Requirements for Bone Marrow–derived Antigen-presenting Cells in Priming Cytotoxic T Cell Responses to Intracellular Pathogens

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Abstract

Bone marrow (BM)-derived antigen-presenting cells (APCs) are potent stimulators of T cell immune responses. We investigated the requirements for antigen presentation by these cells in priming cytotoxic T lymphocyte (CTL) responses to intracellular bacterial and viral pathogens. [Parent→F_1] radiation BM chimeras were constructed using C57BL/6 donors and (C57BL/6 × BALB/c)F_1 recipients. Infection of chimeric mice with either *Listeria monocytogenes* or vaccinia virus expressing the nucleoprotein (NP) antigen from lymphocytic choriomeningitis virus (LCMV) primed H2-D^b–restricted, but not H2-K^d–restricted CTL responses, demonstrating the requirement for BM-derived APCs for successful priming of CTL responses to these pathogens. Surprisingly, this did not hold true for chimeric mice infected with LCMV itself. LCMV-infected animals developed strong CTL responses specific for both H2-D^b– and H2-L^d–restricted NP epitopes. These findings indicate that in vivo priming of CTL responses to LCMV is remarkably insensitive to deficiencies in antigen presentation by professional BM-derived APCs.

Key words: cross-presentation • cytotoxic T cell • bacterial immunity • viral immunity • radiation chimera

Introduction

$CD8^+$ CTLs recognize pathogen-derived peptides that are presented on the surface of infected host cells by MHC class I molecules. As MHC class I molecules are expressed on nearly all nucleated cell types, appropriately stimulated CTLs can lyse a variety of infected host cell types. Such CTLs also produce cytokines such as IFN-γ and TNF-α, which contribute to effective immune responses. A crucial step in the generation of immunity to viral and bacterial intracellular pathogens is thus the priming and activation of pathogen-specific CTL responses.

Antigen presentation by specialized bone marrow (BM)[1]-derived APCs can prime CTL responses in vitro and in vivo. In particular, dendritic cells (DCs) are extremely efficient at presenting antigens and priming T cell responses (for a review, see reference 1). The efficiency of CTL priming by DCs has been attributed in part to the expression of costimulatory molecules, including members of the B7 (CD80/CD86) family (2). Expression of CD80/CD86 molecules is upregulated in cells treated with bacterial components (3). Furthermore, the ability of APCs to costimulate $CD8^+$ T cell activation is enhanced after signals delivered by activated $CD4^+$ helper T cells or by infection of DCs with viral pathogens (4–6). Thus, DCs or other BM-derived APCs infected with intracellular viral pathogens appear to present antigens and prime CTL responses in vivo.

Uninfected BM-derived APCs also prime CTL responses in infected or tumor-bearing animals. This phenomenon, termed cross-priming, was first illustrated in experiments in which CTL responses to minor histocompatibility antigens were primed in animals immunized with cells that expressed the minor H antigens, but not the restricting MHC alleles (7, 8). During cross-priming, antigens from the immunizing cells are acquired and "cross-presented" by the MHC class I molecules on the surface of BM-derived APCs (9, 10). Cross-priming has been implicated in the activation of CTL responses after immuniza-

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[1]*Abbreviations used in this paper:* B6, C57BL/6; BM, bone marrow; CB6, (BALB/c × B6)F_1; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; LLO, listeriolysin O; NP, nucleoprotein; VacNP, vaccinia virus expressing LCMV NP.

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tion of radiation BM chimeras via intramuscular injection of naked DNA (11–13), and via inoculation with nonhematopoietic tumor cells (14, 15). Thus, abundant evidence implicates BM-derived APCs in the priming and cross-priming of CTL responses to a variety of nonreplicating antigens.

Nonprofessional APCs have also been shown to be capable of priming CTL responses under some circumstances. This conclusion stems from experiments demonstrating that fibroblast cell lines can prime murine CTL responses (16–18). In one set of experiments, fibroblasts that expressed lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) or glycoprotein antigens, but not known costimulatory molecules, were shown to directly immunize CTL responses to the LCMV antigens (18). Such priming correlated with the fibroblasts reaching the spleen of inoculated animals, suggesting that priming of CTL responses by nonprofessional APCs selectively occurs within cytokine-rich lymphoid environments (18). These results suggest that infection with viral and intracellular bacterial pathogens that induce inflammation within lymphoid tissues may prime CTL responses independently of antigen presentation by BM-derived APCs.

In this study, we sought to address the role of BM-derived professional APCs in the in vivo priming of CTL responses to bacterial and viral pathogens that replicate within the cytosol of non–BM-derived cell types. To this end, the priming of CTL responses to pathogen-delivered antigens in [Parent→F_1] radiation BM chimeras was evaluated after immunization with *Listeria monocytogenes*, LCMV, or vaccinia virus. Our results indicate that antigen presentation by BM-derived APCs is essential for the priming of CTL responses to *L. monocytogenes* and vaccinia virus. In contrast, CTLs restricted by both BM and parenchymal MHC class I haplotypes were primed during infection with LCMV. These data indicate that LCMV infection uniquely facilitates priming of CTL responses in situations in which there are profound deficiencies in antigen presentation by BM-derived, professional APCs. We discuss potential mechanisms to account for the radiation-resistant, host cell presentation of LCMV.

Materials and Methods

Mice and Construction of Radiation Chimeras. 5–6-wk-old female C57BL/6 (B6) mice were purchased from Taconic Farms. (BALB/c × B6)F_1 (CB6) mice were purchased from The Jackson Laboratory. All mice were housed under specific pathogen-free conditions. To establish chimeras, recipient mice were irradiated with a single dose (1,050 rads) from a ^{137}Cs source. Within 24 h, irradiated recipients were rescued by intravenous injection of 4 × 10^6 T cell–depleted BM cells isolated from the femurs of donor mice. T cells were depleted from BM preparations by incubation with a cocktail of anti-Thy1.2 (clone 30-H12), anti-CD8α (clone 53-6.7), and anti-CD4 (clone GK1.5), followed by lysis with low-tox-M rabbit complement (Cedarlane Labs). Chimeric animals were provided antibiotic water containing 13 mg/liter polymixin B sulfate and 0.025 mg/liter neomycin sulfate for 4 wk after irradiation. At 4 wk after reconstitution, chimerism was evaluated by the staining of PBLs with fluorochrome-labeled antibodies. Similar levels of chimerism were obtained in all three sets of chimeric animals used for the experiments described here. Infections of chimeric animals were done between 6 and 12 wk after BM reconstitution.

Cell Staining and FACS®. Fluorochrome- or biotin-conjugated monoclonal antibodies to H2-K^b (AF6-88.5), H2-K^d (SF1.1.1), and CD3-ϵ (145-2C11) were purchased from BD PharMingen, as was an Fc receptor–blocking antibody (anti-CD16/32; 2.4G2). Streptavidin-Tricolor was used as a secondary reagent to detect biotinylated antibodies (Caltag). After osmotic lysis of erythrocytes, samples of 10^6 cells were incubated on ice with saturating amounts of antibody in staining buffer (PBS, 3% FCS, and 0.02% NaN_3). Stained cells were fixed in a PBS/1% paraformaldehyde solution before analysis on a FACScan™ (Becton Dickinson).

Infectious Agents. *L. monocytogenes* EJL243 was provided by Drs. Eric R. Jensen and Jeff F. Miller (University of California at Los Angeles, Los Angeles, CA). This strain secretes the full-length LCMV NP antigen and is derived from *L. monocytogenes* 10403S (19). For infections, bacteria were thawed from stocks stored at −70°C and grown to mid-log phase (A_{600} ∼ 0.1) in tripticase soy broth (Difco/Becton Dickinson). Log phase bacteria were diluted in PBS for intravenous infection of chimeric mice. CFUs present in inocula were determined by absorbance at 600 nm and by plating on tripticase soy broth/agar plates.

LCMV Armstrong 53b was originally obtained from Dr. Peter Southern (University of Minnesota, Minneapolis, MN). Virus was grown on BHK-21 cells (CCL-10; American Type Culture Collection) and titred on Vero Cl008 cells (CRL-1586; American Type Culture Collection) as described previously (20). Recombinant vaccinia virus encoding the full-length LCMV NP (VacNP) was obtained from Jonathan Yewdell (National Institutes of Health, Bethesda, MD). VacNP was grown on HeLa cells and titred on Vero cells. Mice were infected intravenously with LCMV or vaccinia virus diluted to the indicated PFU/animal in 200 μl PBS.

Peptides. Peptides were synthesized on a Synergy apparatus (Applied Biosystems/PerkinElmer), except for $p60_{(217–225)}$, which was synthesized by Research Genetics. Peptides were stored in water or RPMI 1640 at −20°C as 100–200 μM stocks.

Immunization with DCs. DCs were enriched from spleens of CB6 animals by low density centrifugation and an adherence/deadherence procedure as described previously (21). DCs were pulsed with synthetic listeriolysin O $(LLO)_{(91–99)}$ peptide for 1 h, and washed with RP10 media (RPMI 1640 with 10% heat-inactivated FCS, 2 mM L-glutamine, and antibiotics). DCs were resuspended in PBS and administered intravenously into naive chimeric animals.

CTL Assays. For experiments with *L. monocytogenes*–infected animals, responder splenocytes were harvested from immunized animals and expanded 6 d ex vivo in cultures containing peptide-pulsed stimulator cells. Stimulator cells were splenocytes harvested from naive CB6 mice pulsed for 2 h at 37°C in 1 ml RP10 containing a 1 μM concentration of the indicated stimulatory peptide. Stimulator cells were irradiated (2,500 rads), washed in RP10, and added at 3 × 10^7 cells/flask to T-25 Falcon tissue culture flasks (Becton Dickinson) containing 3 × 10^7 responder splenocytes. Flasks were incubated upright for 6 d in a 7% CO_2 environment at 37°C in 10 ml RP10. Viable lymphoblastoid cells, as determined by trypan blue exclusion, were used at indicated E/T ratios in 4-h ^{51}Cr-release assays. For the assays, P815 ($H2^d$) and EL4 ($H2^b$) target cells were labeled with ^{51}Cr by a

1–2-h incubation at 37°C, then washed and added to 96-well plates in the presence of PBS or peptides diluted in PBS, as described previously (22).

For experiments with LCMV and vaccinia virus–infected mice, splenocyte effectors were harvested and washed twice in PBS. After osmotic lysis of red blood cells, the effector cells were washed twice more in PBS, counted, and resuspended at appropriate concentrations in a 1:1 mixture of RP10 and IMDM plus 10% FCS. For the assay, P815-D^b target cells were labeled by incubation for 1 h with ^{51}Cr with or without 1 μM of the indicated peptides. Labeled target cells were washed and added to dilutions of effector cells for 6-h ^{51}Cr-release assays.

In all CTL assays, supernatants were harvested and counted, and the percentage of specific lysis was determined as described previously (23). Target cells were maintained in RP10.

Results

Preliminary Analysis of Chimeras. We reconstituted the hematopoietic system of lethally irradiated CB6 recipient mice with T cell–depleted BM from B6 donors. Radiation-resistant cells in such chimeric animals express both MHC haplotypes of the recipient ($H2^d$ and $H2^b$), whereas radiation-sensitive BM-derived cells express only the donor haplotype ($H2^b$). The chimerism in BM-derived cell types from naive, chimeric animals was evaluated by fluorescent antibody staining of PBLs 4 wk after reconstitution. The PBLs of irradiated CB6 animals reconstituted with CB6 BM ([CB6→CB6] chimeras) were 98.4 ± 0.4% positive for staining with a monoclonal antibody to H2-K^d. In contrast, this antibody failed to stain 87.3 ± 2.2% of PBLs from animals reconstituted with BM from B6 donors ([B6→CB6] chimeras). The difference between these numbers indicates the approximate proportion of radiation-resistant, recipient-derived PBLs which persisted in the chimeric animals 4 wk after reconstitution (∼11.1%). As discussed below, the proportion of recipient-derived leukocytes was even lower in the spleens of infected [B6→CB6] chimeras (see Fig. 5 and below). These data indicate that BM-derived cell types in the chimeric animals were predominantly donor derived and express only the donor $H2^b$ antigen.

Parenchymal Cell Types Fail to Prime CTL Responses to L. monocytogenes–delivered Antigens. H2-D^b– or H2-K^b–restricted CTL epitopes have not been identified in endogenous *L. monocytogenes* proteins. Thus, to determine the effects of chimerism on priming of CTL responses to this intracellular pathogen, we used a recombinant *L. monocytogenes* strain. The strain used, EJL243, expresses a secreted form of the full-length NP antigen from LCMV, and was previously shown to immunize mice for CTL responses to NP epitopes (19). We infected chimeric animals with an immunizing dose (∼0.1 LD_{50}) of *L. monocytogenes* EJL243, and 7–10 d later isolated immune splenocytes. These cells were expanded in vitro and assayed for lysis of peptide-pulsed target cells. The peptides used for pulsing correspond to H2-K^d–restricted epitopes from two secreted *L. monocytogenes* proteins, LLO and p60, and an H2-D^b–restricted epitope from the LCMV NP antigen. Lysis of P815 ($H2^d$) target cells coated with the $LLO_{(91–99)}$ or $p60_{(217–225)}$ epitopes was readily apparent when effector cells were prepared from [CB6→CB6] animals (Fig. 1, D and E). Thus, CTL responses to these H2-K^d–restricted epitopes were efficiently primed in mice whose BM-derived APCs express both $H2^b$ and $H2^d$ MHC alleles. CTL responses to an H2-D^b–restricted epitope from the NP antigen were also observed in [CB6→CB6] animals, as judged by specific lysis of EL4 ($H2^b$) target cells coated with the $NP_{(396–404)}$ peptide (Fig. 1 F). However, such H2-D^b–restricted responses were observed in only a subset of the [CB6→CB6] animals tested (Fig. 1 F, and data not shown), suggesting that responses to H2-K^d–restricted or other endogenous *L. monocytogenes* epitopes dominate the response in such animals.

An inverse pattern of CTL responses was observed in [B6→CB6] chimeric animals. In these animals, immunization with the recombinant *L. monocytogenes* consistently failed to prime H2-K^d–restricted CTL responses to either

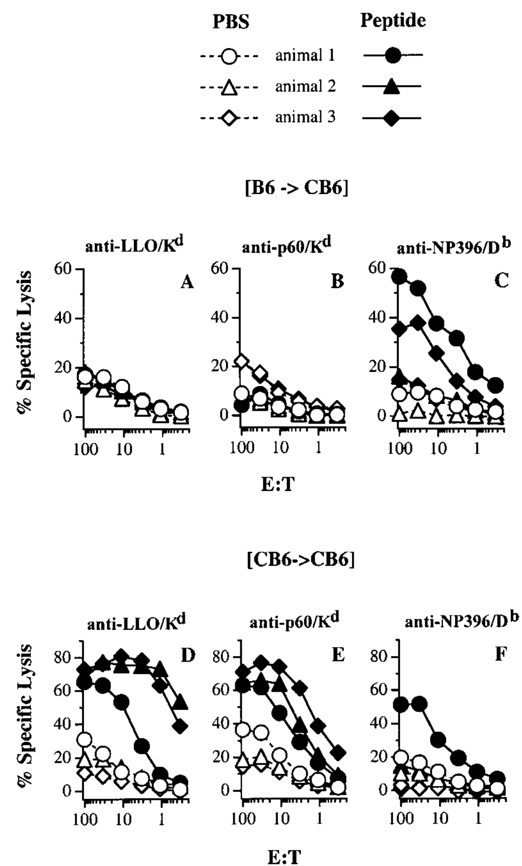

Figure 1. BM MHC haplotype determines CTL priming after *Listeria* infection. Three [B6→CB6] and three [CB6→CB6] chimeras were infected with 500 CFU recombinant *Listeria*. Immune splenocytes were stimulated in vitro with CB6 splenocytes coated with H2-K^d–restricted *Listeria* $LLO_{(91–99)}$ (A and D) or $p60_{(217–225)}$ (B and E) epitopes or with the H2-D^b–restricted $NP_{(396–404)}$ epitope from the recombinantly expressed NP of LCMV. CTL activity was assayed 6 d later using P815 ($H2^d$) (A and B, and D and E) or EL4 ($H2^b$) (C and F) target cells coated with PBS (open symbols) or with 20 nM of the indicated peptide (filled symbols). Symbol types correspond to responder cells from individual mice. Similar results were obtained in four additional experiments.

the $LLO_{(91–99)}$ or the $p60_{(217–225)}$ epitopes (Fig. 1, A and B), whereas CTL responses to the $H2\text{-}D^b$–restricted $NP_{(396–404)}$ epitope were readily detected (Fig. 1 C). These data indicate that chimeric animals whose BM-derived APCs express only $H2^b$ fail to mount detectable $H2\text{-}K^d$–restricted CTL responses to *L. monocytogenes*–delivered antigens. As parenchymal cells of these animals are $H2^{d\times b}$, the selective lack of responsiveness to the $H2^d$ epitopes suggests that such parenchymal cells are quite ineffective at priming CTL responses to *L. monocytogenes*.

Antigen Presentation by BM-derived APCs Is Required for Priming of CTL Responses. A potential caveat to the above experiments is the possibility that [B6→CB6] animals have reduced numbers of $H2^d$-restricted CTL precursors. A paucity of such precursors might arise from differences in thymic selection in [B6→CB6] compared with [CB6→CB6] chimeras. Alternatively, given the requirement for MHC expression in the maintenance of naive T cells (for a review, see reference 24), $H2\text{-}K^d$–restricted CTL precursors might not persist in animals whose BM cells fail to express $H2\text{-}K^d$. Thus, we addressed the issue of whether [B6→CB6] animals fail to select or maintain CTLs specific for $H2\text{-}K^d$–restricted epitopes by assaying for such cells in animals immunized with peptide-pulsed CB6 splenic APCs. The APCs used were low density, DC-enriched preparations pulsed in vitro with the $LLO_{(91–99)}$ peptide. Effector cells from the immunized animals lysed P815 target cells coated with the $LLO_{(91–99)}$ peptide, but not target cells presenting a control $H2\text{-}K^d$–restricted *L. monocytogenes* peptide ($p60_{[217–225]}$). Thus, the immunization procedure selectively primed CTL responses to the immunizing *L. monocytogenes*–derived peptide (Fig. 2). These data demonstrate the existence of CTL precursors specific for the $H2\text{-}K^d$–restricted $LLO_{(91–99)}$ epitope in the [B6→CB6] chimeric animals, and suggest that the failure of *L. monocytogenes* infection to prime $H2\text{-}K^d$–restricted responses in such animals (Fig. 1) is not due to a deficiency in the selection or maintenance of $H2\text{-}K^d$–restricted CTLs. Rather, the data indicate that the priming of CTL responses during infection with *L. monocytogenes*, like the priming of CTL responses in mice vaccinated with naked DNA (11–13), requires antigen presentation by professional, BM-derived APCs.

CTL Responses to LCMV Infection Are Not Dictated by the Donor BM Haplotype. To investigate whether priming of CTL responses to viral infection also requires antigen presentation by BM-derived APCs, we assayed for priming of $H2\text{-}D^b$– and $H2\text{-}L^d$–restricted CTL responses in [CB6→CB6] and [B6→CB6] chimeras immunized with LCMV. Splenocyte effector cells from animals immunized with 10^5 PFU LCMV-Armstrong were tested directly ex vivo for lysis of peptide-pulsed $H2\text{-}D^b$–transfected P815 target cells (P815-D^b). The effector cells from LCMV-immune [CB6→CB6] chimeras efficiently lysed target cells coated with either the $H2\text{-}L^d$–restricted $NP_{(118–126)}$ peptide or the $H2\text{-}D^b$–restricted $NP_{(396–404)}$ peptide (Fig. 3, D and E). This result is consistent with priming of both $H2^b$- and $H2^d$-restricted CTL populations by $H2^{b\times d}$ BM-derived APCs, and was predicted from the results of above experiments using *L. monocytogenes* (Fig. 1, D–F). However, the response pattern of the LCMV-infected [B6→CB6] chimeric animals was surprising. Such animals responded to both the $H2\text{-}D^b$–restricted epitope and the $H2\text{-}L^d$–

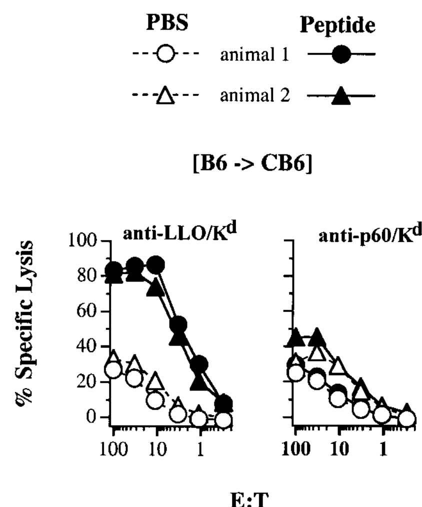

Figure 2. Exogenous CB6 APCs can prime CTL responses to an $H2\text{-}K^d$–restricted *Listeria* epitope in [B6→CB6] chimeras. DCs isolated from spleens of CB6 mice were pulsed with 1 μM $LLO_{(91–99)}$ and used to immunize two naive [B6→CB6] chimeras intravenously. After 3 wk, splenocytes from immunized chimeras were stimulated in vitro with $LLO_{(91–99)}$ (left) or $p60_{(217–225)}$ (right) peptides. Responders were assayed after 6 d in culture for lysis of P815 target cells coated with PBS (open symbols) or 50 nM of the corresponding peptide (filled symbols).

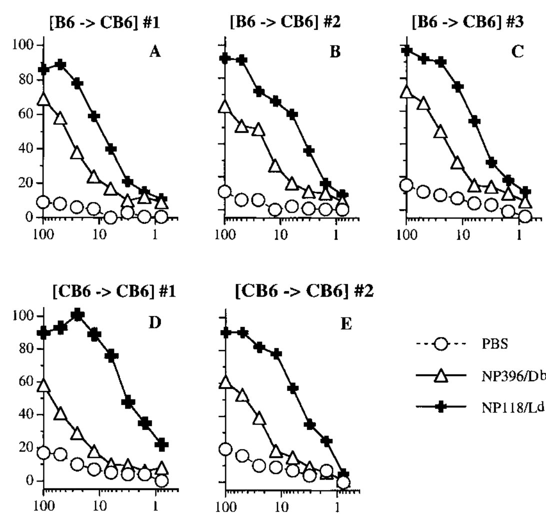

Figure 3. CTL priming during infection with LCMV is not dictated by BM MHC haplotype. Three [B6→CB6] (A–C) and two [CB6→CB6] (D and E) chimeras were infected intravenously with 10^5 PFU LCMV-Armstrong. On day 9 after infection, ex vivo CTL activity was measured directly using P815-D^b target cells pulsed with PBS or with 1 μM of the indicated NP peptide. $NP_{(396–404)}$ is presented by $H2\text{-}D^b$ and $NP_{(118–126)}$ is presented by $H2\text{-}L^d$. The results in this figure are representative of three experiments. A similar response pattern was observed in animals receiving an LCMV inocula of only 200 PFU LCMV-Armstrong per animal.

restricted epitope (Fig. 3, A–C). Identical results were observed when mice were infected with lower doses (~200 PFU) of LCMV. These data indicate that LCMV infection elicits priming of $H2^d$-restricted CTL responses in animals whose BM-derived cells are $H2^b$. Thus, in sharp contrast to the situation described above for responses to *L. monocytogenes* infections, the CTL responses of LCMV-infected chimeric animals are not dictated by the MHC haplotype of the donor BM.

Nonprofessional Antigen Presentation Is Not a Ubiquitous Feature of Viral Infections. The data above suggest that LCMV infection of [B6→CB6] chimeric animals leads to priming of CTL responses that are restricted by parenchymal cell MHC alleles. To determine whether infection with a different viral pathogen could also induce similar priming, we evaluated CTL responses after the infection of chimeric animals with VacNP. As with LCMV infection, CTL activity in spleens of VacNP-infected [B6→CB6] and control [CB6→CB6] animals was assayed directly ex vivo. Expectedly, [CB6→CB6] animals responded well to the L^d-restricted $NP_{(118–126)}$ epitope (Fig. 4, C and D). These animals responded poorly or not at all to the subdominant $H2\text{-}D^b$–restricted NP epitope. In contrast, in [B6→CB6] animals, responses to the $H2\text{-}D^b$ epitope were clearly seen but in no case did we observe responses to the $H2\text{-}L^d$–restricted $NP_{(118–126)}$ epitope (Fig. 4, A and B). This indicates that priming of CTL responses by parenchymal cell types is not detected in the VacNP-infected animals, similar to our findings with *L. monocytogenes*–infected animals (Fig. 1). Furthermore, these results indicate that the apparently BM-independent priming of CTL responses after LCMV infection is not a general feature of infections with viral pathogens and may rather be a specific feature of LCMV infection.

Host-derived APC Populations Are Depleted Over 20-fold in Chimeric Animals and Are Not Enriched upon LCMV Infection. The above data suggest that LCMV infection facilitates priming of CTL responses to antigen expressed by nonprofessional APCs. However, the ability of [B6→CB6] animals to generate strong CTL responses to the $H2\text{-}L^d$–restricted NP epitope might also reflect presentation by residual BM-derived host APCs or selective recruitment or expansion of host-derived professional APCs in the spleens of LCMV-infected animals. To address these issues, we first analyzed $H2\text{-}K^d$ expression on $CD3^+$ and $CD3^-$ splenocytes from LCMV-infected or VacNP-infected chimeric mice. Representative results of this analysis appear in Fig. 5. As expected, nearly all cells from infected [CB6→CB6] animals stained positive with an antibody to $H2\text{-}K^d$. In contrast, only 6.5% of splenocytes from the VacNP-infected [B6→CB6] animal expressed $H2\text{-}K^d$. A similar proportion (5.5%) of cells was $H2\text{-}K^{d+}$ in the LCMV-infected spleen. Furthermore, the majority of host-derived $H2\text{-}K^{d+}$ cells in both sets of infected [B6→CB6] animals were $CD3^+$ T cells. The reduced size (0.2%) of the $H2\text{-}K^{d+}$, $CD3^-$ population in the LCMV-infected [B6→CB6] animal may be explained by dilution of these cells by the three- to fourfold greater expansion of T cells occurring in the spleens of LCMV-infected animals. After accounting for this expansion, the populations of $H2\text{-}K^{d+}$, $CD3^-$ splenocytes are approximately equal in the LCMV-infected and VacNP-infected animals. Thus, we fail to demonstrate any enrich-

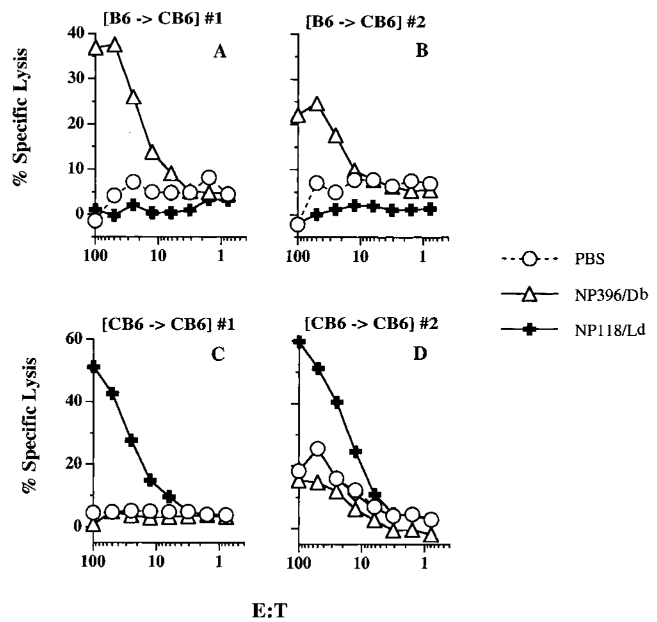

Figure 4. Recombinant vaccinia virus infection elicits CTL responses only to epitopes presented by BM-derived APCs. Two [B6→CB6] (A and B) and two [CB6→CB6] (C and D) chimeric mice were infected intravenously with 5×10^6 PFU of VacNP. 6 d after infection, CTL activity in spleens of infected mice was quantitated using $P815\text{-}D^b$ target cells pulsed with 1 μM of the indicated NP peptides as described in the legend to Fig. 2. Similar results were obtained in two additional experiments, and were seen in animals infected with 7.1×10^6 PFU of VacNP.

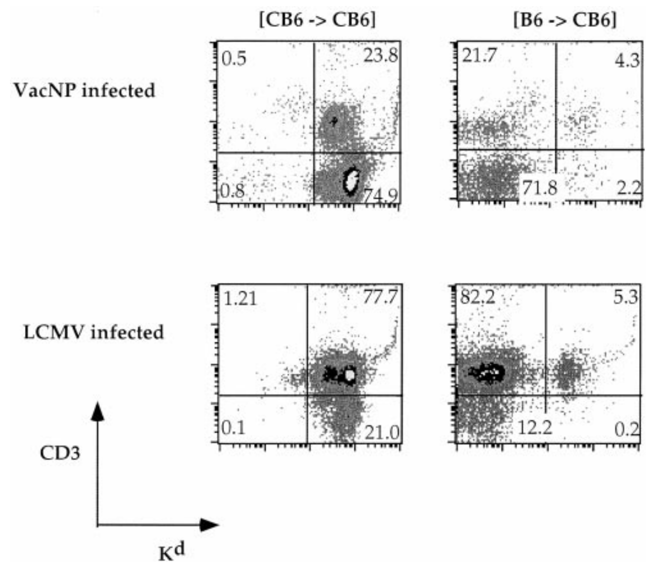

Figure 5. Representation of $H2\text{-}K^d$–expressing APCs is similar in chimeric animals infected with recombinant vaccinia virus or LCMV. Splenocytes from VacNP- or LCMV-infected [B6→CB6] chimeras were harvested on day 6 or 9 after infection, respectively. Cells were stained with antibodies to CD3 and $H2\text{-}K^d$ and analyzed by FACS®.

ment of host-derived BM APCs in LCMV-infected animals. The proportion of chimerism in non-B, non-T cell populations was also evaluated by triple staining splenocytes for CD3, B220, and K^d. The $CD3^+B220^-$, $CD3^-B220^+$, and $CD3^-B220^-$ populations were of equal size in [B6→CB6] and [CB6→CB6] animals, with the $CD3^-B220^-$ population, which includes DCs and macrophages, comprising 3.6–5.8% of total splenocytes. However, the proportion of K^{d+} cells in this $CD3^-B220^-$ population differed substantially between the two groups of animals, with ~99.9% of this population (4.8–5.8% of total splenocytes) staining positive for K^d in the [CB6→CB6] chimeras, but only 3.5–5.5% (0.2–0.3% of total splenocytes) staining for K^d in the [B6→CB6] chimeras. These data suggest that professional K^d-restricted antigen presentation is reduced ~18–30-fold in the [B6→CB6] mice compared with the [CB6→CB6] mice.

Discussion

We used radiation chimeras to investigate the requirement for professional, BM-derived APCs in the priming of CTL responses to antigenic epitopes from three different intracellular pathogens. Our data indicate that [B6→CB6] chimeric animals infected with two of these pathogens generate only CTL responses restricted by the BM MHC haplotype ($H2^b$). Chimeric [B6→CB6] animals infected with recombinant *L. monocytogenes* bacteria or vaccinia virus mounted strong $H2\text{-}D^b$–restricted responses to recombinantly expressed LCMV NP antigen, but were consistently unresponsive to $H2^d$-restricted epitopes from the same NP antigen or from two endogenous *L. monocytogenes* proteins (LLO and p60). The unresponsiveness was not attributable to holes in the T cell repertoire of the radiation chimeras used, as CTL responses to an $H2\text{-}K^d$–restricted *L. monocytogenes*–derived epitope could be induced in the [B6→CB6] chimeras after in vivo priming with peptide-pulsed DCs, and immunization with LCMV showed that the repertoire was intact.

With regard to CTL priming after vaccinia virus infection, our results confirm those reported in a recent study by Sigal et al. (25), who illustrated the importance of BM-derived APCs in the priming and cross-priming of CTL responses to recombinant vaccinia virus and poliovirus infections. Furthermore, older studies had indicated that priming of CTL responses to influenza (26) and vesicular stomatitis virus (27) were abrogated in mice whose macrophages were depleted by in vivo treatment with carrageenan, silica, or liposome-encapsulated toxins. Thus, BM-derived APCs have now been implicated as essential elements in the priming of CTL responses to at least four viral pathogens.

It is well known that wild-type mice infected with *L. monocytogenes* develop robust CTL responses to epitopes from a small number of secreted endogenous or recombinantly expressed bacterial antigens (19, 28). In contrast to the above-mentioned viral infections, mechanisms for priming of CTL responses to this and other bacterial pathogens are largely obscure. However, a study by Shen et al. has shed some light on this subject (29). This study illustrated that a model antigen localized to the *L. monocytogenes* cytosol efficiently primed antigen-specific CTL responses in infected mice. Conversely, this antigen had to be secreted into the cytosol of infected cells to provide CTL-mediated protection during the effector phase of anti-*Listeria* immunity. The authors explained these differences in the requirement for antigen secretion by demonstrating that processing of nonsecreted bacterial antigens can occur within macrophages. Thus, these and other professional APCs are postulated to cross-prime CTL responses to nonsecreted *L. monocytogenes* antigens. In contrast, nonprofessional APCs are unable to access and/or present antigens that are not secreted by cytosolic *L. monocytogenes*, explaining the requirement for antigen secretion in the effector phase of CTL immunity (29). Our results with *L. monocytogenes*–infected chimeric animals are consistent with a central role for BM-derived APCs in the priming of CTL responses *to L. monocytogenes*, as they provide the first direct evidence that antigen presentation by BM-derived APCs is essential for the priming of CTL responses to an intracellular bacterial pathogen.

In sharp contrast to our results with *L. monocytogenes* and vaccinia virus, the priming of CTL responses in LCMV-infected [B6→CB6] chimeric animals shows little requirement for antigen presentation by donor BM-derived APCs. Both $H2^b$- and $H2^d$-restricted CTL responses were invariably observed after infection of [B6→CB6] chimeric animals, even with very low (200 PFU/animal) viral inocula (data not shown). These findings are somewhat at odds with the conclusions of a previous report in which effector cells from chimeric [k×b→d×b] animals failed to lyse LCMV-infected $H2^d$ target cells after in vivo priming with LCMV (30). However, the failure to detect $H2^d$-restricted responses in this case may have been due to other factors, such as the use of infected (as opposed to peptide-pulsed) target cells. In line with this explanation, adoptive transfer experiments in that same study are more consistent with the possibility that $H2^d$-restricted CTL were indeed primed to some extent in the [k×b→d×b] chimeras. Furthermore, our findings with LCMV are confirmed and complemented by results from Sigal and Rock reported in this issue (31). As in our work, they find that CTL responses to $NP118/L^d$ in [b→d×b] BM chimeras are readily detected in LCMV-infected animals (31). This response persisted even after supralethal irradiation of host animals, although even such extreme measures failed to completely eradicate host-derived BM APCs. Thus, it becomes clear that CTL priming during an LCMV infection, particularly to the $NP118/L^d$ epitope, is remarkably insensitive to the level of antigen presentation by BM-derived APCs. Our own staining results indicate that the population of host-derived APCs that persist in [B6→CB6] chimeras is between 1/29 and 1/18 of that seen in [CB6→CB6] chimeras, and that the ratio of chimerism is not altered after infection with LCMV versus VacNP (see Fig. 5, and Results). These data argue against enhanced survival or expansion of recipient-

derived (CB6) professional APCs in the LCMV-infected chimeric mice. However, we cannot yet differentiate between priming of NP118/L^d responses by the small population of host BM–derived APCs versus non–BM-derived host cell types. Such priming is, however, remarkably efficient. Thus, although the proportion of $B220^-CD3^-$ splenocytes expressing K^d is reduced by at least 20-fold in [B6→CB6] animals, there is no observed reduction in the level of CTL priming after LCMV infection (Fig. 3). This contrasts sharply with the situation after *L. monocytogenes* and vaccinia infections and leads us to conclude that unique features of the LCMV infection make this pathogen more efficient at stimulating CTL responses. What might these features be?

First, LCMV may uniquely infect a subset of non–BM-derived cells that constitutively prime host CTL responses. However, this possibility seems unlikely because each of the intracellular pathogens used in this study infects a broad range of non–BM-derived host cell types in vitro. *L. monocytogenes*, for example, has been shown to infect hepatocytes, epithelial, endothelial, and other adherent cell types, such as fibroblasts, in vitro. Furthermore, bacteria have been observed in splenic barrier cells (a fibroblastoid cell type [32]) as well as in hepatocytes in tissue sections from *L. monocytogenes*–infected mice (33). Although the precise nature of all cell types infected by each of these pathogens in vivo remains to be determined, the available evidence suggests that infection of non-BM host cell types, even within lymphoid organs, does not by itself facilitate BM-independent priming of CTL responses to an intracellular pathogen.

Second, LCMV infection may enhance the priming ability of $H2^d$-expressing cells or otherwise lower the threshold for activation of CTL precursors. Such enhancement could affect priming by either BM-derived or parenchymal cell types, perhaps by inducing expression of costimulatory factors that enhance their ability to prime naive CTLs. It is clear from previous work that the priming of CTL responses to LCMV can occur independently of CD28 expression (34). We have also observed CD28-independent priming of CTL responses restricted by both donor and recipient haplotypes after LCMV infection of CB6 radiation chimeras that were reconstituted with CD28-deficient ($H2^b$) BM (data not shown). These observations suggest that additional, CD28-independent, costimulatory factors contribute to priming of CTL responses during LCMV infections, whether restricted by donor or host H2. We hypothesize that selective upregulation of such factors during LCMV infection, but not during *L. monocytogenes* or vaccinia infections, may account for the remarkably efficient priming of CTL responses seen during LCMV infection. Such novel costimulatory factors might act to modify either the non–BM-derived APCs or the T cell. This hypothesis is supported by recent data from Ochsenbein et al. (35). These authors showed that mice immunized with LCMV mount a protective memory response upon secondary challenge with LCMV that is more rapid and robust than the memory response induced by immunization with a recombinant, NP epitope–expressing *L. monocytogenes*. This effect was also seen upon adoptive transfer of equal numbers of immune CTLs from animals primed with LCMV or with NP-expressing *L. monocytogenes*. These results suggest that CTLs immunized during an LCMV infection are more protective on a per cell basis than are CTLs immunized to the same antigen during a *L. monocytogenes* infection. Thus, infection of mice with LCMV, as opposed to *L. monocytogenes* (and presumably vaccinia virus), may induce a prolonged alteration in the threshold required for activation or survival of antigen-specific CTLs. Such mechanisms may also account for the profound differences in the burst size of the primary, CTL response seen after LCMV infection (36, 37) versus *L. monocytogenes* infection (28) of wild-type animals. Further experiments will be required to test the validity of this hypothesis.

The features of host–pathogen interactions that govern the efficiency of CTL priming in vivo are not well understood. Even when antigen is present in central lymphoid organs, in most cases it must be presented by professional APCs to prime effectively. LCMV appears to bend or perhaps even break this rule, as CTL priming to LCMV-delivered antigen is completely insensitive to the profound reduction in antigen presentation by BM APC populations. It will be important to better define the mechanisms for "BM-insensitive" CTL priming during LCMV infection. A better understanding of the mechanisms for CTL priming during infections should facilitate the development of vaccines and immune therapies for important human pathogens.

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