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CD8 T-Cell Recognition of Macrophages and Hepatocytes Results in Immunity to *Listeria monocytogenes*

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CD8 T cells are effective mediators of specific immunity to infection by *Listeria monocytogenes*, a bacterial pathogen that initially infects macrophages in the spleen and liver and subsequently spreads to hepatocytes and unidentified parenchymal cells in the spleen. To identify the in vivo target cells of *L. monocytogenes*-immune CD8 T cells, adoptive transfer assays were performed with bone marrow chimeric or transgenic host mice which had been manipulated to alter the major histocompatibility complex molecules expressed on macrophages or hepatocytes. *L. monocytogenes*-immune CD8 T cells mediate significant immunity in BDF1→β2M−/− chimeras, comparable to that seen in unmanipulated BDF1 recipients. *L. monocytogenes*-immune CD8 T cells also mediate significant antilisterial immunity in parent→F1 chimeras when the CD8 T cells are syngeneic with the bone marrow donor. These data demonstrate that bone marrow-derived macrophages are major targets for *L. monocytogenes*-immune CD8 T cells in adoptive transfer assays. Interestingly, significant immunity was observed in parent→F1 chimeras when the *L. monocytogenes*-immune CD8 T cells were not syngeneic with the bone marrow donor, suggesting that recognition of *Listeria*-infected non-bone-marrow-derived cells such as hepatocytes may also occur in vivo. Consistent with this possibility, H-2K^b-restricted CD8 T cells specific for the listeriolysin O molecule mediate significant immunity in the liver, but not the spleen, in transgenic mice expressing H-2K^b only on hepatocytes. In addition, *Listeria*-specific CD8 T cells lyse *Listeria*-infected hepatocyte-like cells in vitro. Thus, *Listeria*-infected hepatocytes can be recognized by CD8 T cells in vivo and in vitro.

Infection of mice with *Listeria monocytogenes*, a facultative intracellular bacterium, provides a model system for the analysis of both nonspecific (natural) and specific immunity to infection. Natural immunity to *L. monocytogenes* is required for control of acute infection whereas specific immunity is required to sterilize the infection and to resist subsequent challenge with high levels of the pathogen (2, 26). Natural immunity to *L. monocytogenes* requires the recruitment and activation of phagocytes, including neutrophils and macrophages. Depletion of neutrophils, which may be involved in early destruction of *L. monocytogenes*-infected hepatocytes (7), exacerbates acute *L. monocytogenes* infection (1, 8, 11).

Macrophage activation in response to *L. monocytogenes* infection has been characterized in vitro and in vivo. In vitro studies suggest that macrophage activation requires gamma interferon (IFN-γ) produced by natural killer cells in response to macrophage-derived tumor necrosis factor alpha and interleukin 12 (2, 50). These studies are supported by in vivo studies with tumor necrosis factor alpha (47), interleukin-12 (49), and IFN-γ (6)-specific neutralizing antibodies, all of which exacerbate acute infection with *L. monocytogenes*. In addition, a role for interleukin-1 in natural immunity has been documented (44). Subsequent studies demonstrating increased susceptibility of tumor necrosis factor alpha receptor 1 (45), the IFN-γ receptor (22), and IFN-γ (19) gene knockout mice to *L. monocytogenes* infection provide additional evidence that these cytokines play a critical role in macrophage activation in response to acute *L. monocytogenes* infection.

Specific immunity to *L. monocytogenes* infection is mediated by T cells (35), and it has been shown that CD8 T cells expressing αβ T-cell receptors are the most effective mediators of acquired resistance to infection (3, 10, 29, 34, 36). By analogy to natural immunity, CD8 T-cell-mediated antilisterial immunity was assumed to function through IFN-γ-mediated activation of macrophages (25). However, we have recently used IFN-γ gene knockout mice to demonstrate that specific immunity to *L. monocytogenes* can develop and be expressed in the complete absence of IFN-γ (19). These studies, combined with the demonstration that CD8 T cells from perforin gene knockout mice are deficient in antilisterial immunity (24), suggest that lysis of *L. monocytogenes*-infected targets is an important mechanism for specific immunity in vivo.

In the experimental infection of mice, *L. monocytogenes* initially infects fixed tissue macrophages in the spleen and liver. In these cells, which can be induced by cytokines to express potent antimicrobial defenses, some *L. monocytogenes* organisms escape from the phagocytic vesicle into the cytoplasm of the infected cell, primarily through the actions of a secreted virulence factor termed listeriolysin O (LLO) (9). Once in the cytoplasm of the cell, the organism replicates and is capable of movement via the polymerization of host F-actin, a process requiring the Act A protein (30). The ability to move about in the cytoplasm is linked to the ability of the organism to spread between cells, which is accomplished without exposure to extracellular defense mechanisms (41). In this fashion, *L. monocytogenes* is capable of infecting bone marrow (BM)-derived macrophages which enter the spleen and liver in the inflammatory process and non-BM-derived cells, such as hepatocytes, which do not express high levels of defense against microbial infection (15, 43, 48, 51). However, entry of the organism into the cytoplasm of the infected cell exposes it to the major histocompatibility complex (MHC) class I antigen presentation pathway and the attention of CD8 T cells. Localization of *L.*

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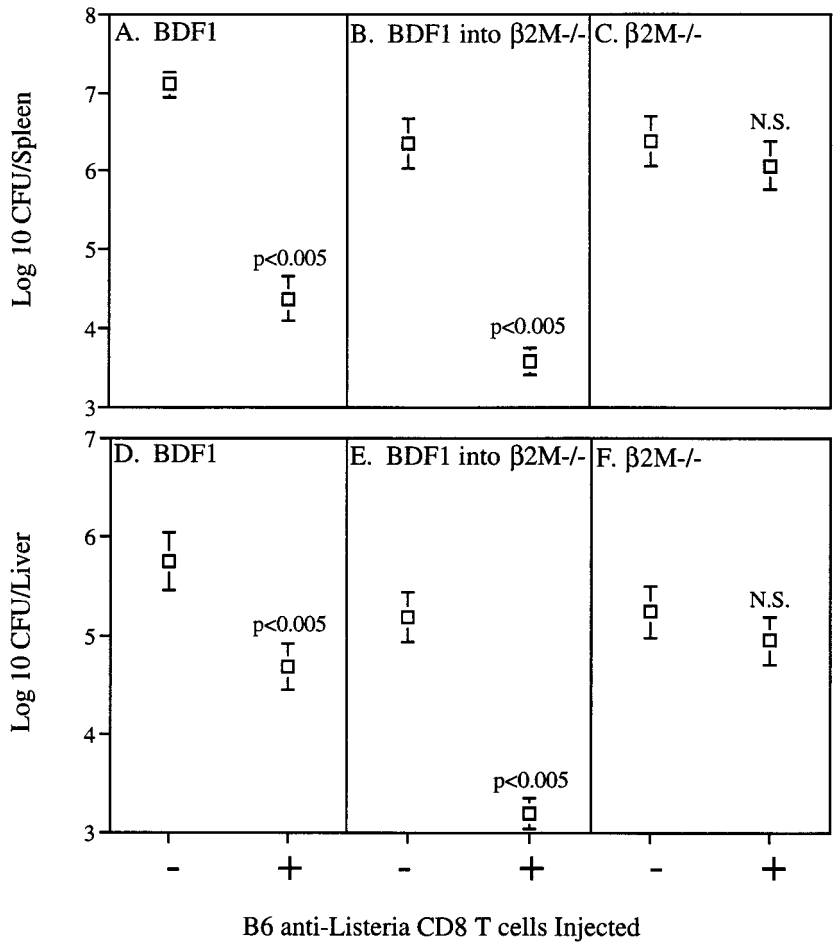

FIG. 1. *L. monocytogenes*-immune CD8 T cells recognize BM-derived cells in vivo. Groups of six BDF1 (A and D) mice, (BDF1→β2M−/−) chimeras (B and E), or β2M−/− mice (C and F) were infected with 5×10^5 *L. monocytogenes* organisms. Three mice in each group received 8×10^7 CD4 T-cell-depleted spleen cells obtained from 7-day-infected (0.1 LD_{50}) B6 mice, 1 h prior to infection. Spleens (A to C) and livers (D to F) were harvested 72 h after infection, and the number of CFU per organ was determined. The results for two independent experiments were pooled and are expressed as the mean number of CFU per organ. Error bars indicate standard errors. Student's *t* test was used in the analysis of the results. N.S., not significant.

monocytogenes in the cytoplasm of the infected cells is thought to explain the finding that CD8 T cells are the most effective mediators of acquired immunity to *L. monocytogenes* infection (5). Consistent with this notion, it has been clearly demonstrated that *L. monocytogenes* antigens are processed and presented by MHC class I molecules in vivo (38).

In vitro evidence demonstrates that *L. monocytogenes*-immune CD8 T cells can recognize and lyse *L. monocytogenes*-infected macrophages (27) and macrophage-like cells (38). However, the target cells for *L. monocytogenes*-immune CD8 T cells in vivo have not been determined. To address this question we have used adoptive transfer of *L. monocytogenes*-immune CD8 T cells into mice which have been manipulated to alter the MHC molecules expressed on BM-derived macrophages or non-BM-derived hepatocytes. Our experiments show that CD8 T-cell recognition of infected BM-derived cells is the major mechanism for the expression of adoptive immunity. In addition, these studies reveal that CD8 T-cell recogni-

TABLE 1. MHC class I expression on PBL from radiation of BM chimeras[a]

Mouse strain MHC	% Positive PBL			
	H-2^{b+d+}	*H-2*$^{b-d+}$	*H-2*$^{b+d-}$	*H-2*$^{b-d-}$
BDF1 (*H-2*$^{b\times d}$)	97.6 ± 0.6	0.1 ± 0.0	2.2 ± 0.3	0.2 ± 0.3
B6→BDF1 (*H-2*^{b}→*H-2*$^{b\times d}$)	8.9 ± 0.9	0.0 ± 0.0	90.9 ± 1.0	0.2 ± 0.2
DBA/2→BDF1 (*H-2*^{d}→*H-2*$^{b\times d}$)	2.4 ± 0.5	96.9 ± 0.6	0.4 ± 0.1	0.3 ± 0.1

[a] PBL were obtained 12 weeks after reconstitution of irradiated (1,000 rad) BDF1 (*H-2*$^{b\times d}$) mice with BM from B6 (*H-2*^{b}) or DBA/2 (*H-2*^{d}) mice. Cells were analyzed for expression of H-2K^{b} and H-2K^{d} by two-color fluorescence-activated cell sorting with specific MAbs. The results are expressed as the mean percentage ± standard errors for six individual mice in each group. Normal BDF1 mice were used as a control.

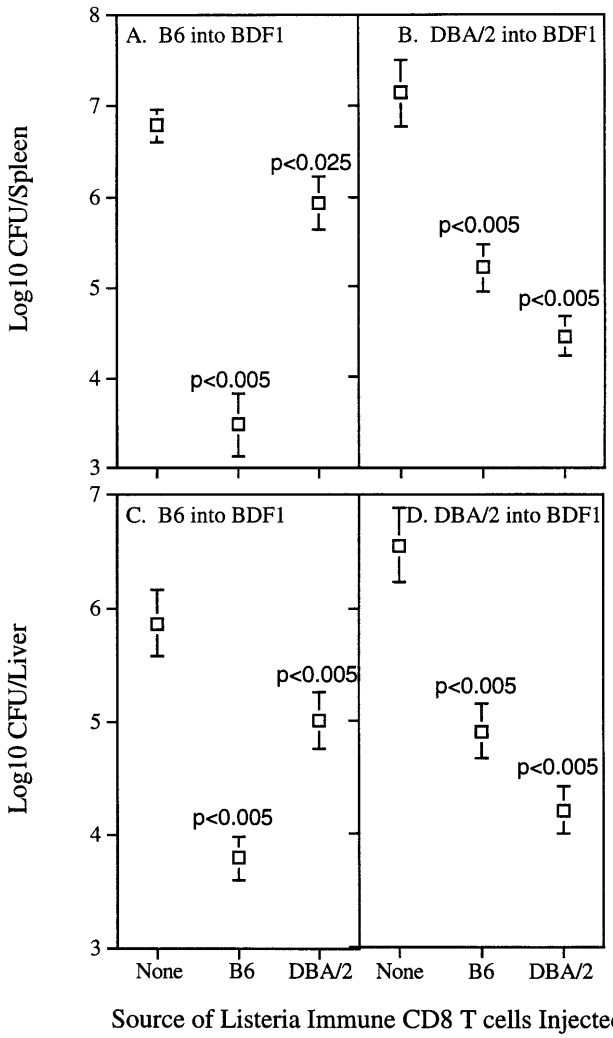

FIG. 2. *L. monocytogenes*-immune CD8 T cells mediate antilisterial immunity in P→F1 BM chimeras. Groups of six B6→BDF1 (A and C) and DBA/2→BDF1 (B and D) chimeras were infected with 5×10^5 *L. monocytogenes* organisms. Three mice in each group received 8×10^7 CD4 T-cell-depleted spleen cells obtained from 7-day-infected (0.1 LD_{50}) B6 or DBA/2 mice 1 h prior to infection. Spleens (A and B) and livers (C and D) were harvested 72 h after infection, and the number of CFU per organ was determined. The results, pooled from three independent experiments, are expressed as the mean number of CFU per organ. Error bars indicate standard errors. Student's *t* test was used in the analysis of the results.

tion of non-BM-derived hepatocytes is relevant to specific immunity against *L. monocytogenes* infection.

MATERIALS AND METHODS

Mice and radiation BM chimeras. Six- to 8-week-old female C57BL/6 (*H-2*^{b}), DBA/2J (*H-2*^{d}), and BDF1 (*H-2*$^{b\times d}$) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). β2M−/− (*H-2*^{b}) mice (31) were originally provided by B. Koller and were bred in the animal facilities at the University of Washington. Alb-Kb mice are DBA/2 (*H-2*^{d}) mice expressing an H-2K^b transgene under control of the albumin promoter. These mice exhibit hepatocyte-specific H-2K^b expression (17) and were provided by D. Nemazee. Alb-Kb mice were bred in the animal facilities at the University of Iowa. For generation of radiation BM chimeras, BDF1 or β2M−/− mice were lethally irradiated (1,000 rads) and reconstituted with 5×10^6 BM cells that had been depleted of Thy-1$^+$ cells by treatment with the T24 antibody (13) and complement. Animals were provided with antibiotic water for 1 month and were housed a minimum of 13 weeks before use to allow reconstitution of the peripheral immune system.

Bacteria. *L. monocytogenes* 43251 was grown in Trypticase soy broth (Becton Dickinson, Cockeysville, Md.) to log phase, and aliquots were frozen and stored at −70°C. Freshly thawed bacterial stocks were allowed to return to log phase by incubation at 37°C for 3 to 4 h, and the bacteria were adjusted to the appropriate dilution for intravenous injection. The 50% lethal dose (LD_{50}) of this strain of *L. monocytogenes* for DBA/2J mice is 10^4 CFU per animal, whereas the LD_{50} for B6 and BDF1 mice is 10^5 CFU per animal.

MAbs, flow cytometry, and T-cell depletion. Peripheral blood was collected from radiated BM chimeras 12 weeks after reconstitution. After lysis of erythrocytes, peripheral blood lymphocytes (PBL) were analyzed for the expression of H-2K^b and H-2K^d MHC class I molecules to assess the degree of chimerism. Binding of biotinylated SF1.1.1, a mouse immunoglobulin G2a monoclonal antibody (MAb) specific for H-2K^d obtained from American Type Culture Collection, was detected with phycoerythrin-conjugated avidin (Becton Dickinson). H-2K^b expression was detected with fluorescein isothiocyanate-conjugated Y3, a mouse immunoglobulin G2a MAb obtained from the American Type Culture Collection. All staining was done at 4°C in the presence of 1% fetal calf serum and 0.02% NaN_3. Cells were analyzed with a fluorescence-activated cell sorter (FACScan; Becton Dickinson). CD4 T-cell depletion of immune splenocytes was carried out by two rounds of incubation with the immunoglobulin M rat anti-mouse CD4 MAb RL172 followed by a rabbit complement as described previously (21). CD4 T-cell depletion of splenocytes was greater than 97% in all experiments. Antibody blocking studies were performed with Y3, anti-H-2K^b, and 28-14-8, a mouse immunoglobulin G2a anti-H-2D^b MAb, obtained from the American Type Culture Collection.

Antigen-specific CD8 T-cell lines and chromium release assays. For generation of LLO-specific CD8 T-cell lines, BDF1 mice were infected intravenously with 10^4 CFU of *L. monocytogenes*. Seven days later, spleen cells were harvested and restimulated in vitro with PHem3.3, an LLO transfectant of P815 (H-2^d) cells (38), as previously described (18) or with EHem8, an LLO transfectant of EL-4 (H-2^b) cells that was generated with the same construct as that for PHem3.3. B3 CD8 T cells, specific for hen ovalbumin amino acids 258 to 265 in the context of H-2K^b, were maintained as described previously (23). CD8 T cells that recognize amino acids 217 to 225 of the *L. monocytogenes* p60 molecule (p60 217-225) in the context of H-2K^d have been previously described (20). A CD8 T-cell line that recognizes influenza virus nucleoprotein amino acids 149 to 157 in the context of H-2K^d was the gift of M. Gavin. Standard ^{51}Cr release assays with nonadherent tumor cell targets were performed for 3 to 4 h as described previously (18). For assays with BNL CL.2 target cells, 2×10^5 BNL CL.2 cells were resuspended in antibiotic-free RP10, seeded in wells of 24-well plates, and allowed to adhere overnight. These cells were infected for 1 h with *L. monocytogenes* at a multiplicity of infection of three *L. monocytogenes* organisms per cell. Unattached bacteria were removed by three washes, and the cells were incubated for 16 h in RP10 plus 50 μg of gentamicin per ml to kill the extracellular bacteria. *L. monocytogenes*-infected and control cells were detached with trypsin-EDTA and labeled with ^{51}Cr. In some of the experiments, the p60 217-225 peptide or LLO 91-99 peptide was added at a final concentration of 1 nM. Spontaneous release in the absence of CD8 T cells was <15% in all experiments.

Adoptive transfer assay. *L. monocytogenes*-immune, polyclonal CD8 T cells were obtained from the spleens of mice infected for 7 days with an LD_{50} of 0.1. Spleen cells were depleted of CD4 T cells and injected in the indicated numbers for each experiment. CD8 T-cell lines were harvested on day 7 after restimulation and washed twice in phosphate-buffered saline (PBS) prior to intravenous injection. BDF1 and chimeric mice were infected intravenously with 5×10^5 *L. monocytogenes* organisms in 0.2 ml of PBS. Alb-Kb mice were infected with 5×10^4 *L. monocytogenes* organisms. Organs were harvested 72 h after infection, and the number of CFU per organ was determined as previously described (18). The detection limits were 50 CFU for the spleen and 100 CFU for the liver. Student's *t* test was employed in the analysis of results. Each group consisted of three animals. Each experiment was performed at least twice, and the results were pooled.

RESULTS

CD8 T-cell recognition of BM-derived macrophages results in antilisterial immunity. *L. monocytogenes* infects BM-derived macrophages and non-BM-derived hepatocytes in vivo and in vitro. To determine if CD8 T-cell recognition of *L. monocytogenes*-infected BM-derived macrophages results in antilisterial immunity in vivo, we constructed radiation BM chimeras where β-2 microglobulin gene knockout mice (β2M−/−, *H-2*^{b}) (31, 52), which express little surface MHC class I, were lethally irradiated and reconstituted with BM from BDF1 (*H-2*$^{b\times d}$) mice (BDF1→β2M−/−). In these mice, only BM-derived cells express surface MHC class I molecules. Chimeric mice were housed for 13 weeks to allow reconstitution with donor BM-derived cells before they were used as hosts in adoptive transfer assays. *L. monocytogenes*-immune CD8 T cells were prepared from B6 (*H-2*^{b}) mice after sublethal infection and were divided into groups of normal BDF1 mice,

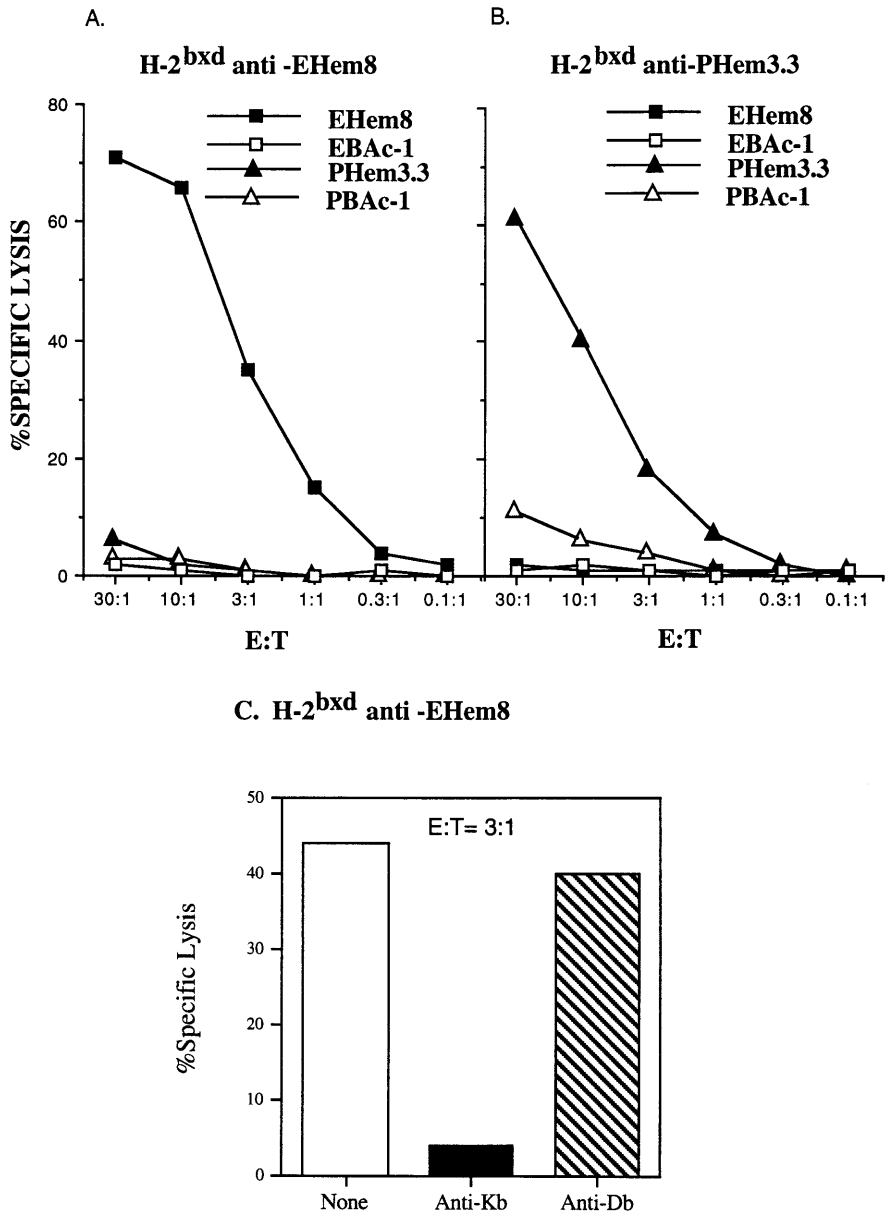

FIG. 3. LLO-specific CD8 T cells do not exhibit MHC class Ib-restricted lysis of LLO transfectants. Spleen cells from 7-day *L. monocytogenes*-infected (0.1 LD_{50}) BDF1 mice were restimulated in vitro with EHem8, an LLO transfectant of EL-4 (A), or PHem3.3, an LLO transfectant of P815 (B). Both CD8 T-cell lines were assayed for lytic activity against EHem8 and PHem3.3 as well as for recognition of the parental cells transfected with the vector without insert, EBAc-1 (EL-4) and PBAc-1 (P815) in a standard 4-h chromium release assay. (C) Recognition of EHem8 is blocked by anti-K^b MAb Y3 but not anti-D^b MAb 28-14-8. E:T, effector-cell-to-target-cell ratio.

BDF1→β2M−/− chimeras, and normal β2M−/− mice. These mice and uninjected controls were challenged with ~5 LD_{50} of virulent *L. monocytogenes*, and the degree of immunity transferred by CD8 T cells was estimated by comparing the numbers of CFU recovered in spleen and liver organ homogenates 3 days later. *L. monocytogenes*-immune CD8 T cells mediated significant antilisterial immunity in normal BDF1 and BDF1→β2M−/− chimeras, reducing the number of CFU in spleens (2 to 3 log 10) and livers (1 to 2 log 10), but failed to reduce the number of CFU in normal β2M−/− mice (Fig. 1). These data demonstrate that CD8 T-cell recognition of BM-derived macrophages results in antilisterial immunity in vivo. The levels of CFU reduction in normal BDF1 and BDF1→β2M−/− chimeras were similar, suggesting that BM-derived macrophages are major targets of CD8 T cells in adoptive transfer assays. These data also show that the chimeric mice exhibit functionally complete replacement of the BM-derived cells that are targets for *L. monocytogenes*-immune CD8 T cells.

CD8 T-cell-mediated immunity in P→F1 BM chimeras suggests recognition of *L. monocytogenes*-infected non-BM-derived target cells. The next experiments were designed to test the hypothesis that immune CD8 T cells recognize *L. monocytogenes*-infected non-BM-derived cells, such as hepatocytes, in a fashion that results in antilisterial immunity. BDF1 mice were lethally irradiated and reconstituted with BM from B6 mice or DBA/2 (*H-2*^{d}) mice (parent [P]→F1 chimeras). Thirteen weeks after reconstitution, the majority of BM-derived cells in

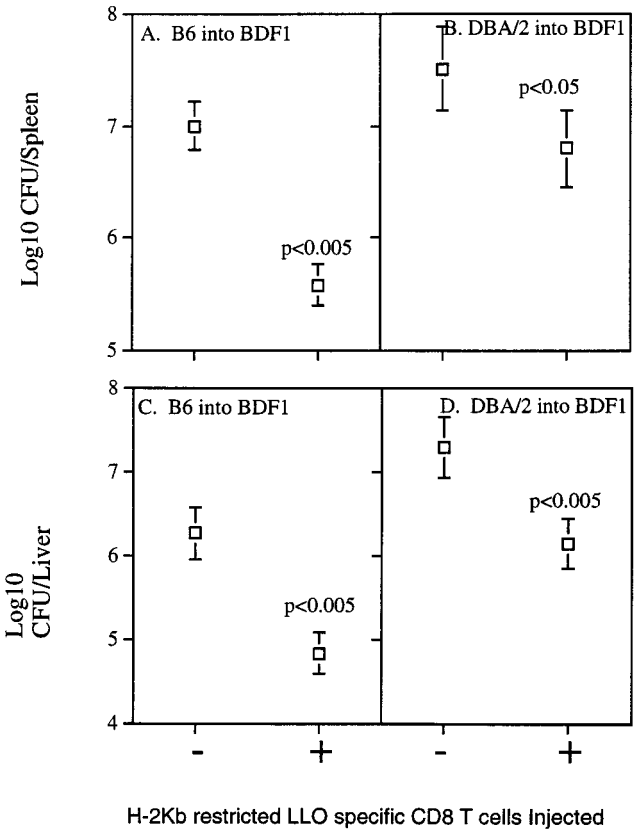

FIG. 4. CD8 T-cell-mediated antilisterial immunity in P→F1 BM chimeras is not dependent on MHC class Ib-presented antigens. Groups of six B6→BDF1 (A and C) and DBA/2→BDF1 (B and D) chimeras were infected with 5×10^5 *L. monocytogenes* organisms. Three mice in each group received 5×10^6 BDF1-derived CD8 T cells specific for LLO in the context of H-2K^b. Spleens (A and B) and livers (C and D) were harvested 72 h after infection, and the number of CFU per organ was determined. The results from two independent experiments were pooled and are expressed as the mean number of CFU per organ. Error bars indicate standard errors. Student's *t* test was used in the analysis of the results.

these chimeras expressed the MHC alleles of the BM donor (*H-2^b* or *H-2^d* [Table 1]) whereas non-BM-derived cells such as hepatocytes express both sets of alleles (*H-2$^{b\times d}$*). In these chimeric mice, adoptive transfer assays with *L. monocytogenes*-immune CD8 T cells from B6 or DBA/2 mice create two experimental situations. In the first situation, *L. monocytogenes*-immune CD8 T cells can recognize the majority of BM-derived macrophages and all non-BM-derived hepatocytes (B6 CD8 T cells and B6→BDF1 chimeras). In the second experimental situation, non-BM-derived cells express MHC alleles that are syngeneic with the *L. monocytogenes*-immune CD8 T cells, but the majority of BM-derived macrophages express a different MHC haplotype (B6 CD8 T cells and DBA/2→BDF1 chimeras). A reduction in the number of CFU in the latter situation would suggest that CD8 T-cell recognition of *L. monocytogenes*-infected non-BM-derived cells results in antilisterial immunity.

In support of the data presented in Fig. 1, transfer of *L. monocytogenes*-immune CD8 T cells syngeneic with BM-derived macrophages results in high levels of antilisterial immunity, reducing the number of CFU in the spleens (2.5 to 3 log 10) and livers (2 to 2.5 log 10) of B6→BDF1 and DBA/2→BDF1 chimeras (Fig. 2). Transfer of *L. monocytogenes*-immune CD8 T cells that were not syngeneic with the majority of BM-derived macrophages also resulted in significant reduction in the number of CFU in the spleens and livers of the chimeric mice. In both sets of chimeric mice, expression of CD8 T-cell-mediated antilisterial immunity, as estimated by CFU recovery, was more effective in the experimental situation where the CD8 T cells were syngeneic with BM- and non-BM-derived cells than in the situation where the majority of BM-derived cells were mismatched with the immune CD8 T cells. These experiments support the hypothesis that CD8 T-cell recognition of *L. monocytogenes*-infected non-BM-derived cells results in antilisterial immunity. However, the biology of CD8 T-cell responses to *L. monocytogenes*, where antigens may be presented by nonpolymorphic MHC class Ib molecules (4, 28, 32, 39) that are shared by these mouse strains (39), and experimental limitations in characterizing the degree of macrophage turnover in radiation BM chimeras could also account for these data.

Adoptive transfer of antilisterial immunity in P→F1 chimeras does not require recognition of MHC class Ib-restricted antigens. To determine whether immunity in P→F1 BM chimeras requires CD8 T-cell recognition of MHC class Ib-restricted *L. monocytogenes* antigens we derived *L. monocytogenes*-antigen-specific CD8 T-cell lines that are restricted by polymorphic MHC class I molecules. Transfection of a construct encoding the *L. monocytogenes* protein LLO into P815 (*H-2^d*) cells renders these cells targets of *L. monocytogenes*-immune CD8 T cells from *H-2^d* mice (38). CD8 T-cell lines specific for an H-2K^d-restricted peptide epitope, amino acids 91 to 99 of the LLO molecule, transfer immunity to *L. monocytogenes* infection in vivo (18). We have recently determined that transfection of the LLO molecule into *H-2^b*-expressing EL-4 cells renders these cells targets for *L. monocytogenes*-immune CD8 T cells from B6 mice and that B6-derived CD8 T-cell lines specific for LLO transfer immunity to *L. monocytogenes* infection in vivo (17a). This system allowed us to infect BDF1 mice and generate CD8 T-cell lines by restimulation with LLO-transfected P815 cells (PHem3.3) or LLO-transfected EL-4 cells (EHem8) and to test these CD8 T-cell lines for the ability to recognize LLO-derived peptides presented by nonpolymorphic MHC class Ib molecules. After several in vitro restimulations, LLO-specific CD8 T cells stimulated with LLO-transfected EL-4 cells specifically lyse EHem8 cells and not EL-4 cells transfected with the vector without insert (EBAc-1) (Fig. 3A). The same spleen cells restimulated with LLO-transfected P815 cells specifically lyse PHem3.3 cells and not P815 cells transfected with the vector without insert (PBAc-1) (Fig. 3B). Interestingly, neither CD8 T-cell line exhibits MHC class Ib-restricted recognition, which would be expected to reveal itself by the ability of each of these CD8 T-cell lines to lyse both of the LLO transfectants but not either control transfectant. Lysis of EHem8 cells is blocked by an antibody specific for the H-2K^b MHC class I molecule and thus exhibits classical MHC restriction (Fig. 3C).

The H-2K^b-restricted, LLO-specific CD8 T-cell line was assessed for the ability to mediate antilisterial immunity in P→F1 BM chimeras because the highest degree of chimerism was seen in PBL from the DBA/2→BDF1 mice (Table 1). Adoptive transfer of these cells into B6→BDF1 BM chimeras resulted in a significant reduction in the number of CFU in the spleen (1.5 log 10) and liver (1.3 log 10) (Fig. 4). To a lesser degree, these cells also mediated the reduction of CFU in the spleens and livers of (DBA/2→BDF1) BM chimeras. Thus, CFU reduction in P→F1 chimeras in which the majority of BM-derived cells are not syngeneic with the *L. monocytogenes*-immune CD8 T cells does not depend on MHC class Ib-restricted antigen presentation.

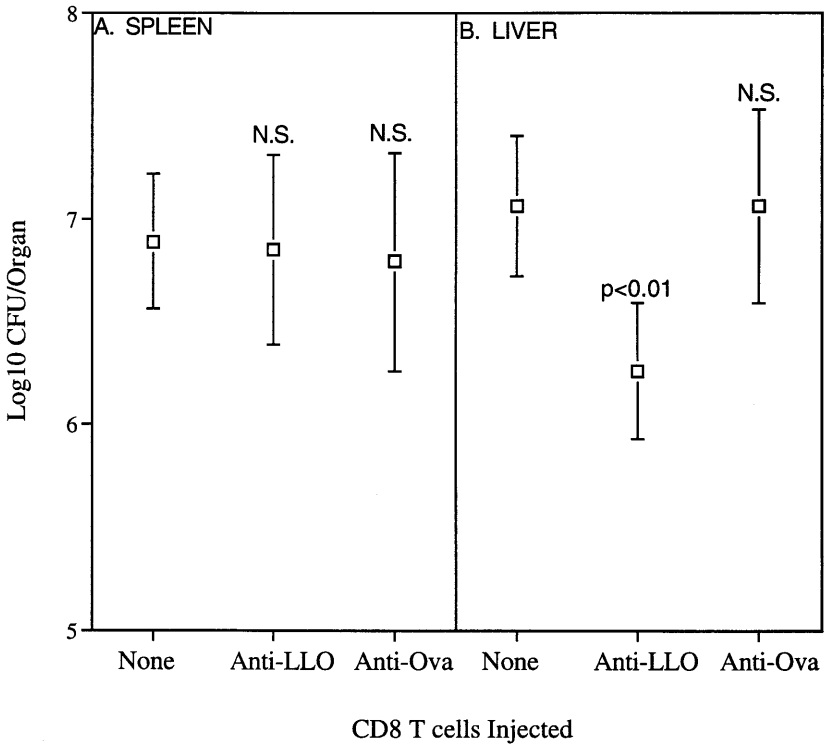

FIG. 5. *L. monocytogenes*-immune CD8 T cells recognize hepatocytes in vivo. Groups of three Alb-Kb mice were injected with 5×10^6 H-2K^b-restricted, LLO-specific CD8 T cells or 5×10^6 H-2K^b-restricted ovalbumin-specific CD8 T cells. These mice and uninjected controls were infected with 5×10^4 CFU of *L. monocytogenes* 1 h later. Spleens (A) and livers (B) were harvested 72 h after infection, and the number of CFU per organ was determined. The results from three independent experiments were pooled and are expressed as the mean number of CFU per organ. Error bars indicate standard errors. Student's *t* test was used in the analysis of the results. N.S., not significant.

CD8 T-cell recognition of hepatocytes results in antilisterial immunity. The degree of chimerism in the P→F1 mice was estimated by analysis of PBL MHC expression and does not rule out the possibility that significant numbers of host-derived macrophages survive the irradiation in specific tissue sites. As evidenced from our studies, tissue macrophages may provide excellent targets for *L. monocytogenes*-immune CD8 T cells. Experimental evidence suggests that splenic antigen-presenting cells, consisting of macrophages and dendritic cells, are essentially replaced by donor BM-derived antigen-presenting cells by 8 weeks after irradiation and BM reconstitution (33). The turnover of liver macrophages after irradiation and BM reconstitution has not been extensively studied and is confused by the heterogeneity of phenotypic markers expressed by these cells. In one study, liver macrophages from radiation BM chimeras were estimated to be 85% donor derived by 21 days after reconstitution, by electron microscopy combined with DNA analysis and determination of antigen-presenting capacity (40). To avoid the complications of incomplete macrophage turnover, we made use of Alb-Kb transgenic mice that exhibit hepatocyte-specific H-2K^b expression (17). Such mice express a functional H-2K^b molecule as evidenced by the peripheral tolerance to H-2K^b exhibited by these animals. Alb-Kb mice were used as hosts for adoptive transfer studies with H-2K^b-restricted LLO-specific CD8 T cells or an H-2K^b-restricted ovalbumin-specific CD8 T-cell clone, B3 (23). Adoptive transfer of the LLO-specific CD8 T-cell line into Alb-Kb mice prior to *L. monocytogenes* infection resulted in a significant reduction in the number of CFU in the liver compared with that in mice which received no CD8 T cells or those that received the ovalbumin-specific CD8 T cells (Fig. 5). There was no reduction in the number of CFU in the spleens of mice receiving the LLO-specific CD8 T cells, in line with the liver-specific expression of H-2K^b in the recipient mice. These data support the hypothesis that CD8 T-cell recognition of *L. monocytogenes*-infected hepatocytes is relevant to antilisterial immunity in vivo.

***L. monocytogenes*-specific CD8 T cells lyse Listeria-infected hepatocyte-like cells in vitro.** To our knowledge, CD8 T-cell-mediated lysis of *L. monocytogenes*-infected hepatocytes has not been demonstrated by in vitro experiments. We have addressed this issue using an embryonic hepatocyte line, of BALB/c (*H-2^d*) origin, BNL CL.2. This cell line has been shown to support *L. monocytogenes* replication (51) and has been studied as a model for the effects of cytokine treatment on the ability of hepatocytes to restrict *L. monocytogenes* replication in vitro (43, 48, 51). Together, these studies demonstrate that cytokine treatment does not promote high-level resistance of BNL CL.2 cells to *L. monocytogenes* replication compared with that of macrophages (48). Similar studies with isolated hepatocytes have yielded contrasting results regarding the antimicrobial activity of cytokine-activated hepatocytes (15, 43). Such studies do not rule out the possibility that lysis of infected hepatocytes by *L. monocytogenes*-immune CD8 T cells may be a relevant effector mechanism in specific immunity to *L. monocytogenes.*

Staining of BNL CL.2 cell for expression of H-2K^d reveals extremely low levels of expression which can be upregulated by IFN-γ treatment (data not shown). Despite this low level of expression, *L. monocytogenes*-infected BNL CL.2 cells are specifically lysed by CD8 T cells that recognize amino acids 217 to 225 of the *L. monocytogenes* p60 molecule (Fig. 6A) or amino

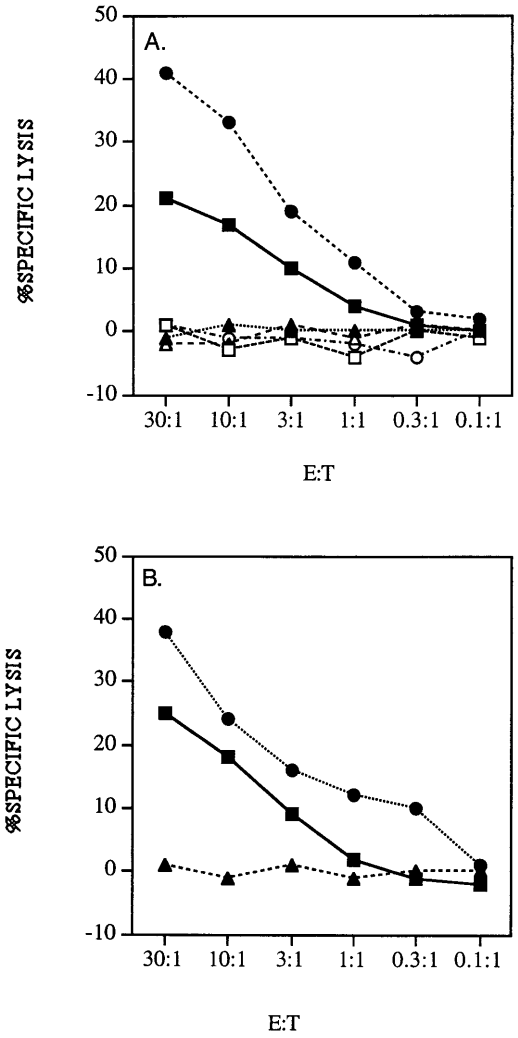

FIG. 6. *L. monocytogenes*-specific CD8 T-cell lysis of an infected hepatocyte cell line. BNL-CL.2 cells were infected with *L. monocytogenes* and used as targets in a standard ^{51}Cr release assay. (A) CD8 T cells specific for *L. monocytogenes* p60 217-225 (filled symbols) or influenza virus NP 149-157 (open symbols) were tested for lysis of uninfected BNL-CL.2 cells (triangles), *L. monocytogenes*-infected BNL-CL.2 cells (squares), or uninfected BNL-CL.2 cells in the presence of 1 nM p60 217-225 synthetic peptide (circles). (B) CD8 T cells specific for LLO 91-99 were tested for lysis of uninfected BNL-CL.2 (▲), *L. monocytogenes*-infected BNL-CL.2 cells (■), or uninfected BNL-CL.2 cells in the presence of 1 nM LLO 91-99 synthetic peptide (●). E:T, effector-cell-to-target-cell ratio.

acids 91 to 99 from the LLO molecule (Fig. 6B), both in the context of H-2K^d. *L. monocytogenes*-infected BNL CL.2 cells are not recognized by CD8 T cells specific for the influenza virus nucleoprotein (amino acid 149 to 157) peptide that is also presented by H-2K^d (Fig. 6A). p60- and LLO-specific CD8 T cells also recognize BNL CL.2 cells in the presence of the appropriate synthetic peptide representing p60 217-225 or LLO 91-99 but not BNL-CL.2 cells in the absence of peptide.

The low level of lysis observed in this experiment is consistent with the level of MHC expression by this hepatocyte-like cell line. IFN-γ treatment of BNL CL.2 cells prior to *L. monocytogenes* infection results in increased lysis (data not shown). In this study, *L. monocytogenes* replication was identical in IFN-γ-treated and untreated BNL CL.2 cells (data not shown) as reported by Wood et al. (51) and Rogers et al. (43). These studies demonstrate that *L. monocytogenes*-specific CD8 T cells can recognize and lyse an *L. monocytogenes*-infected hepatocyte line.

DISCUSSION

Our data clearly demonstrate that CD8 T-cell recognition of *L. monocytogenes*-infected BM-derived macrophages results in antilisterial immunity in adoptive transfer assays. *L. monocytogenes*-immune CD8 T cells consistently mediate the greatest antilisterial immunity when the experimental design allows recognition of BM-derived macrophages. Thus, CD8 T-cell recognition of *L. monocytogenes*-infected macrophages appears to be the primary interaction resulting in antilisterial immunity under adoptive transfer conditions.

These results are consistent with the prevailing model of *L. monocytogenes* infection in which the organisms are initially taken up by fixed phagocytes in the spleen and liver. A significant percentage of these organisms are rapidly eliminated (12), presumably by elimination in the phagolysosome (42). However, some *L. monocytogenes* organisms are able to escape from the phagocytic vesicle into the cytoplasm where they replicate and initiate the process of cell-cell movement that allows spread into nonphagocytes such as hepatocytes. In adoptive transfer assays or in vaccinated mice in which *L. monocytogenes*-immune CD8 T cells are present at the time of infection, recognition of *L. monocytogenes*-infected macrophages should occur relatively early in the infection process. We have recently demonstrated that CD8 T-cell-derived IFN-γ is not required for antilisterial immunity in adoptive transfer assays (19). Other studies demonstrate that CD8 T cells from perforin gene knockout mice are deficient in the ability to transfer antilisterial immunity (24). Although we did not address the issue of effector mechanisms in this study, the data from other studies suggest that CD8 T-cell-mediated lysis of *L. monocytogenes*-infected macrophages or hepatocytes may be an important component of specific immunity. *L. monocytogenes*-infected macrophage-like cells express high numbers of *L. monocytogenes*-derived peptide epitopes as early as 2 to 3 h postinfection in vitro (37). Thus, CD8 T-cell recognition and lysis of *L. monocytogenes*-infected macrophages may occur early after infection, prior to extensive replication and cell-cell spread, effectively converting the pathogenic intracellular infection to a low-grade extracellular infection. Our data demonstrating that CD8 T-cell recognition of BM-derived macrophages is the most effective route of antilisterial immunity are consistent with such a model.

Our data also suggest that CD8 T-cell recognition of *L. monocytogenes*-infected non-BM-derived cells occurs under adoptive transfer conditions. The identity of the non-BM-derived cells in the spleen capable of functioning as targets for *L. monocytogenes* specific CD8 T cells is unclear. However, the use of Alb-Kb mice allowed us to demonstrate antilisterial immunity in an experimental system in which expression of the restricting MHC molecule is absolutely liver specific by PCR analysis and in which significant H-2K^b protein expression is detectable only on hepatocytes (46). Significant macrophage expression of H-2K^b in these mice is unlikely since thymic deletion of H-2K^b-specific CD8 T cells does not occur in (anti-K^b T-cell receptor × Alb-Kb) double transgenic mice (46). The ability of *L. monocytogenes* to spread from BM-derived macrophages, which can be induced by cytokines to express high levels of antimicrobial activity, into hepatocytes, is thought to be an important phase in the pathogenesis of experimental listeriosis (26). In fact, the majority of *L. monocytogenes* is found within hepatocytes at 3 days after infection (14). The ability of hepatocytes to express antimicrobial activity is con-

troversial (15, 43), but they are clearly not as efficient at killing *L. monocytogenes* as activated BM-derived macrophages. Thus, the hepatocyte has been assumed to be a relatively safe haven for the organism during pathogenic infection. The data from our study suggest that CD8 T cells can recognize *L. monocytogenes*-infected hepatocytes in a fashion that results in the expression of antilisterial immunity. Evidence from other infectious disease models suggests that effective CD8 T-cell-mediated immunity directed at hepatocytes can be independent of cell-mediated lysis (16). The present study does not allow us to discriminate between the potential effector mechanisms in CD8 T-cell-mediated, hepatocyte-specific immunity to *L. monocytogenes*.

However, CD8 T-cell recognition of non-BM-derived cells or hepatocytes clearly results in less antilisterial immunity than does recognition of BM-derived macrophages. These results may simply reflect the level of MHC molecule expression on non-BM-derived cells or perhaps a differential ability to process and present antigens through the MHC class I pathway. Alternatively, *L. monocytogenes* gene expression may differ depending on the type of cell infected and thus impact the candidate antigens available for presentation.

Pathogenic infection with *L. monocytogenes* provides a classic model of immune system evasion in which the organism first evades extracellular defense mechanisms by entry into phagocytes. Next, the organism evades the microbicidal activities of macrophages, which are stimulated by CD4 T-cell and NK-cell-derived lymphokines, by escape into the cytoplasm and spread into nonphagocytic cells. However, entry of the organism into the cytoplasm of the infected cells results in activation of specific CD8 T cells which can potentially lyse the infected cells and expose the organism to the same extracellular defense mechanisms that were initially avoided. Our experiments demonstrate the potential of CD8 T cells to mediate antilisterial immunity by recognition of *L. monocytogenes*-infected macrophages and hepatocytes in vivo.

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