absence of exogenous peptide. We incubated RMA-S-Kd cells in the presence of each of the L. monocytogenes-derived epitopes and monitored the stability of surface H2-Kd molecules using the conformation-dependent, H2-Kd-specific Ab SF1-1.1.1 and flow cytometry. All four peptides stabilized surface

\[\text{FIGURE 3. Mitogenic stimulation conserves in vivo hierarchy. L. monocytogenes-immune spleen cells were expanded in vitro in the presence of 2.5 }\mu\text{g/ml Con A. Cell cultures were tested for peptide-specific lysis (a), and the number of epitope-specific CD8}^+\text{ T cells was determined by ELISPOT assay (b). For CTL assays, serial 1:3 dilutions (A–D) and for ELISPOT assays serial 1:5 dilutions (a–d) were used, as described in Materials and Methods.}\]
expression of H2-K\textsuperscript{d} nearly equivalently (Fig. 5), and during the first 2 h after peptide removal there was rapid loss of SF1-1.1.1-reactive H2-K\textsuperscript{d}, perhaps resulting from rapid dissociation of incompletely bound epitopes. As we have shown in previous studies (29), LLO 91–99 and p60 217–225 stabilize H2-K\textsuperscript{d} more effectively than does p60 449–457 between 2 and 6 h after peptide removal (Fig. 5). Remarkably, for mpl 84–92 we observe a rapid loss of SF1-1.1.1-reactive H2-K\textsuperscript{d} (Fig. 5), indicating that mpl 84–92, like p60 449–457, dissociates from H2-K\textsuperscript{d} rapidly, while LLO 91–99 and p60 217–225 dissociate slowly. In previous studies, we used biochemical approaches to determine that the t\textsubscript{1/2} of H2-K\textsuperscript{d} complexes with LLO 91–99 or p60 217–225 is \textasciitilde 1 h. Since mpl 84–92 stabilizes surface H2-K\textsuperscript{d} slightly more effectively than p60 449–457, but much less effectively than LLO 91–99 or p60 217–225, we estimate the t\textsubscript{1/2} of mpl 84–92/H2-K\textsuperscript{d} complexes to be in the range of 1 to 2 h. These findings point to a correlation between MHC/peptide stability and the peptide concentration required for optimal in vitro T cell expansion.

CTL specific for the different H2-K\textsuperscript{d}-restricted epitopes have similarly high avidities

It is not known whether T cell populations specific for dominant and subdominant epitopes have different avidities for their respective epitope/MHC complexes. To determine whether we select for high avidity CTL following in vitro expansion with very low peptide concentrations, we tested different epitope-specific CTL lines for their relative peptide sensitivities. T cell lines were generated by in vitro restimulation of immune splenocytes with naive splenocytes coated...
with each of the epitopes at the concentration that supported optimal proliferation (10^{-6} M for mpl 84–92 and p60 449–457, 10^{-8} for p60 217–225, and 10^{-9} for LLO 91–99). The relative ability of CTL lines to lyse target cells in the presence of decreasing concentrations of each of the peptides was determined (Fig. 6). CTL lines specific for all four epitopes lysed target cells maximally in the presence of their respective peptide at concentrations as low as 10^{-10} M. The similar peptide sensitivities of the generated CTL lines suggest that the avidity of in vitro-challenged CTL lines specific for each of the L. monocytogenes-derived epitopes is similar. This finding is noteworthy because markedly different peptide concentrations were used for in vitro restimulation. We attempted to obtain CTL lines specific for LLO 91–99 and p60 217–225 by restimulation with higher peptide concentrations; however, these lines uniformly could not be maintained beyond two or three restimulations. This indicates that the optimal peptide concentrations determined for short term restimulation are necessary for long term, in vitro expansion.

**CTL specific for immunodominant epitopes have greater TCR diversity than subdominant-specific CTL**

Although the number and range of naive, Ag-specific T cells may be important determinants of the ultimate response magnitude to a particular epitope, few studies have compared the TCR diversity of CTL specific for dominant and subdominant epitopes. We decided, therefore, to stain the four epitope-specific CTL lines with mAbs specific for 13 different Vβ TCR chains. Short term, in vitro peptide-restimulated CTL lines were generated and consisted of $\geq 90\%$ specific CTL, as determined by H2-K^{d} tetramer staining (D.H.B. and E.G.P., unpublished results). As shown in Figure 7, we found remarkable differences between the TCR-Vβ usage of T cell lines specific for immunodominant vs subdominant epitopes: T cell lines specific for both immunodominant epitopes show an extremely diverse TCR-Vβ repertoire, whereas T cell lines specific for the subdominant epitopes express far more restricted TCR-Vβ segments. In the case of mpl 84–92-specific T cells, essentially all TCRs contain the Vβ14 chain. Although the restriction of TCRs specific for p60 449–457 is less stringent, a predominance of TCR-Vβ8 segments is readily and reproducibly detected.

**Discussion**

In this report, we compare the CTL response to four different nonamer peptides derived from the intracellular bacterium L. monocytogenes. All four peptides are presented by H2-K^{d} class I molecules and are bound with high affinity, as determined in competitive binding assays (29, 33). Herein we extend our previous finding that the in vivo T cell response magnitude is distinct for LLO 91–99, p60 217–225, and p60 449–457 to the most recently identified epitope, mpl 84–92. The T cell responses to these four epitopes can be segregated into two dominant responses (LLO 91–99, p60 217–225, and mpl 84–92). The T cell responses to these four epitopes can be segregated into two dominant responses (LLO 91–99, p60 217–225, and mpl 84–92). In the process of investigating the underlying reason for this dichotomy, we have made several important observations. First, we have found that T lymphocyte assays that require peptide-driven, in vitro expansion are subject to misinterpretation because of a previously unsuspected proclivity of dominant (as opposed to subdominant) epitopes to inhibit T cell proliferation. Second, we have uncovered a correlation between the rate of epitope dissociation from MHC class I molecules and the optimal peptide concentration required for in vitro T cell proliferation. Finally, we demonstrate a correlation between the diversity of TCRs expressed by CTL specific for different epitopes and their relative immunodominance. These findings have important implications for our understanding of immunodominance and T cell responses to complex pathogens.

Because of the low frequency of Ag-specific CTL following most infections, studies of CTL generally require in vitro expansion to obtain sufficient numbers of cells. One common method for
expanding T lymphocytes involves in vitro peptide restimulation. This method has been used to generate T cell lines and clones and has also been used to determine the frequencies of epitope-specific T cells by limiting dilution (6, 7, 25). Our studies demonstrate very clearly that the hierarchy of T cell responses following infection cannot be readily determined with assays that depend on in vitro T cell proliferation following peptide restimulation. Our finding that epitopes that stimulate in vitro T cell proliferation at very low concentrations form stable complexes with H2-Kd is unlikely to be coincidental. Stimulator cells coated with peptides that form stable complexes may form more surface complexes, and perhaps more significantly, these complexes will persist on the cell surface for much longer periods of time. In the case of LLO 91–99 and p60 217–225, which dissociate from H2-Kd with a $t_{1/2}$ of ~6 h, it is likely that immunologically relevant numbers of H2-Kd/peptide complexes will be present on peptide-coated stimulator cells much longer than p60 449–457- and mpl 84–92-containing complexes, which decay much more rapidly. Recent studies have shown that T cell lines specific for an HIV-derived epitope are inhibited by and undergo activation-induced cell death in response to in vitro restimulation with high peptide concentrations (31). We see a similar inhibition of in vitro expansion of LLO 91–99-specific CTL upon stimulation with high peptide concentrations. Interestingly, the response to p60 217–225, which is also dominant, is not detectably inhibited by restimulation with high peptide concentrations. The basis for this difference between LLO 91–99 and p60 217–225 is unknown. Our findings extend previous work in this area by demonstrating: 1) CTL with different peptide specificities have distinct responses to changes in peptide concentrations used for in vitro restimulation; 2) CTL specific for subdominant epitopes require high peptide concentrations for optimal in vitro expansion; and 3) CTL specific for dominant epitopes require lower concentrations of peptide for optimal in vitro expansion and can be inhibited by high peptide concentrations.

Analysis of in vitro-expanded T cell lines revealed a highly diverse TCR-Vβ repertoire for immunodominant-specific lines, whereas subdominant epitopes appeared to elicit far more restricted TCR-Vβ repertoire. These findings are similar to a recent analysis of EBV-specific T cell clones isolated from patients with persistent EBV infection (34). Comparison of multiple TCR sequences demonstrated a highly diverse TCR repertoire for T cells specific for an immunodominant EBV epitope, whereas a more restricted TCR repertoire was detected when T cell clones were specific for a subdominant EBV epitope. Interestingly, these differences were also maintained on the level of the TCR-Vβ segments. In other experimental systems, a rather limited TCR repertoire diversity had been described for immunodominant T cell responses (15–17). However, most of the Ags investigated in these studies share homology with self proteins, and a possible role for self homologues in determining the size and quality of the selected TCR repertoire for these particular Ags has been suggested (35). Although our approach only determined how many different Vβ-chains are expressed on CTL lines specific for the different L. monocytogenes epitopes, our study has the advantage of characterizing the majority of T cells (the 13 Vβ-chain-specific Abs should detect ~90% of TCR-Vβ-expressing cells) that respond to each of the epitopes, without the need for cloning. Analyzing the TCR-Vβ usage alone does not allow a definitive statement about the TCR repertoire, since there are clearly additional layers of diversity at the level of CDR3 (complementarity-determining region 3) length and pairing with different TCR α-chains (36). Nevertheless, the TCR-Vβ differences between dominant and subdominant epitopes are striking and are consistent with work in other infectious systems (13). Given these results, it is tempting to attribute T cell response sizes to a relatively greater repertoire of T cells in immunodominant responses and a more restricted repertoire in subdominant responses. However, the correlation between MHC/peptide stability and immunodominance is not readily explained by this model.

FIGURE 7. TCR-Vβ usage of T cell lines specific for dominant and subdominant Listeria epitopes. CTL lines with specificity for each of the four H2-Kd-restricted Listeria epitopes were expanded for 3 wk by in vitro peptide stimulation using optimal peptide concentrations (see Fig. 6). Cells were triple stained with phycoerythrin-conjugated anti-CD8, Cy-Chrome-conjugated anti-CD62L, and a panel of different TCR-Vβ-specific FITC-conjugated mAbs. CD62Llow, CD8+ lymphoblasts were analyzed for their expression of different TCR-Vβ segments using a FACScan flow cytometer. The mean percentage of cells expressing the different TCR-Vβ chains from five different mice and the SD are shown.

| Epitope | TCR-Vβ Segments
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<td>mpl 84-92</td>
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