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Class Ia MHC-Deficient BALB/c Mice Generate CD8+ T Cell-Mediated Protective Immunity Against Listeria monocytogenes Infection

Sarah E. F. D’Orazio,† Dina G. Halme, † Hidde L. Ploegh, † and Michael N. Starnbach‡*

CD8+ T cells are required for protective immunity against intracellular pathogens such as Listeria monocytogenes. In this study, we used class Ia MHC-deficient mice, which have a severe reduction in circulating CD8+ T cells, to determine the protective capacity of class Ib MHC-restricted T cells during L. monocytogenes infection. The Kb−/−/Db−/− mutation was backcrossed onto a C.B10 (BALB/c congenic at H-2 locus with C57BL/10) background, because BALB/c mice are more susceptible to Listeria infection than other commonly studied mouse strains such as C57BL/6. C.B10 Kb−/−/Db−/− mice immunized with a sublethal dose of L. monocytogenes were fully protected against a subsequent lethal infection. Adoptive transfer of Listeria-immune splenocyte subsets into naive Kb−/−/p−/− mice indicated that CD8+ T cells were the major component of this protective immune response. A CD8+ T cell line isolated from the spleen of a Listeria-infected class Ia MHC-deficient mouse was shown to specifically recognize Listeria-infected cells in vitro, as determined by IFN-γ secretion and cytotoxicity assays. Adoptive transfer of this T cell line alone resulted in significant protection against L. monocytogenes challenge. These results suggest that even a limited number of class Ib MHC-restricted T cells are sufficient to generate the rapid recall response required for protection against secondary infection with L. monocytogenes. The Journal of Immunology, 2003, 171: 291–298.

Listeria monocytogenes is a Gram-positive bacterium with a unique intracellular life cycle that allows it to spread from cell to cell without being exposed to the extracellular environment (1). Within 15 min of i.v. inoculation of a mouse, 90% of the bacteria resides in the liver, and most of the remaining inoculum can be found in the spleen (2, 3). During primary infection of mice, Listeria grow in a largely unrestricted fashion for 1–2 days, and then the exponential growth of bacteria is inhibited by innate immune mechanisms. Neutrophils, NK cells, and activated macrophages are critical for early control of Listeria infection. These cells migrate to the spleen and liver during the first 3–4 days of infection; however, they cannot completely eliminate the bacteria (4).

Specific adaptive immunity in the form of CD8+ CTL is necessary for complete clearance of L. monocytogenes. Although CTL appear to recognize Listeria-derived peptide/MHC complexes within the first 24 h of infection (5), their activation, differentiation, and proliferation require several days before T cells are found in significant numbers at sites of infection. Because Listeria survive and multiply within host cells, CTL presumably act by lysing infected cells, releasing the bacteria for subsequent phagocytosis by neutrophils and activated macrophages. Within 10–14 days after a primary infection, organisms are completely cleared from the spleens and livers of BALB/c mice. As a result of this infection, these animals have long-lasting, sterilizing immunity against rechallenge with L. monocytogenes, even when given a inoculum of bacteria that would be lethal to a naive mouse. During subsequent infections, Listeria growth also peaks within the first 1–2 days. However, on the third day after infection, memory T cells begin to appear in the spleen and liver and the bacterial load is completely cleared within 4–5 days (3, 6). No significant role for humoral immunity has been established.

Listeria-specific T cells restricted by both class Ia (K, D, L) and class Ib MHC (Q, T, M) molecules are activated during infection. The Kd molecule presents at least five distinct peptide epitopes to CTL that are derived from three secreted Listeria proteins: listeriolysin O (LLO)91–99, p6091–225, p6094–52, p6047–64, and metalloprotease64–92 (7–11). Adoptive transfer studies and vaccination strategies designed to elicit a single epitope-specific CTL response have shown that T cells specific for either LLO91–99 or p6091–225 can each provide a limited degree of protection against infection with L. monocytogenes (12–14). However, CTL specific for only one Listeria-derived peptide cannot provide the same level of protection as generated during a natural sublethal infection, and it is not known how many different CTL specificities must be activated to achieve full protection.

The class Ib MHC group includes both the H-2-encoded Q, T, and M molecules and proteins encoded outside the MHC locus, such as CD1 and FcRn. Although the number of potential Ag-presenting molecules in the class Ib MHC group far exceeds the number of class Ia MHC molecules, much less is known about the class Ib MHC group. Class Ib MHC molecules generally have lower cell surface expression levels and may be expressed in only a limited number of tissues (15). To date, the only class Ib MHC molecules that have been shown to present Listeria-derived Ags to CTL are M3 and Qa-1b. M3 presents at least three L. monocytogenes-derived peptides to CD8+ T cells, each of which contains an

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3 Abbreviations used in this paper: LLO, listeriolysin O; β2m, β2-microglobulin; BMMΦ, bone marrow-derived macrophage; ICCS, intracellular cytokine staining.
N-terminal formyl methionine: tMIVIL, tMIGWII, and tMIVTLF (16–18). Adaptive transfer of a tMIGWII-specific CTL line into naïve mice before lethal challenge with L. monocytogenes reduced the bacterial load by 10- to 100-fold, suggesting that these T cells can participate in protective immunity against Listeria (19). It is not yet clear whether tMIVIL- or tMIVTLF-specific CTL play any role in clearance of L. monocytogenes infections. Qa-1 restricted CTL have also been detected after Listeria infection, but the antigen recognized by these T cells is not known (20).

Challenge studies using β2-microglobulin (β2m)-deficient mice underscore the importance of CD8+ T cells for clearing L. monocytogenes infections. β2m is required for expression and stability of both class Ia and Ib MHC molecules on the cell surface during thymic selection. In the absence of β2m, functional CD8+ T cells do not mature. As expected, C57BL/6 β2m−/− mice were able to efficiently resolve the infection (21, 22). Kb−/−/Dβ−/− mice, which express no class Ia MHC heavy chains but have normal levels of β2m and class Ib MHC molecules, have greatly reduced numbers of CD8+ T cells in the blood or spleen (23). However, this minor population of CTL appears to contain fully functional effector cells that are capable of lysing specific target cells and secreting cytokines. Thus Kβ−/−/Dβ−/− mice provide a unique model system to explore the role of class Ib MHC-restricted CTL in protective immunity against intracellular bacterial pathogens.

In this study, we backcrossed the Kβ−/−/Dβ−/− mutation onto a C.B10 (BALB/c congenic with C57BL/10 mouse at H-2 locus) background to investigate the contribution of class Ia MHC-restricted T cells using the L. monocytogenes systemic mouse model of infection. We show that the few CD8+ cells T cells present in these mice proliferate extensively during a primary Listeria infection, respond much more rapidly during secondary infection, and are the major component of acquired immunity against lethal challenge with L. monocytogenes.

Materials and Methods

Mice

BALB/CByJl and C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C.B10.H2b/"Il2icd/J (herein referred to as C.B10) mice were originally obtained from The Jackson Laboratory and then bred in a specific pathogen-free barrier facility at Harvard Medical School. C.B10 is a congenic strain identical to BALB/c (H-2b) at all loci except for the H-2 locus, which was derived from a C57BL/10 (H-2b) mouse. They express K β and D β on the surface of all nucleated cells; the H-2 locus of this mouse does not contain an L gene (24). K β−/−/D β−/− mice, generated as previously described (23), were backcrossed onto both the C57BL/6 and the C.B10 strain six times. At the third (N3) and sixth (N6) backcross generation, heterozygotes were brother-sister mated. Homozygotes were identified from the progeny of these matings by screening for wild-type or mutant K β and D β alleles (by PCR of tail DNA) and the presence or absence of K β and D β expression on the surface of peripheral blood cells. N3 and N6 homozygotes (−/− and +/+ ) were brother-sister mated, and the progeny were used for the experiments described in this text. As expected, the K β−/−/D β−/− mice expressed I-A β, but not K β, D β, I-A α, K β, D β, or L α as determined by flow cytometric analysis of peripheral blood cells.

Cell culture

The EL-4 thymoma (H-2b), J774 monocyte-macrophage (H-2b), and L929 fibroblast cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in 7% CO2 in a medium (RP-10) consisting of RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with t-glutamine, HEPES, 50 μM 2-ME, and 10% FCS. Bone marrow-derived macrophages (BMMφ) were harvested from femurs of mice, plated in DMEM (Life Technologies) supplemented with 20% FCS and 20% L929 cell supernatant, and fed every 3–4 days. To generate T cell lines, splenocytes from infected mice were stimulated weekly on syngeneic BMMφ that were infected with Listeria for 4 h and then irradiated (2000 rad). Primary T cell cultures were maintained for 14 days in RP-10 and were then grown in RP-10 supplemented with supernatant from Con A-stimulated rat splenocytes and 50 mM α-methyl mannoside. The LLOp1-9-specific CTL clone 479-2 was generously provided by J. Harty (University of Iowa, Iowa City, IA) and was maintained by weekly stimulation on irradiated BALB/c spleen cells coated with synthetic LLOp1-9 peptide.

Listeria monocytogenes infection

For in vitro infections, L. monocytogenes was grown to early log phase in brain-heart infusion broth (Difco, Detroit, MI), washed twice in PBS, and added to cells at a multiplicity of infection of 2. Contact between the cells and the bacteria was facilitated by centrifugation of the tissue culture dishes at 800 × g for 10 min at 35°C. After 1 h of incubation at 37°C and 7% CO2, the cells were washed three times with warm PBS and then treated with RP-10 containing 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO) to kill extracellular bacteria. For infection of mice, L. monocytogenes was grown to stationary phase, aliquoted, titrated, and stored at –80°C. Before injection, bacteria were thawed on ice, grown to early exponential phase in brain-heart infusion broth, and diluted in PBS. The LD50 of L. monocytogenes strain 10403s is ≈ 1 × 104 CFU in BALB/c mice and ≈ 2 × 104 CFU in C57BL/6 mice. For primary infections, a dose equal to 0.1–0.2 LD50 was suspended in 200 μl of PBS and injected into the tail vein of 6- to 12-wk-old mice. Secondary infections (5–10 LD50) were given by i.v. inoculation 3–5 wk after the primary infection.

Abs/flow cytometry

The purified and/or fluorescently conjugated Abs were purchased from BD Pharmingen (San Diego, CA); anti-CD8α (53-67-2); anti-CD8β (53-85-8); anti-TCRβ (H57-597); anti-CD4 (GK1-5); anti-H-2Kb (AF6-86-55); anti-H-2Dk (SFI-1.1); anti-H-2Dd (KH95); anti-H-2Dd (34-2-12); anti-H-2Ld (24-14-8); anti-I-Ad (AF6-120.1); anti-I-Aβ (AMS-32.1); anti-IFN-γ (R4-6A2, purified and XM1G12, conjugated); and rat IgG1 (R3-34). Cells were suspended in flow cytometry buffer consisting of 0.5% BSA in PBS. Cells were then washed twice with flow cytometry buffer. Fluorescence intensities were measured using a FACScan flow cytometry BD (Biosciences, San Jose, CA), and analysis was performed using CellQuest software (BD Biosciences). Dead cells and monocytes were excluded using forward and side scatter gating. Typically, 10,000 events were collected for murine peripheral blood lymphoproteforming, and 50,000 events were collected for intracellular cytokine staining (ICC). Adapted transfer experiments

Donor female C.B10 Kβ−/−/Dβ−/− mice were immunized with ~1000 CFU of L. monocytogenes (immune groups) or PBS (naive group). Fourteen days later, the spleens of these mice were harvested and pooled in groups of two, and single-cell suspensions were prepared. Mice were lysed with Tris-buffered NH4 Cl, pH 7.2. Male C.B10 Kβ−/−/Dβ−/− recipient mice (groups of four) were each given the equivalent of one-half of a spleen consisting of naive splenocytes, immune splenocytes (no depletion), CD4+ depleted immune splenocytes, or CD8+ depleted immune splenocytes. T cell line S169.8 was split 2-fold 4 days after restimulation and collected for transfer 3 days later. All donor cells were washed twice with PBS and injected i.v. Recipient mice were challenged with 3 × 104 L. monocytogenes 15–60 min after injection of the T cells. Mice were sacrificed 3 or 4 days after infection, and spleens and livers were harvested, homogenized, and diluted in 1% Nonidet P-40 and plated on tryptic soy agar (Difco) containing 10 μg/ml streptomycin (Sigma-Aldrich) to determine the total number of CFU per organ.

Depletion of T cells using Ab-coated magnetic beads

CD4+ and/or CD8+ T cell subsets were depleted from splenocyte suspensions or T cell cultures using mAb-coated magnetic Dynabeads (Dynal Biotech, Lake Success, NY). The extent of CD4+ or CD8+ T cell depletion in each preparation was assessed by staining with PE-conjugated anti-CD4 and APC-conjugated anti-CD8 monoclonal Abs as described above. In two separate experiments, the range of T cell depletion from splenocyte suspensions was 93–97% for CD4+ cells and 42–95% for CD8+ cells.

ELISPOT assay

The frequency of tMIVIL-, tMIGWII-, and tMIVTLF-specific CTL in line S169.8 was determined by ELISPOT assay as described previously (25). Briefly, 96-well filtration plates (0.45-μm pore size cellulose ester membrane; Millipore, Bedford, MA) were coated with 10 μg/ml rat anti-mouse
IFN-γ Ab and then blocked with medium containing 5% FCS. Dilutions of T cells were incubated for 24 h at 37°C and 7% CO₂ in CT medium with either EL-4 cells or EL-4 cells treated with 1 μM synthetic peptide for 1 h (1 × 10⁵ per well). N-Formylated peptides were purchased from Biosynthesis (Lewisville, TX). The plates were then washed with PBS containing 0.25% Tween 20, and any remaining cells were lysed with distilled water. After incubation with a biotinylated rat anti-mouse IFN-γ Ab (XMG1.2; BD PharMingen), the plates were washed with PBS containing 0.25% Tween 20 and incubated with streptavidin-labeled peroxidase (BD PharMingen) in PBS plus 5% FCS for 1 h at room temperature. Plates were developed by adding 3,3′-diaminobenzidine tetrahydrochloride dihydrate (Bio-Rad, Melville, NY) in Tris buffer plus hydrogen peroxide for 30 min at room temperature, and spots were detected on the membranes with the aid of a dissecting microscope. Each spot represents an area in which a single CTL recognized its cognate Ag and was stimulated to locally secrete IFN-γ. The number of Ag-specific cells was determined by subtracting any spots observed for EL-4 cells alone from the number of spots observed for peptide-coated EL-4 cells.

**ICCS**

ICCS was performed using the CytoTox/Cytoperm Plus (with GolgiPlug) kit (BD PharMingen). Briefly, BMMΦ (6 × 10⁶well) were plated in 24-well dishes overnight in antibiotic-free medium and then infected for 4 h as described above. T cells were harvested 10 days after restimulation and were added (1 × 10⁷/well) to the BMMΦ in the presence of 1 μl/ml GolgiPlug. After 6 h of incubation at 37°C and 7% CO₂, the cells were harvested from each well, washed twice in FACS buffer, and incubated with Abs directed against the FcγRIII (Fc Block; BD PharMingen, diluted 1/100), FITC-conjugated anti-TCR, and APC-conjugated anti-CD8 for 30 min on ice. Stained cells were washed twice with FACS buffer and then fixed by incubating in 250 μl of Cytofix for 20 min on ice. Fixed cells were washed twice in FACS buffer and left overnight at 4°C in the dark. The following day, the cells were centrifuged, permeabilized by incubating in 250 μl CytoPerm for 30 min on ice, and washed twice in permeabilization/wash buffer. After staining with either PE-conjugated anti-IFN-γ Ab or PE-conjugated rat IgG1 for 30 min on ice, the cells were washed twice in permeabilization/wash buffer and resuspended in 400 μl of FACS buffer before flow cytometric analysis.

**Cytotoxicity assay**

Target cells were infected with *L. monocytogenes* as described above for a total of 4 h, harvested, and then incubated with sodium [51 Cr]chromate for 1 h at 37°C and 7% CO₂. The cells were washed three times with RPMI 1640 and resuspended in 400 μl of FACS buffer before flow cytometric analysis. The cytotoxicity activity of the T cells was evaluated by measuring ⁵¹ Cr release in the supernatant on a Wallac (Wallac, Gaithersburg, MD) 1470 Wizard gamma counter. Percent specific lysis was calculated using the formula: % specific lysis = 100 × [(release by T cells – spontaneous release) / (maximum release – spontaneous release)].

**Results**

**Generation of C.B10 (BALB/c congenic) class Ia MHC-deficient mice**

We backcrossed the K<sup>b</sup>⁻/⁻D<sup>b</sup>⁻/⁻ mutation onto both BALB/c and C57BL/6 strain backgrounds to generate class Ia MHC-deficient mice that could be used to investigate the role of class Ib MHC-restricted T cells in the clearance of intracellular bacterial pathogens such as *L. monocytogenes*. The K<sup>b</sup>⁻/⁻D<sup>b</sup>⁻/⁻ mutation was generated in a predominantly H-2<sup>d</sup> strain background (23); thus, backcrossing onto C57BL/6 mice was a simple process that only required screening for the presence of K<sup>b</sup> and D<sup>b</sup> alleles and the absence of wild-type alleles (see Materials and Methods). However, BALB/c mice encode a functional L gene product and Q, T, and M molecules that may or may not be same alleles found in an H-2<sup>d</sup> locus. Therefore, to simplify the screening process at each backcross generation, we elected to use C.B10 mice, a BALB/c-congenic strain identical with BALB/c (H-2<sup>d</sup>) at all loci except for the H-2 locus, which was derived from a C57BL/10 (H-2<sup>b</sup>) mouse.

Because there were no previously published reports describing *Listeria* infection of C.B10 mice, we performed a series of experiments to verify that C.B10 mice are comparable to BALB/c mice in their innate susceptibility to *L. monocytogenes*. The kinetics of primary sublethal infection in each of these mouse strains is shown in Fig. 1A. In both C.B10 and BALB/c mice, the bacterial load peaked at ~4 days postinfection. During the next few days, the number of organisms in both the spleen and the liver slowly decreased. By 14 days after infection, the bacteria were completely cleared at each of the time points indicated. Spleens and livers were harvested, and homogenized, and dilutions were plated to determine the total number of CFU per organ. — — —, Limit of detection for this assay (50 CFU). Average values ± SD are given. B, Fifteen C.B10 and BALB/c mice were challenged with 2 × 10⁷ CFU *L. monocytogenes* and monitored for survival during a 5-day period. Time of death was recorded for each mouse, and the number of survivors found each day postinfection is indicated as a percent of the total number of mice in each group. C, Groups of four mice were immunized with either 1 × 10⁵ *L. monocytogenes* (immune mice) or PBS (naive mice). All mice were challenged 17 days later with a lethal dose of *Listeria* (~10⁷ LD₅₀). Three days postinfection, the total CFU per liver (■) or spleen (□) was determined. Average values ± SD are shown.

![FIGURE 1. C.B10 and BALB/c mice are equally susceptible to *L. monocytogenes* infection. A, Mice were infected with 2 × 10⁷ CFU of *L. monocytogenes*. Groups of three mice were sacrificed at each of the time points indicated. Spleens and livers were harvested, and homogenized, and dilutions were plated to determine the total number of CFU per organ. — — —, Limit of detection for this assay (50 CFU). Average values ± SD are given. B, Fifteen C.B10 and BALB/c mice were challenged with 2 × 10⁷ CFU *L. monocytogenes* and monitored for survival during a 5-day period. Time of death was recorded for each mouse, and the number of survivors found each day postinfection is indicated as a percent of the total number of mice in each group. C, Groups of four mice were immunized with either 1 × 10⁵ *L. monocytogenes* (immune mice) or PBS (naive mice). All mice were challenged 17 days later with a lethal dose of *Listeria* (~10⁷ LD₅₀). Three days postinfection, the total CFU per liver (■) or spleen (□) was determined. Average values ± SD are shown.](http://www.jimmunol.org/Downloadedfrom)
cleared from these mice. As shown in Fig. 1B, similar survival curves were observed when C.B10 or BALB/c mice were infected with a lethal dose of *Listeria*, with all mice succumbing within 4 to 5 days. In addition, we showed that as a result of primary sublethal infection, C.B10 mice develop an enhanced recall response against subsequent lethal challenge with *L. monocytogenes* (Fig. 1C). A significant degree of protection was observed when comparing the number of organisms present in naive or immune C.B10 and BALB/c mice after secondary infection. These results suggest that C.B10 mice have the same natural susceptibility to *Listeria* infection and the same ability to induce protective acquired immunity as BALB/c mice.

The role of CD8$^+$ T cells has been explored extensively in both BALB/c and C57BL/6 mice during the past few decades. However, susceptible mouse strains such as BALB/c have a slower clearance rate and a lower LD$_{50}$ for *Listeria* than resistant strains such as C57BL/6. To confirm that C57BL/6 K$^{b/-}$D$^{b/-}$ mice were also naturally more resistant to i.v. *Listeria* infection than C.B10 K$^{b/-}$D$^{b/-}$ mice, we challenged both strains of mice with $8 \times 10^7$ CFU of *L. monocytogenes* (Fig. 2). Twenty-four hours postinfection, all mice had significant bacterial loads in the spleen and liver. However, 2 days later, the C57BL/6 K$^{b/-}$D$^{b/-}$ mice already had a reduced bacterial burden, whereas the number of *Listeria* remained high in the C.B10 K$^{b/-}$D$^{b/-}$ mice (Fig. 2). Because the bacterial load was higher in the C.B10 K$^{b/-}$D$^{b/-}$ mice, we chose to study this mouse model of *Listeria* infection to fully appreciate the role of CD8$^+$ T cells in the complete clearance of an intracellular pathogen. Although Ag-specific T cells are activated early during an infection, it is generally believed that migration of CTL to sites of infection requires several days of differentiation and proliferation. Therefore, in the C57BL/6 K$^{b/-}$D$^{b/-}$ mice, components of innate immunity such as neutrophils, NK cells, and activated macrophages were most likely responsible for clearing *Listeria* within the first 3 days of infection.

Protective CD8$^+$ T cell immunity can be generated in class Ia MHC-deficient mice

CD8$^+$ memory T cells primed during initial infection are thought to be absolutely required for protection against subsequent challenges with *L. monocytogenes*. We immunized C.B10 K$^{b/+}$D$^{b/+}$ or K$^{b/-}$D$^{b/-}$ mice with a sublethal dose of *Listeria* to determine whether the few CD8$^+$ T cells present in the class Ia MHC-deficient mice could be sufficiently stimulated to provide protective immunity. Control groups of mice received i.v. injections of PBS. Three weeks later, all mice were challenged with a lethal dose of *L. monocytogenes*. Three days after infection, naive mice had very high numbers of *Listeria* in their spleens and livers (Fig. 3). These mice were moribund and would have succumbed to infection within 24 h if they had not been sacrificed. However, both groups of immunized mice appeared healthy and had at least 3 orders of magnitude fewer bacteria present in their spleen and liver (Fig. 3). These findings suggest that protective immunity was generated in class Ia MHC-deficient mice. This recall response presumably consisted of memory T cells capable of responding to secondary infection much more rapidly than during a primary infection.

Previous studies using BALB/c mice have shown that only the CD8$^+$ T cells in the spleen are required to passively transfer *Listeria*-specific immunity to naive mice (26, 27). To confirm that the acquired immunity we observed in K$^{b/-}$D$^{b/-}$ mice was mediated by CD8$^+$ T cells, we transferred immune splenocytes depleted of various T cell subsets into naive K$^{b/-}$D$^{b/-}$ mice and then challenged the mice with a lethal dose of *Listeria*. The immune splenocytes were harvested from mice infected 2 weeks earlier with a sublethal dose of *L. monocytogenes*. This cell population should therefore include mainly memory T cells, not primary effector T cells. Adoptive transfer of immune splenocytes afforded a significant level of protection (Fig. 4), whereas transfer of naive splenocytes resulted in death of the animals within 4 days. Depletion of CD4$^+$ T cells had no significant effect on the level of protection observed. However, depletion of CD8$^+$ T cells resulted in a greatly increased bacterial load (3 orders of magnitude) in the livers of C.B10 K$^{b/-}$D$^{b/-}$ mice (Fig. 4). Taken together, these data suggest that during a primary *Listeria* infection class Ib MHC-restricted CD8$^+$ T cells are primed that provide protective immunity against subsequent lethal challenge with *L. monocytogenes*.

Expansion of CD8$^+$ T cells in class Ia MHC-deficient mice

Many different cell types in the spleen are capable of responding to *Listeria* infection, and the resulting inflammation causes significant splenomegaly, particularly during primary infection when the

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**FIGURE 2.** *L. monocytogenes* is cleared more quickly from the spleens and livers of C57BL/6 K$^{b/-}$D$^{b/-}$ mice than from C.B10 K$^{b/-}$D$^{b/-}$ mice. Groups of four K$^{b/-}$D$^{b/-}$ mice backcrossed six times onto either the C57BL/6 (■) or C.B10 (□) strain background were infected i.v. with $\sim 8 \times 10^3$ *L. monocytogenes*. Spleens and livers were harvested at the indicated time points, and the total number of CFU was determined for each organ. Average values ± SD are shown. *, $p < 0.05$; **, $p < 0.02$ as determined by Student’s $t$ test.

**FIGURE 3.** Class Ia MHC-deficient mice generate protective immunity against secondary lethal challenge with *L. monocytogenes*. C.B10 K$^{b/-}$D$^{b/-}$ mice (□) or wild-type C.B10 control mice (■) were immunized with $1 \times 10^8$ CFU of *L. monocytogenes* (immune group) or PBS (naive) i.v. Three weeks later, all mice were challenged with $6 \times 10^6$ CFU of *L. monocytogenes*. Three days later, the mice were sacrificed, and spleens and livers were harvested aseptically, homogenized, diluted, and plated to determine the total number of CFU per spleen or liver. Each square represents the number of bacteria found in an individual mouse; horizontal bars indicate average values for each group. Representative data from one of three independent experiments are shown.
bacterial load is higher (6). CD8+ T cell expansion occurs in conjunction with the proliferation of other splenocytes; thus, a net increase in the percentage of CD8- cells found in the spleen is not usually observed. However, a direct comparison of the number of T cells and the number of total splenocytes found during Listeria infection clearly indicated that CD8+ cells were proliferating to a greater extent in Kb+/Db−/− mice than in Kb+/Db+/+ mice (Fig. 5A). In Kb+/Db−/− mice, the total number of splenocytes increased ~2-fold during primary infection, but there was a 5-fold increase in CD8+ cells. During this same time period, CD4+ cells increased only 2-fold, corresponding to the overall 2-fold increase in total splenocytes. In wild-type mice, the increase in both CD4+ and CD8+ cells was proportional to the total increase in splenocytes observed 5–7 days after infection. Similar kinetics of T cell expansion was seen after secondary infection with L. monocytogenes (Fig. 5B). No significant elevation of total splenocytes was seen in wild-type or class Ia MHC-deficient mice 3–6 days after secondary Listeria challenge. However, the number of CD8+ cells in Kb+/−/Db−/− mice increased >4-fold by day 6 after infection. This suggests that a significant portion of the class Ib MHC-restricted CD8+ T cells present in Kb+/−/Db−/− mice recognized Listeria-specific Ags and proliferated in response to this interaction.

Isolation of a Listeria-specific, class Ib MHC-restricted CD8+ T cell line

To begin to identify the specific CD8+ T cell populations responsible for protection in the class Ia MHC-deficient mouse model of listeriosis, we attempted to isolate CD8+ T cell lines from the spleens of Listeria-infected Kb+/−/Db−/− mice. Two weeks after a sublethal infection, splenocytes were harvested and stimulated in vitro with irradiated, syngeneic, Listeria-infected BMMφ. The resulting T cell line, S169, was restimulated weekly using the same protocol. Analysis of cell surface markers by flow cytometry indicated that line S169 was a heterogeneous mixture of ~61% CD8+ T cells and 36% CD4+ T cells (Fig. 6). To enrich for CD8+ T cells, line S169 was depleted of CD4+ T cells using mAb-coated magnetic beads and then restimulated as described above. The resulting T cell line was designated S169.8 and was shown to be 93% CD8+ (Fig. 6).

FIGURE 4. Adoptive transfer of CD8+ T cells from immune class Ia MHC-deficient mice is required to protect naive mice from lethal challenge with L. monocytogenes. Groups of four Kb+/−/Db−/− mice were given splenocytes (0.5 spleen equivalent per mouse i.v.) prepared from Kb+/−/Db−/− immune mice infected 14 days earlier with 1 × 10⁵ CFU of L. monocytogenes. Splenocytes were depleted of various T cell subsets using mAbs attached to magnetic beads. One hour later, the recipient mice were infected with 3 LD₅₀ of L. monocytogenes. Four days postinfection, the mice were sacrificed, and the total number of CFU per liver was determined. Average values (±SD) from one of two separate experiments are shown. †, Organs were from mice that were found dead 4 days postinfection; *, p < 0.05 compared with immune splenocytes as determined by Student’s t test.

FIGURE 5. CD8+ T cell expansion in the spleen after primary or secondary L. monocytogenes infection is disproportionate in Kb+/−/Db−/− mice. Splenocytes obtained from groups of three mice at each of the indicated time points were stained with FITC-conjugated anti-CD8 or PE-conjugated anti-CD4 and analyzed by flow cytometry. The total number of CD4+ or CD8+ cells per spleen was calculated from the percentages obtained by FACS analysis. A, Mice were given either PBS (naive group) or 2 × 10⁶ CFU of L. monocytogenes. Data represent the average increase for each population of cells compared to the average number of cells present in naive spleens. B, Mice were infected with 2 × 10⁶ CFU of L. monocytogenes. After 17 days, some mice received PBS (immune, uninfected group), and the rest were challenged with 1.3 × 10⁶ CFU of L. monocytogenes. Data represent the average increase in cell number compared to the number of cells present in immune uninfected spleens. Data from one of two separate experiments are shown in each panel.

FIGURE 6. Line S169.8 is enriched for CD8+ T cells. Six days after in vitro restimulation, lines S169 and S169.8 were triple-stained with anti-TCR-β, anti-CD4, and anti-CD8 mAbs as described in Materials and Methods. TCR-β+ cells were gated and analyzed by FACS for cell surface expression of CD4 and CD8 as shown in the dot plots. Numbers in the upper left and lower right quadrants indicate the percentage of CD8 single-positive and CD4 single-positive cells, respectively.
Effector function of line S169.8 was assessed by measuring IFN-γ secretion and cytotoxicity. More than 80% of S169.8 T cells expressed high levels of IFN-γ 6 h after exposure to Listeria-infected BALB/c BMMφ as determined by intracellular cytokine staining (Fig. 7A). S169.8 T cells exposed to uninfected macrophages did not secrete IFN-γ. Listeria-infected or uninfected J774 (H-2d) macrophage-like cells were also used as targets in a chromium release assay to assess the lytic capacity of line S169.8. Significantly greater lysis of Listeria-infected J774 cells was observed compared with lysis of uninfected cells (Fig. 7B). A control cell line (479-2), which recognizes LLO93–99 peptide in the context of K, showed less specific lysis of infected J774 cells than did line S169.8. Similar results were obtained when primary BMMφ from either BALB/c mice or C.B10 K b mice were used as target cells in chromium release assays (data not shown).

To determine whether line S169.8 recognized one of the three known Listeria-derived peptide epitopes that bind to M3 molecules, we quantified fMIVIL, fMIGWI, and fMIVTLF reactivities by ELISPOT analysis. No fMIVIL- or fMIVTLF-specific IFN-γ-secreting cells were detected in line S169.8 (Fig. 7C). A small number (~0.4%) of fMIGWII-specific T cells were observed; however, the dominant Ag recognized by line S169.8 did not appear to be one of these three formylated peptides. Intracellular cytokine staining was used to verify these results and revealed that 0.01% of the cells recognized either fMIVIL or fMIVTLF peptide and 0.11% of the cells recognized fMIGWII (data not shown).

**FIGURE 7.** T cell line S169.8 specifically recognizes a L. monocytogenes-derived Ag. A, S169.8 cells were incubated with either uninfected BALB/c BMMφ (unstimulated) or BALB/c BMMφ that had been previously infected with L. monocytogenes for 4 h (Lm-BMMφ). IFN-γ secretion was assayed by ICCS. Dot plots shown are gated on TCR-β cells. Numbers in the upper right quadrants represent the percent of CD8 T cells that secreted IFN-γ in response to the stimulus indicated above each plot. B, Uninfected (∆, ○) or L. monocytogenes-infected (■, □) J774 cells were used as targets in a 51Cr release assay to measure the cytotoxicity of line S169.8. The CTL clone 479-2, which recognizes LLO93–99 in the context of K, was used as a positive control for infection of the target cells. C, The number of fMIVIL-, fMIGWI-, and fMIVTLF-specific T cells present in line S169.8 was measured by IFN-γ ELISPOT assay. The number of S169.8 cells added per well is indicated on the x-axis. Data from one of two separate experiments are shown in each panel.

**FIGURE 8.** Adoptive transfer of line S169.8 cells confers protection against challenge with L. monocytogenes. Groups of class Ia MHC-deficient mice were injected i.v. with either PBS (no T cells), 2 × 10^6 S169.8 T cells, or 2 × 10^6 S169.8 cells. Fifteen minutes later, all groups of mice were infected i.v. with 3 × 10^4 CFU of L. monocytogenes. Three days postinfection, the mice were sacrificed, and spleens and livers were harvested, homogenized, and plated to determine the total number of CFU per spleen (∆) or liver (■). Each square represents the number of bacteria found in an individual mouse; horizontal bars indicate average values for each group. *, p < 0.01 as determined by Student’s t test. Data are from one of two independent experiments.

**Line S169.8 T cells confer protection against Listeria**

To determine whether the CD8+ T cells in line S169.8 could alter the course of a systemic Listeria infection, we adoptively transferred T cells to naive class Ia MHC-deficient mice. Thirty minutes later, the mice were infected with 3 LD_{50} of L. monocytogenes. A control group of mice received no T cells. Three days postinfection, the mice were sacrificed, and the bacterial load was assessed by plating dilutions of spleen and liver homogenates. As shown in Fig. 8, adoptive transfer of S169.8 T cells conferred protection against Listeria challenge. Transfer of 2 × 10^6 T cells resulted in 3 logs fewer bacteria present in the spleen and at least 2 logs fewer bacteria present in the liver compared with mice that received no T cells. The bacterial load in mice that received 10-fold fewer T cells (2 × 10^5) was not significantly different from the number of Listeria organisms found in mice that were not given T cells. These results indicate that line S169.8 contains CD8+ T cells that recognize Ags expressed during infection of mice and that these T cells can play a role in the clearance of L. monocytogenes.

**Discussion**

Although it has been demonstrated that several class Ib MHC molecules can present Ags to T cells, it has not been clear whether these T cells play a significant role in adaptive immune responses. In this report, we describe the generation of a class Ia MHC-deficient congenic BALB/c mouse strain and show that an enhanced CD8+ T cell recall response can be stimulated in these animals that protects against subsequent challenge with the intracellular bacterial pathogen L. monocytogenes. L. monocytogenes was chosen as a representative intracellular pathogen for these studies because it is a well-characterized bacterium with a simple, reproducible mouse model that is absolutely dependent on CD8 T cells for clearance. However, there are several other organisms such as Chlamydia trachomatis, Brucella abortus, and Trypanosoma cruzi that more readily infect BALB/c mice than other commonly studied mouse strains such as C57BL/6 (28–30). The highly susceptible class Ia MHC-deficient BALB/c mouse described in this report will be useful for further characterizing the role of CD8+ T cells in the clearance of these intracellular pathogens.

Many different strains of mice can be infected systemically with L. monocytogenes. We chose to use the BALB/c mouse model because BALB/c mice have higher bacterial loads during the later...
stages of infection than other mouse strains. The full magnitude and function of CTL responses will likely be required to effectively clear *L. monocytogenes* in these highly susceptible animals. A genetic basis for the differences in host susceptibility to *Listeria* infection has been well established. At least three loci, including the *Hc* gene coding for C5, appear to be involved in determining the level of innate resistance to infection with *L. monocytogenes* (31–33). Because these differences are observed as early as 1–3 days postinfection, the mechanisms underlying the susceptibility most likely involve innate immunity or resistance rather than specific adaptive immune responses.

Seaman et al. (34) previously showed that backcrossing the K\(^{b/-}\)D\(^{b/-}\) mutation onto the more naturally resistant C57BL/6 background resulted in animals that were able to fully clear *L. monocytogenes* infections despite the lack of K\(^b\) or D\(^b\)-restricted CD8\(^+\) T cells. However, no K\(^b\)- or D\(^b\)-binding antigenic epitopes derived from *L. monocytogenes* have been identified to date. Harty and Bevan (35) described in unpublished results that a K\(^b\)-restricted LLO-specific CD8\(^+\) T cell line could transfer some degree of protective immunity to C57BL/6 mice, but the contribution of Ag-specific populations of class Ia MHC-restricted CD8\(^+\) T cells in C57BL/6 mice has not been firmly established. In fact, one could hypothesize that the majority of the CD8\(^+\) T cell response stimulated during *L. monocytogenes* infection of mice of a H-2\(^b\) haplotype is class Ia-MHC-restricted. If that were the case, it would not be surprising that elimination of class Ia MHC-restricted CD8\(^+\) T cells did not affect the ability of C57BL/6 mice to clear *L. monocytogenes* infection. Because the bacterial burden found in either wild-type or K\(^b/-\)D\(^b/-\) C57BL/6 mice 4–7 days after infection is relatively low, it is easy to imagine that only a small number of circulating class Ib MHC-restricted CD8\(^+\) T cells would be needed to clear the infection. In contrast, studies using the more sensitive BALB/c mice have identified several protective antigenic epitopes presented by K\(^b\) molecules. It was previously thought that activation of these class Ia MHC-restricted CD8\(^+\) T cells was absolutely required for clearance of *Listeria* infection in BALB/c mice. Therefore, an important question remained to determine whether class Ib MHC-restricted CD8\(^+\) T cells would also be sufficient to clear the higher bacterial loads found in BALB/c mice after infection with *L. monocytogenes*.

We show here that class Ia MHC-deficient BALB/c mice immunized with a sublethal dose of *L. monocytogenes* are fully protected against a subsequent lethal challenge with *Listeria*. Despite the fact that these mice have a 5- to 10-fold decrease in the number of circulating CD8\(^+\) T cells (23), the class Ib MHC-restricted T cells present in these mice are sufficient to provide effective adaptive immunity. Only the CD8\(^+\) subset of immune splenocytes from K\(^b/-\)D\(^b/-\) mice was required to adoptively transfer immunity to naive mice, suggesting that the animals did not simply use alternate compensatory mechanisms to clear *Listeria* in the absence of class Ia MHC-restricted CTL. In the adoptive transfer experiment presented here, we used splenocytes harvested from mice infected 14 days earlier with *L. monocytogenes*. Most adoptive transfer studies utilize splenocytes harvested only 6–7 days after infection. T cells present in the spleens of 6-day-immune mice are mostly primary effector cells that have been expanded after activation. In contrast, 2 wk after infection, most of the primary effector T cells have undergone apoptosis, and the remaining *Listeria*-specific T cells are presumably memory cells.

Interestingly, CD8\(^+\) T cells proliferated to a greater extent in the K\(^b/-\)D\(^b/-\) mice than in wild-type mice. Urdahl et al. (36) recently showed that CD8\(^+\) T cells that are positively selected on class Ia MHC-deficient hemopoietic cells express a different set of activation markers than classically restricted CD8\(^+\) T cells. Thus, it is possible that class Ib MHC-restricted T cells exist in a partially activated state and have an inherent ability to proliferate faster or receive proliferation signals earlier than class Ia MHC-restricted T cells. If this were the case, one might not expect to detect the rapid growth of these CD8\(^+\) cells in a wild-type mouse, because the class Ib MHC-restricted cells represent only a small fraction of the total CD8\(^+\) T cell population. Alternatively, class Ib MHC-restricted T cells may traffic differently than class Ia MHC-restricted T cells, with more cells migrating from the periphery to the spleen. It could be suggested that the reduced number of CD8\(^+\) T cells in K\(^b/-\)D\(^b/-\) mice are simply expanding to fill lymphoid space. However, both CD4\(^+\) and CD8\(^+\) T cells reside primarily in the periarteriolar lymphoid sheath of the spleen, and we saw no relative increase in the number of CD4\(^+\) cells after *L. monocytogenes* infection. Our results with wild-type C.B10 mice were similar to a previous study in BALB/c mice which showed that infection-induced splenomegaly results in an approximate doubling of splenocytes, with a corresponding 2-fold increase in CD4\(^+\), CD8\(^+\), and NK cells (6).

To begin to identify specific class Ib MHC-restricted T cells capable of protecting against *Listeria* infection, we isolated a CD8\(^+\) T cell line (S169.8) from a class Ia MHC-deficient BALB/c-congenic mouse that had recovered from a *L. monocytogenes* infection. This T cell line displayed two *Listeria*-specific effector functions, cytotoxicity and IFN-\(\gamma\) secretion. Adoptive transfer of these T cells to naive mice resulted in significant protection against infection with *L. monocytogenes*. In fact, the level of protection observed in spleens (~3.0 logs; Fig. 8) was almost the same as the protection that can be afforded by prior immunization with a sublethal dose of *Listeria*.

The nature of the *L. monocytogenes* Ag recognized by line S169.8 T cells and the identity of the class Ib MHC molecule presenting this Ag are not yet known. Only three class Ib MHC-binding antigenic epitopes derived from *Listeria* have been described to date, and each of these is a formylated peptide presented by M3 molecules. Although a small percentage of line S169.8 recognizes formylated MIGWII peptide (Fig. 7C), the major reactivity present in the T cell line does not appear to be one of the three known M3-binding peptides. Preliminary experiments indicate that line S169.8 does not recognize an Ag presented by CD1d (S. E. F. D’Orazio and M. N. Starnbach, unpublished observations).

Because previous studies have suggested that there is not an enhanced recall response for either Qa-1\(^b\)-restricted (37) or M3-restricted (38, 39) CD8\(^+\) T cells during secondary *Listeria* infection, it is possible that line S169.8 recognizes an Ag presented by one of the less well-characterized class Ib MHC molecules. We are currently generating T cell clones derived from line S169.8 and plan to use these clones to identify antigenic epitopes, determine MHC restriction, and assess the degree of TCR diversity found in this protective CD8\(^+\) T cell line. One of the challenges in studying class Ib MHC-restricted T cells has been that they often comprise only a small fraction of the total lymphocytes in wild-type mice. Isolation of these T cells during infection can be difficult when a large class Ia MHC-restricted T cell response occurs simultaneously. Although class Ib MHC-restricted T cells are not found in large numbers in lymphoid organs, they may still have an important role in the clearance of intracellular pathogens in wild-type mice. It has been suggested that some class Ib MHC-restricted T cells may function as a critical link between the early innate immune response, and the specific adaptive immune response that occurs later (40). By eliminating the dominant class Ia MHC-restricted T cell response, the mice described in this report may be useful for identifying new
class Ib MHC molecules that present unique classes of Ags during infection.

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References