Quantitation of CD8$^+$ T Cell Expansion, Memory, and Protective Immunity After Immunization with Peptide-Coated Dendritic Cells

Sara E. Hamilton and John T. Harty

*J Immunol* 2002; 169:4936-4944; doi: 10.4049/jimmunol.169.9.4936

http://www.jimmunol.org/content/169/9/4936

**Why *The JI*?**

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References** This article cites 31 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/169/9/4936.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Dendritic cells (DCs) are potent APCs for naive CD8⁺ T cells and are being investigated as vaccine delivery vehicles. In this study, we examine the CD8⁺ T cell response to defined peptides from *Listeria monocytogenes* (LM), lymphocytic choriomeningitis virus, and murine CMV coated singly and in combination onto mature bone marrow-derived DCs (BMDCs). We show that immunization of mice with 2 × 10⁸ mature BMDCs coated with multiple MHC class I peptides generates a significant Ag-specific CD8⁺ T cell response in both the spleen and nonlymphoid organs. This immunization resulted in a peptide-specific hierarchy in the magnitude of CD8⁺ T cell priming and noncoordinate kinetics in response to different peptide epitopes. Kinetics were not exclusively due to specific characteristics of the MHC class I molecule, and were not altered in an Ag-independent manner by concurrent LM infection. Mice immunized with listeriolysin O 91–99-coated BMDCs are protected against high dose challenge with virulent LM. This protection was enhanced by diversifying the memory CD8⁺ T cell compartment, even in the absence of a large increase in Ag-specific CD8⁺ memory T cells. *The Journal of Immunology, 2002, 169: 4936–4944.*

Due to their potent capacity to stimulate T cells, dendritic cells (DCs) are being investigated in vaccine and therapeutic approaches. DCs pulsed with tumor-derived peptide, proteins, or RNA have been shown to activate tumor-specific CD8⁺ T cells and reduce tumor load (1–3). Induction of virus-specific CD8⁺ T cells and TCR-transgenic CD8⁺ T cells by peptide-pulsed DCs has also been demonstrated (4, 5). The direct visualization of virus-infected DCs interacting with naive T cells in draining lymph nodes was also recently described (6). However, little quantitative data exists as to the magnitude and kinetics of the endogenous CD8⁺ T cell response after immunization with bone marrow-derived DCs (BMDCs) coated with multiple MHC class I peptides.

Activation of Ag-specific CD8⁺ T cells results in proliferation and differentiation into effector cells which are able to secrete inflammatory cytokines (IFN-γ and TNF-α) and cytotoxic compounds like perforin and granzymes (7, 8). These effector cells will contract in a programmed fashion (9), resulting in stable memory levels related to the peak of initial expansion (7, 9). Although a pathogen may contain many epitopes that stimulate CD8⁺ T cell precursors, populations of CD8⁺ T cells that differ in Ag specificity expand and contract with tightly regulated coordinate kinetics regardless of the magnitude of response (7, 9–12). Also upon activation, CD8⁺ T cells appear to acquire as yet undefined characteristics enabling them to migrate to and enter nonlymphoid tissues (13). These characteristics may have important ramifications for the ability of memory CD8⁺ T cells in different locations to perform effector functions and protect the host from subsequent infections.

Our goal was to assess in quantitative fashion the ability of peptide-coated DCs to stimulate CD8⁺ T cell memory and protective antibacterial responses. Thus, we determined the kinetics of primary CD8⁺ T cell responses to DCs pulsed with several MHC class I-restricted peptides derived from *Listeria monocytogenes* (LM). Next, we examined the relationship between DC immunization and protective immunity against LM.

**Materials and Methods**

Mice and bacteria

Six- to 8-wk-old female BALB/c (H-2d MHC) mice were purchased from the National Cancer Institute (Frederick, MD). Virulent LM strains used in this study were recombinant strain HSL236 (listerylosin O (LLO)⁺, nuclear protein (NP)⁺ expressing a secreted fusion protein containing the lymphocytic choriomeningitis virus NP118–126 epitope and HSL235 (LLO⁺, NP⁻) which expresses the fusion protein without NP118–126 (14). Growth and maintenance of all LM strains were as described (15). Mice were infected i.v. with the number of bacteria indicated in figure legends.

**Generation of peptide-coated BMDCs**

CD11c⁺ BMDCs were generated as previously described with modifications (16). Briefly, RBC-depleted BALB/c (H-2d) bone marrow was subjected to complement depletion after incubation with mAbs 3.168 (CD8⁺-specific), 34-5-3 (I-A⁻d specific), RL172 (CD4⁺-specific), RA3-3A1/6.1 (B220/CD45R-specific), and RB6-8C5 (neutrophil-specific). All mAbs were purified from culture supernatant by protein G (Amersham Pharmacia, Piscataway, NJ). Remaining cells were plated at 1 × 10⁷/ml in RP10 (17) supplemented with 1000 U/ml GM-CSF (BD Pharmingen, San Diego, CA) and 25 U/ml rIL-4 (PeproTech, Rocky Hill, NJ) and incubated for 5–7 days with 75% media replacement every other day. LPS (500 ng/ml; Sigma-Aldrich, St. Louis, MO) was then added for 1–2 days to induce maturation, and 1 μM peptide (except as indicated in Fig. 1D) was added to cultures 3 h before harvest. Cells were then washed extensively before injection. The resulting cell populations consisted of 40–80% CD11c⁺ cells. These cells were also H-2Ld⁺, B7.1⁺, B7.2⁺, CD80⁺, I-A⁻d⁺, and CD11b⁻. Based on the percentage of CD11c⁺ cells (determined before
Immature DCs were generated by the culture conditions stated above but were not exposed to LPS. To remove any contaminating mature DCs, cells were stained with PE-conjugated anti-B7.2 (BD Pharmingen) followed by incubation with anti-PE microbeads (Miltenyi Biotec, Auburn, CA). B7.2 high cells were removed by passage through an MS®/RS® MACS separation column. The negative fraction was collected and surface-stained for CD11c. Peptide coating and injection was as described for mature BMDC.

Detection of Ag-specific CD8+ T cells

The number of CD8+ T cells specific for NP118–126 or murine CMV (MCMV)168–176 in the context of H-2Ld and LLO91–99 or p60 217–225 in the context of H-2Kd was determined by intracellular cytokine staining (ICS). 5 days after immunization, peptide-stimulated splenocytes from immunized mice were incubated for 5–6 h in 2 μl/ml brefeldin A at 37°C with or without synthetic peptides at 200 nM. For staining, cells were placed on ice, washed, and incubated with Ab directed against the FcγRII/III receptors (2.4G2) and FITC-conjugated anti-CD8 mAb (eBioscience, San Diego, CA), fixed, permeabilized, and stained with PE-conjugated anti-IFN-γ mAb according to manufacturer’s instructions (eBioscience). Data was acquired on a FACScan flow cytometer (BD Biosciences, San Jose, CA) using Summit software. One-hundred thousand events were collected for analysis with FlowJo software (TreeStar, San Carlos, CA). The gate for IFN-γ+ cells was selected on an unstimulated sample for each mouse. This value was subtracted from the peptide stimulated values to determine the frequency of Ag-specific CD8+ T cells. Total numbers of epitope-specific CD8+ T cells per sample, and the total number of splenocytes per animal.

51Cr-release assays

LL091–99 or p60 217–225 specific CD8+ T cell-mediated cytotoxicity was determined in a 6-h 51Cr-release assay using BMDC (H-2d MHC) targets coated with or without 1 μM peptide as described for cell injection. 51Cr-labeled (PerkinElmer, Boston, MA) target cells (1 × 104) were plated with DC peptide-coated BMDCs. Data points represent individual mice from two independent experiments. A, Representative FACs profiles are shown with numbers representing the frequency of Ag-specific CD8+ T cells in the spleen 7 days after immunization. B, Total number of Ag-specific CD8+ T cells in the spleen 7 days after infection with LM. Data are means ± SD of two to three mice per group. C, Total number of Ag-specific CD8+ T cells in the spleen 7 days after immunization with the indicated number of peptide-coated DCs. Data points represent individual mice from two independent experiments. D, Total number of Ag-specific CD8+ T cells in the spleen 7 days after infection with LM and indicated concentrations of LLO91–99 or NP118–126. Data points are means ± SD of two experiments with two to three mice per group. Symbols refer to B, C, and D. LOD, limit of detection; ND, not done; BLD, below limit of detection.
CD8⁺ T cell response in the spleen 7 days after immunization by ICS for IFN-γ. The Ag-specific CD8⁺ T cell response of representative mice, either infected with LM strain HSL236 or immunized with 2 × 10⁵ peptide-coated BMDCs, is shown in Fig. 1A. As shown previously (20), few CD8⁺ T cells from immune mice make IFN-γ in the absence of peptide stimulation. In contrast, ~0.6–3.3% of CD8⁺ T cells from LM- or peptide-coated BMDC-immunized mice produced IFN-γ in response to LLO91–99 or NP118–126 stimulation. This response is Ag-specific as seen by the lack of IFN-γ production following stimulation with peptides not present during the in vivo immunization. The total number of Ag-specific cells ranged from 3 × 10⁶ to 6 × 10⁶ per spleen at 7 days after immunization with peptide-coated BMDC. Interestingly, the magnitude of response to LLO91–99 after immunization with peptide-coated BMDCs was comparable to that seen after LM infection (6 × 10⁵ compared with 7–8 × 10⁵ LLO91–99 CD8⁺ T cells/spleen). However, the response to NP118–126 was consistently lower than the response to LLO91–99 (ranging from ~2- to 10-fold in different experiments) (Fig. 1C). This is in contrast to LM infection, where LLO91–99- and NP118–126-specific CD8⁺ T cell responses are similar in magnitude (Fig. 1B). The hierarchy of response after BMDC immunization was not a result of insufficient quantities of peptide, as coating of BMDC with 1 μM of either LLO91–99 or NP118–126 was found to be saturating for stimulating CD8⁺ T cell responses in vivo (Fig. 1D). The magnitude of the CD8⁺ T cell responses to BMDCs was also not enhanced by the addition of an MHC class II epitope (OVA 323–339) (data not shown). The Ag-specific CD8⁺ T cell response after delivery of 2 × 10⁴ to 2 × 10⁶ peptide-coated BMDCs was dose-dependent (Fig. 1C). Because the spleen is normally composed of 1–2 × 10⁶ DCs, it is likely that injection of 2 × 10⁶ peptide-coated BMDCs is saturating given the available space in the lymphoid compartment. Based on this rationale, mice in the following experiments were immunized with a nonsaturating dosage of 2–2.5 × 10⁵ peptide-coated BMDCs (Fig. 1C).

BMDCs can stimulate CD8⁺ T cells of multiple Ag specificities provided that peptides are presented by different MHC class I molecules

Due to efficient Ag capture ability, and the high number of MHC class I molecules on the cell surface, DCs most likely present a number of different antigenic determinants simultaneously in vivo. We asked if the presentation of multiple epitopes by DCs impacted the magnitude of the CD8⁺ T cell response. BMDCs were coated with LLO91–99, p60 217–225 (both H-2Kd restricted), or NP118–126 (H-2Ld restricted) alone or in combination, and the CD8⁺ T cell response in the spleen was determined 7 days after immunization of BALB/c mice. We found that multiple peptides could be presented effectively by the same BMDC populations in the case of LLO91–99 and NP118–126 (Fig. 2A) or p60 217–225 and NP118–126 (Fig. 2C). Importantly, the magnitude of the CD8⁺ T cell response against BMDC coated with single or multiple peptides was similar. Because the number of injected BMDCs was not saturating (Fig. 1C), this data suggests that the same DC is capable of stimulating CD8⁺ T cell responses of more than one Ag specificity.

In contrast to this result, coating BMDCs with two peptides restricted by the same MHC class I molecule (p60 217–225 and LLO91–99), resulted in a reduced CD8⁺ T cell response to the p60 217–225 epitope compared with mice injected with BMDCs coated with p60 217–225 alone (Fig. 2B). This result was not due to exclusion of the p60 217–225 epitope from binding to H-2Kd on the BMDC surface, because both p60 217–225- and LLO91–99-specific CD8⁺ T cell lines caused significant ⁵¹Cr release from BMDC targets (Fig. 3, A and B). Although there was a reduction in p60 217–225-specific ⁵¹Cr release when BMDC targets were also coated with LLO91–99, this data still suggests that both p60 217–225 and LLO91–99 epitopes were presented by peptide-coated BMDCs. However, because the Ag density required for activation of naive CD8⁺ T cells may be much greater than for previously activated T cell lines (21), the requisite number of p60 217–225:Kd complexes may not have been available to stimulate naive CD8⁺ T cells in vivo. The in vitro data also suggests that if peptide titrations were performed, conditions might be found in which both p60 217–225 and LLO91–99 specific CD8⁺ T cells may be stimulated in vivo. However, dilution of LLO91–99 could also decrease the CD8⁺ T cell response to this epitope (Fig. 1D).

**FIGURE 2.** CD8⁺ T cell responses to BMDCs presenting multiple MHC class I peptides. BALB/c mice were immunized i.v. with BMDCs coated with LLO91–99 (H-2Kd-restricted), p60 217–225 (H-2Kd-restricted), NP118–126 (H-2Ld-restricted), or combinations of these peptides. Total number of Ag-specific CD8⁺ T cells in the spleen was determined 7 days after BMDC injection by ICS for IFN-γ. A and B, BMDCs were coated with the indicated peptides. Bars represent means ± SD of three mice per group and are representative of three independent experiments. C, BMDCs were coated with the indicated peptides and injected. Indicated groups received a second injection a few minutes after the first of an equivalent number of either nonpeptide-coated BMDCs or BMDCs coated with p60 217–225 and NP118–126. Data are means ± SD of three mice per group and are representative of two independent experiments. Symbols refer to A–C. BLD, below level of detection.
We also found that coating BMDCs separately with p60 217–225 or LLO91–99 and then injecting these cells sequentially (within a few minutes) into the same animal partially restored CD8+/H11001 T cell priming against p60 217–225 (Fig. 2C). However, responses to both epitopes were reduced in these mice which may be due to a limitation in the space available in the spleen for this number of peptide-coated BMDCs (twice that injected in previous experiments). This notion is supported by data demonstrating a decrease in the number of LLO91–99 and NP118–126 specific CD8+/H11001 T cells if mice received a second injection of “cold” BMDC which were not coated with peptide (Fig. 2C). Although this experimental design prevents peptide transfer between BMDC loaded with either p60 217–225 or LLO91–99 during in vitro peptide loading and injection, it is a possibility that some degree of peptide transfer occurs in vivo after BMDC migrate to the spleen. Together, the data in Figs. 2C and 3B suggest that competition for the H-2Kd molecule during in vitro loading as well as competition for access to peptide-loaded DC in vivo may influence the eventual CD8+/H11001 T cell response. Although studies with TCR-transgenic systems suggest that in vivo CD8+ T cells may compete for access to the APC or for peptide:MHC complexes on the cell surface (4, 22, 23), our results demonstrate that BMDCs coated with multiple peptides can effectively stimulate CD8+/H11001 T cells provided that the peptides are presented by different MHC class I molecules.

**Differential kinetics of CD8+ T cells responding to BMDCs coated with peptides presented by H-2Kd and H-2Ld**

During bacterial or viral infection in wild-type mice, CD8+ T cells with different Ag specificities and MHC restriction display coordinate expansion and contraction leading to stable memory (7, 10–12). We examined the pattern of CD8+ T cell expansion and contraction after immunization with BMDCs coated with LLO91–99 (H-2Kd restricted) or NP118–126 (H-2Ld restricted) (Fig. 4, A and B). Five days after injection, the NP118–126 specific response had peaked at $3.4 \times 10^5$ CD8+ T cells per spleen. Although the number of LLO91–99-specific CD8+ T cells was similar on day 5 (3.0 x 10^5 CD8+ T cells), this response did not reach peak levels until day 7. In contrast, the number of NP118–126-specific CD8+ T cells dropped between 3- and 5-fold by day 7. These kinetics
were observed in both mice injected with single (Fig. 4) and double (Fig. 5) peptide-coated BMDCs. Between days 12 and 14, both populations of CD8⁺ T cells had declined to memory levels that could be detected for at least 100 days following immunization (Fig. 4 and data not shown).

The noncoordinate kinetics of the CD8⁺ T cell response to LLO91–99 and NP118–126 after BMDC immunization are unique given that these and other epitopes elicit coordinate T cell expansion and contraction phases during bacterial or viral infection. (7, 10–12). The kinetics measured after BMDC immunization may be due to unique properties of the specific MHC class I molecule presenting peptide (H-2Ld vs H-2Kd). To ask whether the noncoordinate kinetics observed after immunization with LLO91–99, and NP118–126-coated BMDC were MHC class I molecule-specific, numbers of Ag-specific CD8⁺ T cells were measured after immunization of mice with BMDCs coated with MCMV168–176 (H-2Ld-restricted) or p60 217–225 (H-2Kd-restricted) (Fig. 4, C and D). We found that although the magnitude of Ag-specific CD8⁺ T cell responses depended on the epitope examined, similar kinetic patterns were measured in response to NP118–126, p60 217–225, and MCMV168–176, but not LLO91–99. Although this experiment examines only four peptides, it demonstrates that noncoordinate kinetics after peptide-coated BMDC immunization are not restricted to peptides presented by H-2Ld.

Another possible mechanism for the noncoordinate responses observed in Fig. 4 may be that the NP118–126:Ld complex is less stable than the LLO91–99:Kb complex. H-2Ld has a shorter surface half-life compared with other H-2d MHC class I molecules due to an unusually weak association with B₂-microglobulin and peptide (24). We compared the surface half-life of peptide:MHC class I molecule complexes by coating BMDCs with peptide, washing away unbound peptide, and combining these cells with Ag-specific T cell lines over the next three days (Fig. 4E). No additional peptide was added to BMDCs after day 0, and on each day BMDCs were washed to remove any free peptide before addition to Ag-specific CD8⁺ T cell lines. This experiment is designed to functionally measure the number of peptide:MHC class I molecule complexes on the BMDC surface by measuring CD8⁺ T cell activation. Surprisingly, the ability of peptide-coated BMDCs to stimulate NP118–126 and LLO91–99-specific CD8⁺ T cell responses decreased similarly over time, while a stronger decline in T cell stimulation was seen in BMDC coated with p60 217–225. However, we do not know from this experiment if the initial number of LLO91–99:Kb and NP118–126:Ld complexes formed was different, which may impact the rate of decline in the CD8⁺ T cell response. Because this experiment also uses in vitro restimulated CD8⁺ T cell lines, it is also possible that the Ag level for activation of naive CD8⁺ T cells in vivo differs from that measured with previously activated T cell lines. However, this experiment suggests that the stability of the NP118–126:Ld complex is not responsible for the differential kinetics measured in vivo between NP118–126- and LLO91–99-specific CD8⁺ T cell responses.

**LM infection does not alter CD8⁺ T cell kinetics in response to BMDCs presenting multiple MHC class I peptides**

CD8⁺ T cell expansion kinetics can be affected by the inflammatory response to infection (25). This may be due to bacterial products and/or proinflammatory cytokines that promote the maturation of DCs to a highly stimulatory state for naive T cells or to the production of growth-promoting cytokines that drive CD8⁺ T cell proliferation upon activation (20, 25). We asked whether LM infection would alter the magnitude or kinetics of the CD8⁺ T cell response to peptide-coated mature BMDCs. Mice were either immunized with mature BMDCs coated with LLO91–99 and NP118–126, infected with LM strain HSL235 (LLO⁺, NP⁻), or were coinfected with LM and peptide-coated mature BMDCs. Because strain HSL235 does not express the NP118–126 epitope, an enhanced CD8⁺ T cell response to BMDC presenting this epitope in infected mice would reflect a role for inflammation. In contrast to this notion, the NP118–126-specific response was similar in magnitude and kinetics in mice that received BMDCs alone or in the presence of LM infection (Fig. 5A, filled symbols). This was also true if animals received LM infection 1 day before peptide-coated BMDC immunization, which would allow more time for a vigorous inflammatory response to be generated (data not shown). These experiments suggest both that LPS-matured BMDCs are optimal for stimulating naive CD8⁺ T cells, and that the primary purpose of infection-induced inflammation in vivo is to establish the maturation of APCs (20).

In contrast, the LLO91–99-specific CD8⁺ T cell response peaked on day 7 at ~8.3 × 10⁵ cells for both BMDC- and LM-
immunized animals. This response was enhanced \((2.3 \times 10^6)\) LLO91–99 specific cells in animals which were immunized with peptide-coated BMDCs and LM. This increased response is presumably due to increased levels of Ag in these mice, although the measured response was more than additive. When the same experiment was performed using LM strain HSL236 (LLO\(^+\), NP\(^+\)), both LLO91–99- and NP118–126-specific CD8\(^+\) T cell responses were enhanced in animals which received peptide-coated BMDC and LM (Fig. 5, B and C). These experiments demonstrate that infection-induced inflammation is not sufficient to cause the coordinate regulation of CD8\(^+\) T cell kinetics.

**Ag-specific CD8\(^+\) T cells are present in the lung and liver after DC or LM immunization**

One explanation for the inability of inflammation to alter Ag-specific CD8\(^+\) T cell numbers in the spleen is that infection with LM may change the migratory properties of CD8\(^+\) T cells, resulting in their accumulation in nonlymphoid organs. Previous work has shown that populations of Ag-specific CD8\(^+\) T cells are present in many nonlymphoid tissues after infection with bacteria or viruses, and that these cells may contain unique properties compared with Ag-specific T cells isolated from the spleen or lymph nodes (13, 26). To assess the magnitude of Ag-specific CD8\(^+\) T cells were quantitated by intracellular staining for IFN-\(\gamma\) in the spleen, liver, and lung (Fig. 6A). We found that immunization with peptide-coated BMDC or infection with LM caused trafficking of Ag-specific CD8\(^+\) T cells to tissues. The magnitude of LLO91–99- and NP118–126-specific CD8\(^+\) T cells was similar between organs, although the frequency of CD8\(^+\) T cells isolated from each organ was significantly different as previously reported (13) (data not shown). As a control, immature BMDC defined as being CD11c\(^+\) but B7.2 low or negative (Fig. 6B), were also injected into mice after coating with LLO91–99 and NP118–126. Five days after immunization, no Ag-specific response could be detected in any of the organs examined (Fig. 6C). If immature BMDCs were plated for 24 h in LPS, peptide-coated, and then injected into mice, CD8\(^+\) T cell responses were detected in the spleen, lung, and liver (data not shown) demonstrating that these cells are capable of stimulating a response when matured in vitro. This experiment shows that

---

**FIGURE 6.** Ag-specific CD8\(^+\) T cells are present in nonlymphoid organs after peptide-coated mature BMDC immunization. BALB/c mice were immunized i.v. with either BMDCs (coated with LLO91–99 and NP118–126 peptide) or \(2.3 \times 10^6\) CFU of LM strain HSL235 (LLO\(^+\), NP\(^+\)). On day 7 after immunization, mice were perfused with PBS containing heparin and the indicated organs were removed. After lymphocyte isolation (see Materials and Methods), the frequency of Ag-specific CD8\(^+\) T cells was determined by ICS for IFN-\(\gamma\). Histograms shown are of IFN-\(\gamma\) staining after gating on CD8\(^+\) T cells. Data are representative of six mice in two independent experiments. B, Surface staining of immature and mature BMDCs before injection. The indicated background staining was performed with an isotype-matched control. C, BALB/c mice were immunized i.v. with immature BMDC coated with LLO91–99 and NP118–126 peptide. On day 5 after immunization, mice were perfused, the indicated organs were removed, and lymphocytes were analyzed for the frequency of Ag-specific CD8\(^+\) T cells as described above. Histograms shown are of IFN-\(\gamma\) staining after gating on CD8\(^+\) T cells. Data are representative of three mice.
immature BMDC do not stimulate CD8+ T cell responses in vivo, and that detection of Ag-specific CD8+ T cells in the spleen correlates with detection in nonlymphoid organs. Together, these experiments demonstrate that infection is not required for Ag-specific CD8+ T cells to migrate into nonlymphoid organs, suggesting that peptide-coated BMDC immunization is likely to generate both central and effector memory CD8+ T cell populations (13, 27).

**Protective immunity is generated in BMDC immunized mice**

We next asked whether the CD8+ memory T cells generated by immunization with peptide-coated BMDC were capable of protecting mice from high dose challenge with LM. Twenty-eight days after injection with either mature BMDCs coated with LLO91–99 and NP118–126 or LM, mice contained ~1.5 x 10^5 LLO91–99 (LM- and BMDC-immunized) and ~3.0 x 10^4 NP118–126- (BMDC-immunized only) specific CD8+ T cells in the spleen (Fig. 4). On day 30, mice were infected with ~10 LD_{50} (for a naive mouse) of HSL235 (LLO+, NP+) or HSL236 (LLO+, NP+). These strains of LM are isogenic, demonstrate equivalent virulence (14), and equivalent sensitivity to CD8+ T cell protective immunity in vivo (8). Bacterial numbers in the liver and spleen 3 days after infection showed that BMDC-immunized animals challenged with HSL235 (LLO+, NP+) had ~1000- and ~25-fold fewer organisms in the liver and spleen, respectively, compared with naive animals (Fig. 7, A and B). Mice challenged with HSL236 (LLO+, NP+) had a ~600-fold or more decrease in bacterial numbers compared with naive mice in both the spleen and liver. LM-immune animals had numbers of bacteria similar to BMDC-immunized mice challenged with HSL236 (LLO+, NP+) (Fig. 7, A and B). These results demonstrate that stimulation of NP118–126-specific memory CD8+ T cells in addition to LLO91–99-specific memory T cells (after HSL236 challenge only) resulted in enhanced immunity. This was particularly true in the spleen where a 50-fold decrease in bacteria load was measured between BMDC-immunized animals challenged with HSL236 (LLO+, NP+) compared with those challenged with HSL235 (LLO+, NP+) (Fig. 7B). Enhanced immunity in this situation is somewhat surprising, considering that the same low number of NP118–126-specific CD8+ T cells alone were unable to consistently protect mice from HSL236 infection (data not shown). However, a large expansion of LLO91–99- (all animals) and NP118–126- (HSL236 challenge of BMDC-immunized mice only) specific CD8+ T cells was observed at 5 days after infection (Fig. 7C). Interestingly, both populations of specific CD8+ T cells expanded to ~10 x 10^6 cells per spleen despite the difference in the type of primary immunization (BMDC vs LM) and the number of memory T cells before infection (Fig. 7C). These experiments demonstrate strong protection of mice from a high dose of LM infection by prior immunization with peptide-coated BMDCs.

**Discussion**

The utility of peptide-coated DC-based vaccines will depend on a thorough understanding of the magnitude, kinetics, and protective capacity of the CD8+ T cell response engendered. Our results show that immunization with a single dose of peptide-coated BMDCs is sufficient to stimulate substantial CD8+ T cell expansion and stable Ag-specific memory cell populations similar to that obtained after infection. These CD8+ T cells provide high level protection from a normally lethal dose of LM.

Because DCs efficiently acquire Ag and have numerous MHC class I molecules on their surface, it is likely they have the ability to present multiple peptide determinants simultaneously in vivo. We have shown that multiple peptides can be presented by peptide-coated BMDCs provided these peptides are not presented by the same MHC class I molecule (Fig. 2, A and B). Support for this conclusion has recently been published by Probst et al. (28), in which no competition was seen between CD8+ T cells of differing Ag specificities after adoptive transfer of DCs presenting multiple Ags. Data examining CD8+ T cell priming in H-2b mice has also indicated that there is no significant difference in the primary host response to single- vs double-peptide-coated DC immunization (4). Because our experiments were conducted with a dose of BMDCs that is not saturating, the results suggest that DCs can engage in stimulation of naive T cells of multiple Ag specificities, either simultaneously or in sequence.

In contrast to this data, we saw strong inhibition of p60 217–225-specific responses when BMDCs were simultaneously coated with LLO91–99 (both presented by H-2K\(^b\)). Both p60 217–225
and LLO91–99 have been reported to bind with high affinity to the H-2Kd molecule and to stabilize the peptide:Kd complex similarly (29, 30). However, our data suggest that there is competition for the Kd molecule in vitro between these two peptides. In addition to this competition, we also measured competition in vivo when double the normal number of CD11c+ cells were injected (half of which were not coated with peptides) (Fig. 2C). This experiment suggests competition for access to peptide-coated BMDCs because we did not detect any inhibition of the CD8+ T cell response when multiple epitopes were loaded onto the entire population of BMDCs. However, additional experiments would need to be done to assess the contribution of this mechanism compared with the previously demonstrated competition for peptide:MHC complexes on the cell surface (4, 22, 23).

After infection with pathogens, there is coordinate expansion and contraction of Ag-specific CD8+ T cell populations regardless of the amount of epitope generated during infection (7, 9, 10, 12). Intriguingly, we observed noncoordinate responses between LLO91–99 and NP118–126-specific CD8+ T cells after peptide-coated BMDC immunization (Fig. 4, A and B). Although the number of naive CD8+ T cell precursors specific for LLO91–99 and NP118–126 could be different, it is unlikely that there are significantly fewer NP118–126 precursors because the number of LLO91–99 and NP118–126-specific CD8+ T cells after HSL236 (LLO7, NP12) infection is comparable and follows similar kinetics (Fig. 1B and data not shown). Secondly, the largest CD8+ T cell response measured is to the NP118–126 epitope after LCMV infection (7). Thus, it is unlikely that the precursor frequency for this epitope is abnormally low. The instability of H-2Ld compared with other MHC class I molecules may limit the duration of CD8+ T cell stimulation in vivo (31). However, examination of other peptides bound by H-2Ld and H-2Kd showed that noncoordinate kinetics were not restricted to H-2Ld-presented peptides (Fig. 4). Additionally, when MHC:peptide stability was examined in vitro there was no measurable difference in the rate at which BMDC coated with LLO91–99 or NP118–126 lost the ability to stimulate CD8+ T cell lines (Fig. 4E). Although these experiments suggest that there is no gross difference in the stability of LLO91–99:Kd and NP118–126:Ld, we do not know if a difference in the rate of these peptides causes a difference in the number of peptide: MHC class I complexes formed. Similarly, we do not know precisely how the sensitivity to epitope levels of naive CD8+ T cells in vivo differs from our T cell lines. The noncoordinate response observed between LLO91–99 and NP118–126-specific CD8+ T cells was unchanged by either LM or LCMV infection unless additional Ag was present in the infecting pathogen (Fig. 5A and data not shown). Therefore, the presence of a robust innate inflammatory environment does not alter the Ag-specific CD8+ T cell kinetic after peptide-coated BMDC immunization. An additional difference between immunization with peptide-coated BMDC and infection may relate to the duration of Ag presentation. Peptide-coated BMDC immunization results in uniformly high level presentation which will last as long as the BMDC survive and maintain surface peptide:MHC expression. In contrast, DC-mediated presentation after infection may occur over a longer interval because it may take a week or longer to clear the infection. Increasing the duration of Ag presentation by DC could alter both the number of precursors recruited and time to achieve a peak response. However, despite these differences in CD8+ T cell kinetics, both infection and peptide-coated BMDC immunization result in stable memory populations.

It has been clearly demonstrated that numerous Ag-specific CD8+ T cells are present in nonlymphoid organs after infection (13). Recently, it was shown that specific Ag is itself not required in peripheral organs for recruitment of previously activated CD8+ T cells after influenza virus infection (26). We show that Ag-specific CD8+ T cell responses can be observed in the lung and liver after peptide-coated mature BMDC immunization. In contrast, no responses were observed in any organs after vaccination with immature peptide-coated BMDCs (Fig. 6). This demonstrates that infection is not a requirement for CD8+ T cell trafficking to peripheral organs and suggests that migration to all nonlymphoid tissues may be programmed upon initial activation. Additional experiments will be needed to determine whether organ-specific differences exist in the CD8+ T cell response to peptide-coated BMDCs, what characteristics allow CD8+ T cell entry into these organs, and what impact individual populations have on protective immunity.

Finally, because CD8+ T cells play a critical role in protection against many viral and intracellular bacterial pathogens, we examined the protective capacity of memory CD8+ T cells generated by immunization with peptide-coated BMDCs. Memory cells specific for a single CD8+ T cell epitope protected mice from high dose challenge with virulent LM, demonstrating that CD4+ T cell responses are not required for robust protective immunity in this system. Diversification of the memory compartment did result in enhanced immunity despite small increases in the absolute number of Ag-specific CD8+ memory T cells (Fig. 7). These results indicate that in the design of vaccination strategies using Ag-loaded DC, strong consideration should be given to the unique characteristics of the specific peptide:MHC complex(es) of interest and to creating a diverse memory T cell compartment in the host.

**Acknowledgments**

We thank E. Gutierrez for technical support and V. Badovinac and S. Perman for critical reading of the manuscript.

**References**


