Cytosolic Localization of *Listeria monocytogenes* Triggers an Early IFN-γ Response by $CD8^+$ T Cells That Correlates with Innate Resistance to Infection[1]

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IFN-γ is critical for innate immunity against *Listeria monocytogenes* (*L. monocytogenes*), and it has long been thought that NK cells are the major source of IFN-γ during the first few days of infection. However, it was recently shown that a significant number of $CD44^{high}CD8^+$ T cells also secrete IFN-γ in an Ag-independent fashion within 16 h of infection with *L. monocytogenes*. In this report, we showed that infection with other intracellular pathogens did not trigger this early IFN-γ response and that cytosolic localization of *Listeria* was required to induce rapid IFN-γ production by $CD44^{high}CD8^+$ T cells. Infection of C57BL/6 mice with an *Escherichia coli* strain expressing listeriolysin O (LLO), a pore-forming toxin from *L. monocytogenes*, also resulted in rapid IFN-γ expression by $CD8^+$ T cells. These results suggest that LLO expression is essential for induction of the early IFN-γ response, although it is not yet clear whether LLO plays a direct role in triggering a signal cascade that leads to cytokine production or whether it is required simply to release other bacterial product(s) into the host cell cytosol. Interestingly, mouse strains that displayed a rapid $CD8^+$ T cell IFN-γ response (C57BL/6, 129, and NZB) all had lower bacterial burdens in the liver 3 days postinfection compared with mouse strains that did not have an early $CD8^+$ T cell IFN-γ response (BALB/c, A/J, and SJL). These data suggest that participation of memory $CD8^+$ T cells in the early immune response against *L. monocytogenes* correlates with innate host resistance to infection. ***The Journal of Immunology,*** **2006, 177: 7146–7154.**

Listeria monocytogenes is an intracellular bacterial pathogen that has been used for decades as a model organism to study cell-mediated immunity. Systemic infection can be established via i.v. administration of bacteria in most of the commonly studied inbred strains of mice. However, there is a significant difference among mouse strains in the degree of susceptibility to *L. monocytogenes*, as measured by both bacterial burden following sublethal infection and LD_{50} values. For example, in a previous study we showed that 2–5 × 10^4 CFU of *L. monocytogenes* strain 10403s caused death of all BALB/c mice within 72 h, while C57BL/6 mice, which have an LD_{50} that is at least 50-fold higher, all survived infection with this dose of *L. monocytogenes* (1). Complement (C5) deficiency and a *sst1*-dependent macrophage function have been implicated in innate host susceptibility to *L. monocytogenes* infection (2, 3). However, host resistance appears to be a multigenic trait (1), and further genetic differences between mouse strains have yet to be identified.

IFN-γ is a critical cytokine involved in control of *L. monocytogenes* infection during both the innate and adaptive immune responses. NK cells and NKT cells are known to secrete IFN-γ and are thought to limit exponential growth of the bacteria primarily by activating macrophages during the first few days of infection (4, 5). IFN-γ secretion by these cells is also thought to promote a Th1 type response against *Listeria* by increasing MHC class I expression. The adaptive immune response against *L. monocytogenes*, which peaks at 6–8 days postinfection, is dominated by $CD8^+$ T cells, which have two main effector functions: specific lysis of infected cells and secretion of IFN-γ. $CD8^+$ T cells are essential for complete clearance of the bacterial burden and provide long-lasting sterilizing immunity against *L. monocytogenes* (6, 7). Intriguing recent studies suggest that $CD8^+$ T cells can also function during the innate phase of immunity (8–10). In fact, Berg et al. showed that on a cell-for-cell basis, $CD8^+$ T cells may contribute as much IFN-γ as NK cells during the first 3 days of *L. monocytogenes* infection (11). They further demonstrated that IFN-γ-secreting $CD8^+$ T cells were found in the T cell zones of the white pulp of the spleen close to infected macrophages while NK cells were limited to red pulp regions and did not colocalize with foci of infection (12). These results suggest that $CD8^+$ T cell derived IFN-γ may be more effective at bacterial control than NK cell-derived cytokine.

The subpopulation of $CD8^+$ T cells capable of rapidly secreting IFN-γ in response to *L. monocytogenes* infection has been partially characterized. Berg et al. (11) showed that the early IFN-γ response by $CD8^+$ T cells is IL-12 and IL-18 dependent and Ag independent and that the cells appeared to have a memory phenotype ($CD44^{high}Ly6C^{high}CD62L^{low}$). In an adoptive transfer experiment, more than half of the transferred memory T cells secreted IFN-γ within 16 h of infection with *L. monocytogenes*, suggesting that the early IFN-γ response may be an inherent characteristic of memory $CD8^+$ T cells (8). It has been suggested that memory $CD8^+$ T cells have higher levels of IL-12 and IL-18 receptors on their surface, thus accounting for the ability to rapidly secrete IFN-γ in response to IL-12 and IL-18 production by *L. monocytogenes*-infected macrophages and/or dendritic cells (DC)[3] (8).

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Received for publication March 22, 2006. Accepted for publication August 28, 2006.

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[1] This work was supported by National Institutes of Health grants from the National Center for Research Resources (P20 RR20171; to S.E.F.D.), the National Institute of Allergy and Infectious Disease (AI055962; to M.N.S.), and by a pilot grant from the Harvard Digestive Diseases Center (DK34854; to S.E.F.D.).

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[3] Abbreviations used in this paper: DC, dendritic cell; BMMϕ, bone marrow-derived macrophage; CDC, cholesterol-dependent cytolysin; ICCS, intracellular cytokine staining; LB, Luria-Bertani; LLO, listeriolysin O; MOI, multiplicity of infection.

0022-1767/06/$02.00

In this study, we examined the bacterial components that trigger the early IFN-γ response. We showed that cytosolic localization of *L. monocytogenes* was required to induce IFN-γ secretion by memory $CD8^+$ T cells and that other bacterial pathogens did not trigger this response. We found that only half of the mouse strains we tested (including C57BL/6, 129, and NZB) were capable of mounting an early IFN-γ response by memory $CD8^+$ T cells. Furthermore, the early IFN-γ response appeared to correlate with innate host resistance to *L. monocytogenes* since the $CD8^+$ T cells from the most susceptible mouse strains (BALB/c, A/J, and SJL) were not capable of rapidly secreting IFN-γ. These results suggest that initial clearance of *L. monocytogenes* infection occurs by different mechanisms in the commonly studied BALB/c and C57BL/6 mouse models.

Materials and Methods

Mice

BALB/c/By/J, C57BL/6/J (B6), C.B10-$H2^b$/LilMcd/J (C.B10), BALB × B6 F_1, 129/3/J, NZB, A/J, and SJL mice were obtained from The Jackson Laboratory and were maintained in a specific-pathogen free facility at either Harvard Medical School or the University of Kentucky. C.B10 is a congenic strain identical to BALB/c ($H\text{-}2^d$) at all loci, except for the H-2 locus, which was derived from a C57BL/10 ($H\text{-}2^b$) mouse.

Bacteria and viruses

Frozen aliquots of *L. monocytogenes* 10403s, *Salmonella typhimurium*, *Listeria innocua*, and *Bacillus subtilis* were thawed on ice and then incubated in brain heart infusion broth shaking at 37°C until early exponential phase. Dilutions prepared in PBS were used to infect either tissue culture cells or mice. A groin wound isolate of *Streptococcus pyogenes* was provided by Dr. J. Ribes (U.K. Hospital, Lexington, KY) and was grown on TSA II agar plates containing 5% sheep RBC (Difco). The *L. monocytogenes* listeriolysin O (LLO) deletion strain DP-L2161 (13) was provided by D. Higgins (Harvard Medical School, Boston, MA). Heat-killed *Listeria* were prepared by incubating exponential phase *L. monocytogenes* at 65°C for 3 h. Loss of viable colony-forming units was verified by plating on brain heart infusion agar. *Chlamydia trachomatis* L2 was isolated as previously described (14), stored at −80°C, and thawed immediately before use. Wild-type *Vaccinia* virus provided by B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD) was plaque purified and titered after growth in human TK-143B cells.

Infection of mice

For bacterial infections, mice were inoculated i.v. in the lateral tail vein using a total volume of 200 μl. The LD_{50} for *L. monocytogenes* 10403s is $\sim 1 \times 10^4$ for BALB/c mice and $\sim 2 \times 10^5$ for C57BL/6 mice. To determine bacterial burden, mice were sacrificed, and their spleens and livers were harvested aseptically and homogenized in 0.2% Nonidet P-40. Serial dilutions were plated on tryptic soy agar containing 10 μg/ml streptomycin, and the total number of colony-forming units was determined following overnight incubation at 37°C. *Vaccinia* virus preparations were treated with an equal volume of 0.25 mg/ml trypsin for 30 min at 37°C and diluted in PBS. Mice were infected either i.p. or i.v. with 1×10^6 PFU of virus.

Infection of bone marrow-derived macrophages (BMMϕ) and DC

To generate BMMϕ, bone marrow cells were harvested from the femurs of mice and plated in BMM-20 medium, consisting of DMEM (11960; Invitrogen Life Technologies) supplemented with L-glutamine, 20% FCS, and 20% L929 supernatant. On day 4, cells were washed once with PBS and replenished with BMM-20. BMMϕ were then fed with BMM-10 every 3–4 days and maintained in culture for up to 6 wk as described previously (15). $CD11c^+$ DC were isolated from mouse spleen by negative selection with Ab-coated magnetic beads. Cells were infected at a multiplicity of infection (MOI) of 1 (Mϕ) or 5 (DC) for 30 min at 37°C in 7% CO_2. Contact between the cells and the bacteria was facilitated by centrifugation of the tissue culture dishes at 600 × *g* for 5 min at 35°C. The cells were washed three times with prewarmed PBS, and then RP-10 containing 25 μg/ml gentamicin was added to kill extracellular bacteria.

Abs/flow cytometry

$CD8^+$ or $CD11c^+$ cell populations were enriched from mouse spleen by negative selection using IMag mixtures of Ab-coated magnetic beads according to the manufacturer's instructions (BD Biosciences). Fluorescently conjugated anti-murine Abs directed against IFN-γ (clone XMG1.2), CD8α (53-6.7), TCRβ (H57-597), and CD44 (IM7) and were purchased from eBioscience. Biotin-labeled anti-mouse IL-12Rβ1 (clone 114) and PE-Cy5 conjugated streptavidin were purchased from BD Biosciences. Purified mAbs against murine IL-12 (PeproTech) and murine IL-18 (clone 93-10C; Medical & Biological Labs) were used at a final concentration of 1 μg/ml. Cells were suspended in FACS buffer consisting of HBSS with 0.5% BSA and 0.1% sodium azide. Fluorescence intensities were measured using a FACSCalibur flow cytometer, and analysis was performed using CellQuest software (BD Biosciences). Dead cells and monocytes were excluded using forward and side scatter gating.

Intracellular cytokine staining (ICCS)

Intracellular staining for IFN-γ was performed using the Cytofix/Cytoperm (with GolgiPlug) kit, according to the manufacturer's instructions (BD Biosciences). For ex vivo assays, spleens were harvested 18 h postinfection, and 5×10^6 splenocytes were plated in RP-10 in 24-well dishes (2 ml/well) in the presence of GolgiPlug (2 μl/well) with no further in vitro stimulation. For in vitro assays, 5×10^5 BMMϕ were infected for 1 h, and then 5×10^6 naive splenocytes were added to each well. The cells were coincubated at 37°C in 7% CO_2 for 18–20 h, with Golgi Plug added to the medium during the last 4 h of incubation. Typically, 50,000 lymphocytes (based on forward and side scatter gating) were collected and analyzed.

Infection with Escherichia coli *expressing LLO and OVA*

E. coli DH5α harboring pDP3616 (*E. coli* OVA; Amp^R) and *E. coli* DH5α harboring both pDP3615 (Cm^R) and pDP3616 (*E. coli* LLO/OVA) were provided by D. Higgins. pDP3616 encodes a truncated form of OVA under the control of an IPTG-inducible T7 promoter; pDP3615 contains the *hly* gene encoding LLO (lacking the secretion signal sequence) under the control of a constitutive *tet* promoter (16). The *E. coli* strains were grown on Luria-Bertani (LB) agar plates containing 10 μg/ml chloramphenicol and/or 50 μg/ml carbenicillin, and a fresh colony was used to inoculate 2 ml of LB broth containing the appropriate antibiotics. Cultures were grown to stationary phase overnight at 37°C with aeration and then diluted 1/100 into fresh medium. Expression of OVA was induced by adding 1 mM IPTG to mid-log phase cultures ($OD_{600} = 0.5$) and growth was continued for an additional 2 h at 37°C with aeration. Equivalent numbers of bacteria were washed once in PBS and used to infect tissue culture cells or mice. Serial dilutions of the inocula were plated on LB agar containing appropriate antibiotics to determine the total number of colony-forming units present.

Cytotoxicity assays

Effector cell populations were generated by harvesting splenocytes from immune mice (14 days postinfection) and incubating for 5 days in vitro (37°C in 7% CO_2) in the presence of irradiated (2000 rad) syngeneic peptide-coated (final concentration of 100 nM) splenocytes. Synthetic peptides corresponding to the $LLO_{91–99}$ and $OVA_{257–264}$ epitopes were purchased from Bio-Synthesis. Target cells (10^6) were prepared by coincubating either EL-4 thymoma ($H\text{-}2^b$) or P815 mastocytoma ($H\text{-}2^d$) cells with the appropriate peptide epitope (final concentration of 100 nM) and 100 μCi of sodium [^{51}Cr]chromate for 1 h at 37°C in 7% CO_2 in a total volume of 150 μl. Target cells were washed three times with RPMI 1640, suspended in RP-10 at 1×10^5 cells/ml, and distributed into 96-well plates (100 μl/well). Serial dilutions of the effector cell cultures (100 μl/well) were added, and the plates were incubated for 4 h at 37°C in 7% CO_2. The assay plates were centrifuged for at 500 × *g* for 5 min, and 100 μl of the supernatant from each well was applied to a Luma plate (PerkinElmer) and allowed to dry overnight at room temperature. The amount of ^{51}Cr released into the supernatant of each well was evaluated (without the addition of scintillation fluid) using a TopCount (PerkinElmer) gamma counter. Spontaneous release (SR) was determined by incubating target cells in RP-10 alone (no effector cells); maximum release (MR) was determined by incubating target cells in RP-10 with 0.1% Triton X-100. Percent-specific lysis (SL) was calculated using the following formula: percentage of SL = 100 × (release by effector T cells − SR)/(MR − SR)).

Results

Only L. monocytogenes *infection triggers the early IFN-γ response*

Previous work has demonstrated that $CD44^+CD8^+$ T cells in C57BL/6 mice secrete significant amounts of IFN-γ within 16 h of

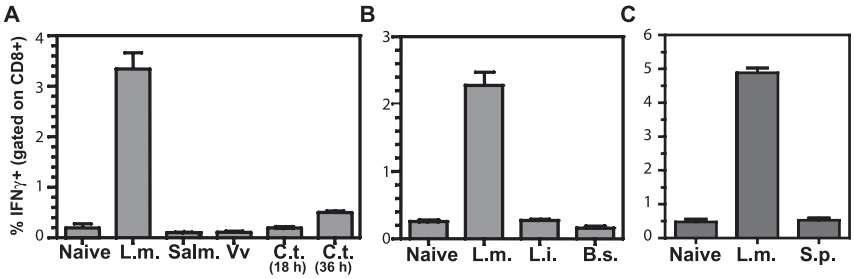

FIGURE 1. The early IFN-γ response is not induced during infection with all intracellular microbial pathogens. *A*, C57BL/6 mice were injected i.v. with either 10^6 wild-type *L. monocytogenes* (L.m.), 10^6 *S. typhimurium* (Salm.), 10^6 PFU of wild-type *Vaccinia* virus (Vv), or 10^6 IFU of *C. trachomatis* (C.t.). *B*, C57BL/6 mice were infected (i.v.) with 10^6 CFU of either L.m., *L. innocua* (L.i.), or *B. subtilis* (B.s.). *C*, C57BL/6 mice were infected (i.v.) with either 10^6 CFU of L.m. or 10^7 CFU of *S. pyogenes* (S.p.). Control groups of mice received PBS (naive). Spleens were harvested 18 h later, and the total number of IFN-γ-secreting cells was determined ex vivo by ICCS. Average values $\pm$ SD for groups of three mice each are shown.

infection with the intracellular bacterial pathogen *L. monocytogenes* (8, 11). Although these memory T cells responded to infection in an Ag-independent manner, they appeared to play a role in the clearance of *L. monocytogenes*. To determine whether this early IFN-γ response was involved in the clearance of other intracellular pathogens, we infected C57BL/6 mice i.v. with *S. typhimurium*, *C. trachomatis*, and *Vaccinia* virus. Splenocytes were harvested from these mice 18 h after infection and incubated for 4 h in vitro in the presence of brefeldin A to block protein secretion. The number of CD8$^+$ T cells secreting IFN-γ ex vivo was assessed by intracellular cytokine staining. Consistent with the earlier findings of Berg et al. (11), ~3% of the CD8$^+$ T cells in *L. monocytogenes*-infected mice secreted IFN-γ (Fig. 1*A*). However, none of the mice infected with *Salmonella*, *Vaccinia*, or *Chlamydia* demonstrated an increase in IFN-γ-secreting CD8$^+$ T cells over the background level detected in naive mice. Since *C. trachomatis* replicates much more slowly than *Salmonella* or *Vaccinia* virus, we also examined splenocytes harvested from C57BL/6 mice 36 h postinfection with *C. trachomatis*. Although the level of IFN-γ^+CD8$^+$ T cells was slightly higher at 36 h compared with 18 h postinfection, it was not significantly different from the number of IFN-γ^+ T cells found in the spleens of naive mice (Fig. 1*A*).

One difference between *L. monocytogenes* and the other intracellular bacterial pathogens we tested is cell wall composition. *L. monocytogenes* is a Gram-positive organism, whereas *Salmonella* spp. are Gram-negative and *C. trachomatis* has a Gram-negative-like cell wall. Thus, it was possible that the presence of a Gram-positive cell wall component, such as lipoteichoic acid, was responsible for triggering the early IFN-γ response. To test this possibility, we infected C57BL/6 mice i.v. with three other Gram-positive organisms: a nonpathogenic *Listeria* (*L. innocua*), the closely related *Bacillus subtilis*, or *Streptococcus pyogenes*. As shown in Fig. 1, *B* and *C*, there was no increase in the number of IFN-γ^+CD8$^+$ T cells in these mice 18 h postinfection. These results indicate that not all Gram-positive bacteria are capable of triggering the early IFN-γ response. Taken together, these data suggest that *L. monocytogenes* infection is unique in its ability to cause rapid secretion of IFN-γ by CD8$^+$ T cells.

Cytosolic localization of L. monocytogenes *is required to trigger the early IFN-γ response*

Upon infection of host cells, *L. monocytogenes* rapidly escapes from phagocytic or endocytic vacuoles into the host cell cytosol. In contrast, *C. trachomatis* and *S. typhimurium* are intracellular bacteria that survive and multiply within phagocytic vacuoles. If cytosolic localization were required to trigger the early IFN-γ effect, then it would not be surprising that *Lm* was the only one of these three pathogens that triggered rapid IFN-γ secretion by CD8$^+$ T cells. To test this, we infected C57BL/6 mice with either 10^9 HK *Lm* or 10^8 CFU of either *L. innocua* or an LLO deletion mutant strain of *L. monocytogenes* (ΔLLO *L. monocytogenes*). Since LLO is not actively secreted by any of these bacterial strains, the organisms should remain within host cell vacuoles during infection of mice. As shown in Fig. 2, no increase in the number of IFN-γ^+CD8$^+$ T cells was observed within 18 h of infection with any of these strains. These results suggested that the *Listeria* product responsible for triggering the early IFN-γ effect must be localized to the host cell cytosol.

E. coli *expressing cytosolic LLO can trigger the early IFN-γ response*

Recombinant LLO has been shown to induce cytokine production in vitro when incubated with mouse splenocytes (16, 17). Thus, we considered the possibility that LLO itself could trigger the early IFN-γ response by memory CD8$^+$ T cells in the absence of other *L. monocytogenes* components. To test this, we infected mice with *E. coli* strains expressing either LLO and the heterologous antigenic protein OVA (*E. coli*-OVA/LLO) or OVA alone (*E. coli*-OVA). *E. coli* expressing recombinant LLO do not actively secrete LLO and thus do not have the ability to escape from phagocytic vacuoles. However, degradation of *E. coli* strains expressing LLO is thought to release preformed stores of active hemolysin that form pores in the phagocytic membrane, releasing abundant bacterial proteins into the host cell cytosol (18). *E. coli* strains overexpressing target Ags such as OVA in combination with LLO have

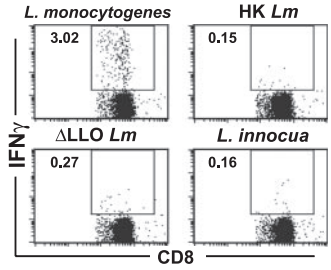

FIGURE 2. Cytosolic localization of *L. monocytogenes* is required to trigger the early IFN-γ response. C57BL/6 mice were injected i.v. with either 10^6 wild-type *L. monocytogenes*, 10^9 heat-killed *L. monocytogenes* (HK *Lm*), 10^8 CFU of a LLO deletion mutant strain of *L. monocytogenes* (ΔLLO *Lm*), or 10^8 *L. innocua*. Spleens were harvested 18 h later, and the total number of IFN-γ-secreting cells was determined directly ex vivo by ICCS. Primary dot plots from a representative mouse from each group of three mice (gated on TCRβ^+CD8$^+$ lymphocytes) are shown. Numbers in the *upper left corners* indicate the percentage of IFN-γ^+CD8$^+$ T cells.

been shown to deliver a K^b-restricted OVA epitope (SIINFEKL) to the host cell cytosol for processing and presentation to $CD8^+$ T cells and thus have been used as an intracellular vaccine delivery system in both murine and human DC (19, 20).

As shown in Fig. 3*A*, infection with 10^7 CFU of either *E. coli* strain did not trigger the early IFN-γ response. However, increasing the dose to 10^8 CFU resulted in an increase in IFN-γ^+ $CD8^+$ T cells in mice infected with *E. coli*-OVA/LLO but not in mice infected with *Ec*-OVA. To verify that significant amounts of bacterial proteins were being released into the host cell cytosol during these infections, we also looked for activation of either SIINFEKL or LLO_{91-99}-specific $CD8^+$ T cells following infection with the recombinant *E. coli* strains. Two weeks postinfection, splenocytes were harvested from these mice and incubated in vitro in the presence of irradiated syngeneic peptide-coated splenocytes to culture SIINFEKL-specific $CD8^+$ T cells. As shown in Fig. 3*B*, T cells capable of lysing SIINFEKL-coated target cells were isolated from C57BL/6 (H-2^b) mice infected with either 10^7 or 10^8 CFU of *E. coli*-OVA/LLO. This suggests that abundant amounts of OVA protein or partially digested peptides derived from OVA were released into the host cell cytosol and were processed and presented to naive $CD8^+$ T cells. OVA-specific $CD8^+$ T cells were not detected in the cultures derived from mice infected with *E. coli*-OVA, indicating that expression of LLO was required to release the Ag into the host cell cytosol.

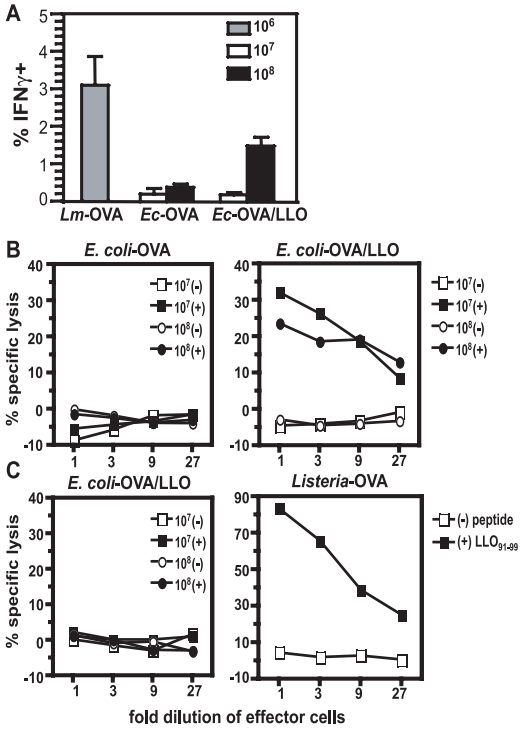

FIGURE 3. Infection with *E. coli* expressing LLO triggers the early IFN-γ response in C57BL/6 mice. C57BL/6 mice were infected (i.v.) with the number of CFU indicated of either *L. monocytogenes* JJL-OVA (*Lm*-OVA), *E. coli* DH5α carrying pDP3616 (*Ec*-OVA), or *E. coli* DH5α carrying both pDP3616 and pDP3615 (*Ec*-OVA-LLO). *A*, Spleens were harvested 18 h postinfection, and the total number of IFN-γ^+ cells (gated on $CD8^+$ T cells) was determined by ICCS. Average values ± SD from one of three experiments are shown. *B*, Cytotoxic activity of splenocyte cultures derived from C57BL/6 mice infected 2 wk previously with either 10^7 (squares) or 10^8 (circles) CFU of the indicated *E. coli* strain was determined using a ^{51}Cr release assay. The percent-specific lysis of EL-4 (open symbols) or 100 nM SIINFEKL-coated EL-4 (closed symbols) target cells at various E:T cell ratios is shown. *C*, Cytotoxic activity of splenocyte cultures derived from BALB/c mice infected 2 wk previously with either 10^7 (squares) or 10^8 (circles) CFU of the indicated *E. coli* strain was determined using a ^{51}Cr release assay. The percent-specific lysis of P815 (open symbols) or 100 nM LLO_{91-99}-coated P815 (closed symbols) target cells at various E:T cell ratios is shown. Representative data from one of three separate experiments are shown.

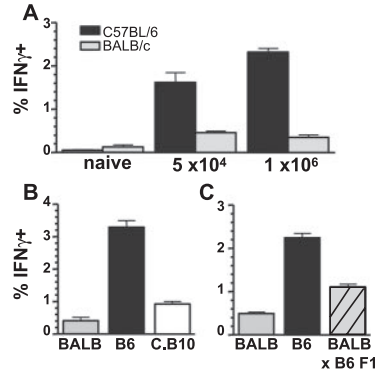

FIGURE 4. $CD8^+$ T cells do not rapidly secrete IFN-γ during *L. monocytogenes* infection of BALB/c mice. *A*, Mice were infected (i.v.) with either 5×10^4 or 1×10^6 CFU of wild-type *L. monocytogenes*; control groups of mice received injections of PBS. Representative data from one of three separate experiments are shown. *B*, Groups of three BALB/c (▨), C57BL/6 (■), and C.B10 mice (□) or (*C*) BALB/c × C57L/6 F_1 mice (▨) were infected i.v. with 10^6 CFU of wild-type *L. monocytogenes*. Splenocytes were harvested 18 h later, and the total number of IFN-γ-secreting cells was determined ex vivo by ICCS. The average number of $CD8^+$ T cells that expressed IFN-γ ± SD for each group is shown.

To assess the amount of LLO being delivered into the cytosol by the *E. coli*-OVA/LLO strain, we also infected BALB/c (H-2^d) mice and setup $CD8^+$ T cell cultures using irradiated syngeneic LLO_{91-99}-coated splenocytes as stimulator cells. The T cell cultures derived from mice infected with either 10^7 or 10^8 CFU of *E. coli*-OVA/LLO were not capable of lysing LLO_{91-99}-coated target cells, while cultures derived from a control group of mice infected with *L. monocytogenes* showed significant lysis of the peptide-coated target cells (Fig. 3*C*). These results suggest that although LLO was expressed by *E. coli*-OVA-LLO in vivo and appeared to form enough pores in phagocytic membranes to release proteins that were abundantly expressed by the bacteria, large amounts of LLO protein were not likely to be released into the host cell cytosol during infection of mice.

The early IFN-γ response occurs in C57BL/6 but not BALB/c mice

Many different strains of mice can be infected i.v. with *L. monocytogenes*, however, innate resistance to *Listeria* infection varies considerably between mouse strains. For example, the LD_{50} for *L. monocytogenes* 10403s is 1×10^4 in BALB/c mice and 50-fold higher (2×10^5) in C57BL/6 mice. We postulated that rapid production of IFN-γ by memory $CD8^+$ T cells would contribute significantly to innate resistance to *Listeria* infection and that susceptible BALB/c mice might lack this early IFN-γ response. To test this, we infected groups of BALB/c and C57BL/6 mice with 5 LD_{50} of *L. monocytogenes* and examined splenocytes 18 h postinfection to determine the total number of IFN-γ-secreting $CD8^+$ T cells. As shown in Fig. 4*A*, BALB/c mice given 5×10^4 bacteria (5 LD_{50}) did not show an increase in the number of IFN-γ-secreting $CD8^+$ T cells compared with naive mice, while the comparable dose in C57BL/6 mice resulted in a significant increase in IFN-γ^+CD8^+ T cells. Although the two strains of mice were each

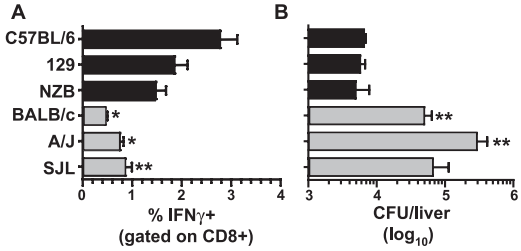

FIGURE 5. Rapid production of IFN-γ by CD8$^+$ T cells correlates with host resistance to *L. monocytogenes* infection. *A*, Mice were infected (i.v.) with 10^6 CFU of *L. monocytogenes* 10403s. Spleens were harvested 18 h postinfection, and the total number of IFN-γ^+CD8$^+$ T cells was determined by ICCS. *B*, Mice were infected (i.v.) with 2×10^3 CFU of *L. monocytogenes* 10403s, and the total number of bacteria found in the liver was determined 3 days later. Mean values $\pm$ SD from one of two separate experiments are shown. Means significantly different compared with average values for C57BL/6 mice are indicated (*, $p < 0.005$; **, $p < 0.026$).

given an dose equal to 5 LD_{50}, and thus, should have established equivalent infection in vivo, it was possible that a certain threshold of organisms was required to trigger the early IFN-γ response. However, BALB/c mice that received 1×10^6 *L. monocytogenes* still did not show a significant increase in IFN-γ^+CD8$^+$ T cells 18 h postinfection. These data suggested that CD8$^+$ T cells were not involved in innate immune responses in BALB/c mice.

To further characterize the genetic difference between BALB/c and C57BL/6 mice involved in the early IFN-γ response, we tested both C.B10 and BALB/c $\times$ C57BL/6 F_1 mice. C.B10 is a BALB/c (H-2^d) congenic strain that differs from BALB/c mice only at the MHC locus, which is derived from a C57BL/10 (H-2^b) mouse. C.B10 mice infected with 1×10^6 CFU of *L. monocytogenes* showed a slight but not significant increase in the number of IFN-γ^+CD8$^+$ T cells 18 h postinfection (Fig. 4*B*). This result suggested that the early IFN-γ response is not linked to the MHC locus since C.B10 mice displayed the same phenotype as BALB/c mice. F_1 progeny derived from a BALB/c $\times$ C57BL/6 cross showed an intermediate phenotype. Eighteen hours after infection, these mice had 3-fold more IFN-γ^+CD8$^+$ T cells than BALB/c mice but fewer than C57BL/6 mice (Fig. 4*C*). These data indicated that the genetic difference between BALB/c and C57BL/6 mice responsible for induction of the early IFN-γ response displayed an incomplete dominance pattern.

The early IFN-γ response correlates with innate host resistance against Listeria *infection*

We further examined whether or not the early IFN-γ response by CD8$^+$ T cells correlated with innate resistance to *L. monocytogenes* infection by testing four additional strains of mice. As shown in Fig. 5*A*, the mouse strains could be classified into two groups—early IFN-γ responders and nonresponders. C57BL/6, 129, and NZB mice each displayed a significant increase in the number of IFN-γ^+CD8$^+$ T cells 18 h after infection with *L. monocytogenes*. As observed for the BALB/c mice, an early IFN-γ response was not detected in either A/J or SJL mice (Fig. 5*A*). To assess the level of innate resistance or susceptibility to *Listeria* infection, we infected each of these mouse strains with 2×10^3 CFU of *L. monocytogenes*. Three days later, the total number of CFU present in the liver was determined. BALB/c and A/J mice (early IFN-γ nonresponder strains) each had significantly higher bacterial burdens in the liver compared with C57BL/6 mice (Fig. 5*B*). In addition, SJL mice had higher bacterial titers in the liver compared with either C57BL/6, 129, or NZB mice, although the difference was not statistically significant. Thus, the bacterial load early during the course of *Listeria* infection inversely correlated with the presence of innate CD8$^+$ T cells capable of secreting IFN-γ.

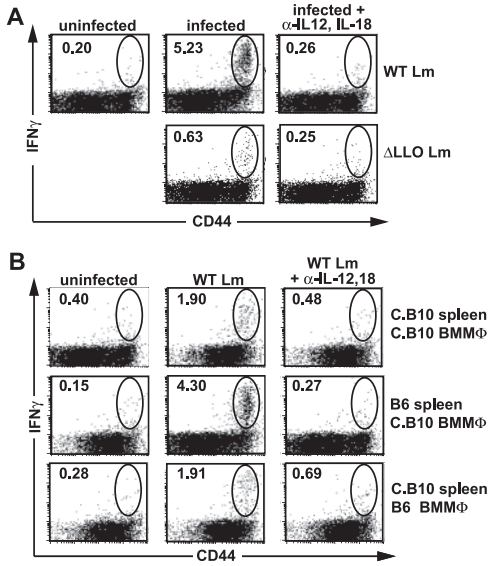

FIGURE 6. CD8$^+$ T cells from both C57BL/6 and C.B10 mice are capable of rapidly secreting IFN-γ in vitro following exposure to *L. monocytogenes*-infected BMMϕ. The early IFN-γ response was reconstituted in vitro by preinfecting BMMϕ with *Listeria* and then adding splenocytes from a naive mouse. Anti-murine IL-12 and IL-18 Abs were added at a final concentration of 1 μg/ml where indicated. The total number of IFN-γ-expressing CD44^{high}CD8$^+$ T cells was determined by ICCS 18 h later. Primary data plots (gated on CD8β^+ lymphocytes) for one of two separate experiments are shown. Numbers in the *upper left corner* indicate the percentage of CD8$^+$ T cells in each oval. *A*, C57BL/6 BMMϕ (5×10^5) were infected with either 10^6 CFU of *L. monocytogenes* 10403s (WT Lm) or 10^8 CFU of *L. monocytogenes* DP-L2161 (ΔLLO Lm), and then 5×10^6 splenocytes from a naive C57BL/6 mouse were added. *B*, Infected or uninfected BMMϕ isolated from either C57BL/6 or C.B10 mice were coincubated with 5×10^6 whole splenocytes from a naive C57BL/6 or C.B10 mouse as indicated.

Listeria*-infected macrophages isolated from either early IFN-γ responder or nonresponder mice can trigger CD8$^+$ T cells to rapidly secrete IFN-γ*

To begin to identify the cell type responsible for host differences in triggering the early IFN-γ effect, we designed an in vitro assay to recapitulate the response observed in mice. BMMϕ isolated from a C57BL/6 mouse were infected with *L. monocytogenes*, and 1 h later, splenocytes from a naive C57BL/6 mouse were added. After 14–16 h of coincubation, GolgiPlug (brefeldin A) was added to block protein secretion, and the number of CD8$^+$ T cells expressing IFN-γ was determined by intracellular cytokine staining 4 h later. As shown in Fig. 6*A*, ~5% of the CD8$^+$ T cells from naive C57BL/6 spleen were producing IFN-γ 16 h after exposure to *L. monocytogenes*-infected BMMϕ. All of the IFN-γ^+CD8$^+$ cells expressed high levels of CD44, suggesting that they were memory T cells. Addition of anti-IL-12 and anti-IL-18 mAbs completely blocked the IFN-γ response, indicating that it was TCR independent (Fig. 6*A*). Thus, the IFN-γ-secreting cells detected in the in vitro assay had the same characteristics that have been described for the innate CD8$^+$ T cell response detected ex vivo. When the BMMϕ were infected with an LLO deletion mutant strain of *L. monocytogenes*, only a few IFN-γ^+CD8$^+$ T cells were detected (Fig. 6*A*). Since LLO was not actively secreted by this strain, these data suggest that there may be an alternate pathway

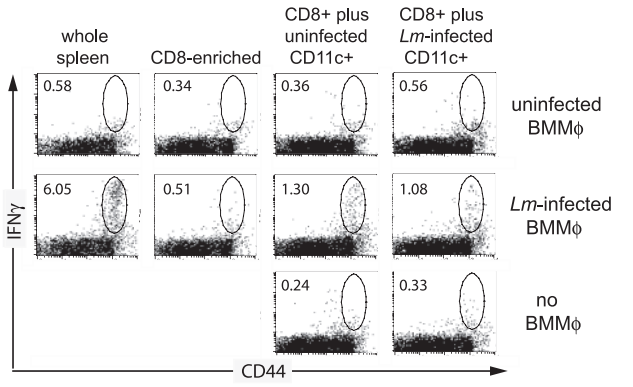

FIGURE 7. Both DC and macrophages are required to induce $CD8^+$ T cells to rapidly secrete IFN-γ during *L. monocytogenes* infection. C57BL/6 BMMϕ seeded in 24-well dishes were infected for 1 h with *L. monocytogenes* and then either 5×10^6 whole splenocytes or 1×10^6 $CD8^+$-enriched splenocytes from a naive C57BL/6 mouse were added. Control wells either lacked BMMϕ or contained uninfected BMMϕ. As indicated, some wells also contained either uninfected or *Listeria*-infected (1 h) $CD11c^+$ DC enriched from naive C57BL/6 spleen. The total number of IFN-γ-expressing $CD44^{high}CD8^+$ T cells was determined by ICCS 18 h later. Primary data plots (gated on $CD8\beta^+$ lymphocytes) for one of three separate experiments are shown; either 5,000 whole splenocytes or 10,000 $CD8^+$-enriched splenocytes were analyzed in each plot. Numbers in the *upper left corner* indicate the percentage of $CD8^+$ T cells in each oval.

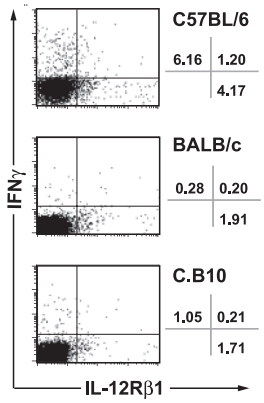

FIGURE 8. $CD8^+$ T cells that rapidly secrete IFN-γ in response to *Listeria* infection do not express high levels of IL-12Rβ1. Mice ($n = 3$) were infected (i.v.) with 10^6 CFU of *L. monocytogenes*, and the total number of IFN-γ-secreting $CD8^+$ T cells was determined ex vivo 18 h postinfection by ICCS. Primary dot plots for IL-12Rβ1 and intracellular IFN-γ staining (gated on $CD8^+$ lymphocytes) for a representative mouse from each group are shown. Numbers to the *right* indicate the percentage of cells in each quadrant.

leading to low levels of IL-12/IL-18-dependent IFN-γ secretion that does not require LLO-mediated cytosolic localization of the bacteria. However, as shown in vivo, LLO was required to induce the majority of the memory $CD8^+$ T cells to secrete IFN-γ.

We next mixed cells from C57BL/6 and C.B10 mice to determine whether the infected macrophage or the responding $CD8^+$ T cell was responsible for the mouse strain differences we observed. We used C.B10 mice to avoid MHC mismatch during the assay, since C.B10 mice, like BALB/c mice, do not demonstrate the early IFN-γ effect (Fig. 5*B*). When naive C.B10 splenocytes were incubated with *Listeria*-infected C.B10 BMMϕ in vitro, a 4-fold increase in the number of IFN-γ^+CD8^+ T cells was detected (Fig. 6*B*), compared with the 22-fold increase in IFN-γ^+ T cells detected for C57BL/6 mice (Fig. 6*A*). These data indicate that as in vivo, C.B10 memory $CD8^+$ T cells do not display a robust early IFN-γ response. C.B10 splenocytes showed the same low level IFN-γ response when incubated with heterologous C57BL/6 BMMϕ; however, C57BL/6 splenocytes rapidly produced IFN-γ in response to C.B10-infected BMMϕ (Fig. 6*B*). Therefore, the responding splenocyte population, rather than the infected macrophage, seemed to be responsible for determining whether or not a robust early IFN-γ response would occur.

Both DC and macrophages are required to induce $CD44^{high}CD8^+$ T cells to rapidly secrete IFN-γ

To verify that the responding $CD8^+$ T cells were the critical cell population that determined whether the early IFN-γ response would be induced, we used $CD8^+$-enriched splenocytes as a source of T cells in the in vitro assay rather than whole naive spleen. Surprisingly, $CD8^+$ T cells isolated from C57BL/6 mice did not secrete IFN-γ following exposure to *L. monocytogenes*-infected autologous BMMϕ (Fig. 7). This suggested that an additional population of cells found in C57BL/6 spleen was required to trigger the early IFN-γ response. Accordingly, we found that adding $CD11c^+$ splenocytes to the in vitro assay resulted in a partial reconstitution of the early IFN-γ response (Fig. 7). Coincubation of either uninfected or *Listeria*-infected $CD11c^+$ cells with naive $CD8^+$ T cells and *Listeria*-infected BMMϕ resulted in the same percentage (~1.0%) of IFN-$\gamma^+CD44^{high}CD8^+$ T cells 18 h later, suggesting that the DC did not need to be directly infected with *L. monocytogenes*. As shown in Fig. 7, coincubation of infected DC alone with $CD8^+$ T cells did not trigger the $CD44^{high}CD8^+$ T cells to secrete IFN-γ, indicating that both macrophages and DC were required to induce memory $CD8^+$ T cells to rapidly secrete IFN-γ. The maximum number of T cells rapidly secreting IFN-γ (~6% of $CD8^+$ T cells) was achieved only in the presence of whole naive spleen. Collectively, these results suggest that interactions between a *Listeria*-infected macrophage and a DC population are required to induce $CD44^{high}CD8^+$ T cells to rapidly secrete IFN-γ and that interactions with other cell types in the spleen may enhance this response.

$CD8^+$ T cells that rapidly secrete IFN-γ do not express higher levels of IL-12 receptor

A recent study suggested that the memory $CD8^+$ T cells capable of rapidly secreting IFN-γ in C57BL/6 mice may have higher levels of IL-12 and IL-18 receptors on their surface (8). We did not observe a difference in IL-12Rβ1 staining on the surface of naive C57BL/6 splenocytes compared with C.B10 splenocytes. For both strains of mice, ~2.0% of splenic $CD8^+$ T cells were IL-12R$\beta1^+$ (data not shown). Eighteen hours after infection with *L. monocytogenes*, the number of IL-12R$\beta1^+$ cells increased to ~4% of splenic $CD8^+$ cells in C57BL/6 mice while the number of IL-12R$\beta1^+CD8^+$ T cells in the spleens of BALB/c or C.B10 mice stayed the same (Fig. 8). However, the majority of $CD8^+$ cells that expressed IFN-γ ex vivo following *L. monocytogenes* infection did not express high levels of IL-12Rβ1 (Fig. 8). These results indicate that high levels of IL-12R on the cell surface are not required in order for $CD44^{high}CD8^+$ T cells to rapidly secrete IFN-γ and suggest that there are other differences between C57BL/6 and BALB/c splenocytes that account for the differential ability to rapidly secrete IFN-γ in response to *Listeria* infection.

Discussion

It has long been known that BALB/c mice are more susceptible to *L. monocytogenes* infection than C57BL/6 mice (21). We previously showed that 3 days postinfection with *L. monocytogenes*,

C57BL/6 mice have well-defined granulomas in the liver, while the more susceptible BALB/c mice showed only neutrophil-rich microabscesses that were characteristic of an acute inflammatory response (1). Granuloma formation is a CD4$^+$ T cell-dependent process that requires secretion of both TNF-α and IFN-γ (22, 23). In this report, we show that early expression of IFN-γ by CD44^{high}CD8$^+$ T cells directly correlates with innate resistance to *L. monocytogenes* infection. Our data are consistent with the hypothesis that rapid production of IFN-γ by memory CD8$^+$ T cells leads to accelerated granuloma formation and walling off of bacteria in the resistant C57BL/6 mice. In strains of mice that lack the early IFN-γ response, acute inflammation continues, and the *Listeria* are able to spread from cell-to-cell, resulting in a larger bacterial burden. NK cells are also a significant source of IFN-γ, and we did find that a greater percentage of NK cells from C57BL/6 mice compared with BALB/c mice were also secreting IFN-γ 18 h after infection with *L. monocytogenes* (our unpublished observation). However, Berg et al. (12) recently showed that IFN-γ-secreting memory CD8$^+$ T cells, and not NK cells, colocalized with foci of *L. monocytogenes* infection in both the spleen and the liver. These observations suggest that IFN-γ secreted by memory CD8$^+$ T cells may be more effective in promoting granuloma formation at sites of infection.

The early IFN-γ effect was not observed following infection with other pathogens, suggesting that a unique product made by *L. monocytogenes* may be responsible for triggering this event. We showed here that infection with *L. monocytogenes* strains that were not actively secreting LLO did not trigger the early IFN-γ effect. Furthermore, infection with an *E. coli* strain expressing LLO did result in rapid IFN-γ expression in C57BL/6 mice. LLO is a pore-forming, cholesterol-dependent cytolysin that is essential for *L. monocytogenes* to escape from phagocytic vacuoles in most host cell types (24, 25). It is not yet clear whether LLO itself triggers the response or whether LLO is needed simply to allow for cytosolic localization of another bacterial product. The fact that we observed a weaker response during infection with *E. coli* OVA/LLO where the cytosolic LLO concentration was limiting (Fig. 3*C*) strongly suggests that LLO is directly responsible for triggering the early IFN-γ effect. However, it is also possible that a bacterial product, such as DNA, that is common to both *E. coli* and *L. monocytogenes* induces the response and that much more of this bacterial product is released into the cytosol during *Listeria* infection than during *E. coli* OVA/LLO infection.

A significant amount of work from Mitsuyama's laboratory (16, 17, 26, 27) has shown that coincubation of murine splenocytes with purified recombinant cholesterol-dependent cytolysins (CDC) such as LLO can trigger secretion of various cytokines, including IL-6 and IFN-γ. These in vitro data suggest that LLO alone can induce a population of cells in the spleen to secrete IFN-γ within 24 h. Nomura et al. (17) further showed that neutralization of IL-12 and IL-18 resulted in an inhibition of IFN-γ production by spleen cells exposed to purified LLO in vitro. Therefore, cytokine production induced by LLO was an IL-12- and IL-18-dependent process, as has been observed for the early IFN-γ response by memory CD8$^+$ T cells. However, in these earlier in vitro studies, IFN-γ expression was measured by RT-PCR or ELISA, and the splenocyte populations producing the IFN-γ were not identified.

One mechanism by which LLO could result in IFN-γ secretion would be to form pores in the cell membrane, allowing for calcium and/or potassium flux in the cell, triggering a signaling pathway that leads to cytokine production (28). However, LLO has an acidic pH optimum and is thought to be significantly more cytolytic in phagocytic vacuoles than in the host cell cytosol or in tissue culture medium with a neutral pH (29, 30). Alternatively, another domain of LLO, not involving the C-terminal active site, could be important for triggering a signaling cascade that results in IFN-γ production. Investigators from Mitsuyama's laboratory (31, 32) showed that truncated versions of either recombinant LLO or the related toxin pneumolysin that lacked the C-terminal active site were still capable of inducing spleen cells to secrete IFN-γ in vitro. In another study, Gekara et al. (33) pretreated LLO with cholesterol to block cytolytic activity and showed that this did not affect the ability of LLO to induce tyrosine phosphorylation in J774 cells, a process that appeared to be dependent on oligomerization of LLO and aggregation of lipid rafts. These data suggest that the cytokine-inducing activity of CDC proteins may be localized to the N terminus and argue against the hypothesis that LLO triggers IFN-γ production simply by forming pores that allow for calcium flux. However, in these in vitro studies, cells were treated exogenously with purified recombinant forms of LLO in tissue culture medium, and it is not clear how much of the toxin may have been taken up by splenocytes or whether LLO acted only from outside the cell. Since exogenous treatment of cells with large quantities of LLO will likely result in cell damage that does not normally occur during intracellular infection, the mechanisms of LLO-induced cytokine production following in vitro treatment of cells may differ significantly from the effects of LLO in the host cell cytosol.

Recent work has demonstrated that cytosolic localization of *L. monocytogenes* activates multiple signaling pathways in macrophages (34–37). One downstream effect of these signaling events is the production of IFN-β, a type I IFN that has long been associated with antiviral immunity and more recently has been shown to have immunomodulatory effects on NK cells and T cells. Mice that lack the ability to either produce or respond to IFN-β are significantly more resistant to *L. monocytogenes* infection (38, 39), and it has been suggested that induction of IFN-β may be deleterious to the host as it actually promotes *L. monocytogenes* pathogenesis (40). Although there is some evidence to indicate that type I IFN can enhance expression of IFN-γ by naive CD8$^+$ T cells (41), other data suggest that IFN-β production inhibits IFN-γ expression during infection of mice (42). However, most of this work has been done in the context of viral infections that do not elicit a robust IL-12 response. Thus, it remains to be determined whether early production of IFN-β following cytosolic localization of *L. monocytogenes* is involved in the early IFN-γ response by memory CD8$^+$ T cells.

It is possible that more than one pathway can lead to rapid IFN-γ production by memory CD8$^+$ T cells during *L. monocytogenes* infection. Kambayashai et al. (9) previously showed that CD44^{high}CD8$^+$ T cells from C57BL/6 mice rapidly produced IFN-γ following injection with either LPS or dsRNA alone and that the secretion of IFN-γ required IL-12 and IL-18 release by macrophages. Those data suggest a role for TLR-mediated signaling in the rapid production of IFN-γ. However, we did not observe an early IFN-γ effect by CD8$^+$ T cells following infection with either *E. coli* or *S. typhimurium*, presumably because injection with large doses of purified LPS can result in the triggering of signaling pathways that are not activated during infection with live LPS-containing bacteria. A recent study showed that incubation of BMMϕ with a variety of recombinant CDC toxins (including LLO) resulted in TNF-α and IL-6 mRNA expression in a TLR4-dependent manner, and the authors suggested that CDC toxins may in fact act as TLR4 agonists (43). However, it is not known whether the basal amount of LLO secreted by extracellular *L. monocytogenes* would be great enough to trigger TLR4 signaling on the surface of host cells during in vivo infection. As shown in Fig. 6*A*, we did see a slight increase in IFN-γ production by CD44^{high}CD8$^+$ T cells when C57BL/6 BMMϕ were infected in

vitro with 10^8 CFU of a LLO mutant strain of *L. monocytogenes*. This response may represent the basal level of activation that occurs following interaction of bacterial products with surface-exposed TLR, while infection with LLO-expressing *Listeria* or *E. coli* results in a greater level of IFN-γ production via a different signal transduction cascade that is triggered in the host cell cytosol.

Although *L. monocytogenes* was the only microorganism we tested that triggered the early IFN-γ effect, another bacterial pathogen has been shown to cause rapid production of IFN-γ by $CD8^+$ T cells. Lertmemongkolchai et al. (10) showed that incubation of spleen cells from C57BL/6 mice with the Gram-negative bacterium *Burkholderia pseudomallei* resulted in significant production of IFN-γ by $CD44^{high}CD8^+$ T cells within 15 h of infection. *B. pseudomallei* encodes a cluster of genes that is similar to the *Shigella ipa/mxi/spa* loci, and it is thought that these genes are responsible for promoting phagosome escape and intracellular survival of *Burkholderia* in macrophages (44). It is not yet clear whether this early IFN-γ effect is triggered by a *B. pseudomallei* protein with functional similarities to LLO or whether cytosolic localization of *Burkholderia* simply allows for detection of another bacterial product that may be found in both bacterial species.

Results from our in vitro assays indicate that both macrophages and DC are essential to induce $CD44^{high}CD8^+$ T cells to rapidly secrete IFN-γ. Although innate production of IFN-γ by memory $CD8^+$ T cells is known to be dependent on both IL-12 and IL-18, it is not yet clear which cell type is the primary source of these cytokines. Liu et al. (45) showed that splenic DC harvested from C57BL/6 mice 6 h postinfection with *L. monocytogenes* expressed more IL-12 mRNA than DC harvested from *Listeria*-infected BALB/c mice, suggesting that DC may in fact be the key splenocyte population that determines whether a mouse will be an early IFN-γ responder or nonresponder. Further work will be needed to determine which cell type(s) must come in direct contact with LLO (and/or other bacterial products derived from *Lm*) to trigger the early IFN-γ effect.

Our data clearly shows that memory $CD8^+$ T cells have innate immune functions in C57BL/6 mice that are lacking in the more susceptible BALB/c mice. These results may help to explain differences in both innate and adaptive immune responses that have been observed by a number of investigators using the BALB/c and C57BL/6 mouse models of *Listeria* infection. In the future, it will be interesting to determine whether memory $CD8^+$ T cells in humans are also capable of responding to *Listeria* infection by rapidly secreting IFN-γ and whether the ability to mount an early IFN-γ response correlates with increased susceptibility to systemic infection with *L. monocytogenes*.

Acknowledgments

We thank Darren Higgins for bacterial strains, Denise McElroy and Greg Bauman for technical assistance and Russell Vance for critical review of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Boyartchuk, V. L., K. W. Broman, R. E. Mosher, S. E. F. D'Orazio, M. N. Starnbach, and W. F. Dietrich. 2001. Multigenic control of *Listeria monocytogenes* susceptibility in mice. *Nat. Genet.* 27: 259–260.
2. Boyartchuk, V., M. Rojas, B.-S. Yan, O. Jobe, N. Hurt, D. M. Dorfman, D. E. Higgins, W. F. Dietrich, and I. Kramnik. 2004. The host resistance locus sst1 controls innate immunity to *Listeria monocytogenes* infection in immunodeficient mice. *J. Immunol.* 173: 5112–5120.
3. Gervais, F., M. Stevenson, and E. Skamene. 1984. Genetic control of resistance to *Listeria monocytogenes*: regulation of leukocyte inflammatory responses by the Hc locus. *J. Immunol.* 132: 2078–2083.
4. Andersson, A., J. W. Dai, J. P. D. Santo, and F. Brombacher. 1998. Early IFN-γ production and innate immunity during *Listeria monocytogenes* infection in the absence of NK cells. *J. Immunol.* 161: 5600–5606.
5. Bancroft, G. J. 1993. The role of natural killer cells in innate resistance to infection. *Curr. Opin. Immunol.* 5: 503–510.
6. Kaufmann, S. H. E., E. Hug, and G. D. Libero. 1986. *Listeria monocytogenes* reactive T lymphocyte clones with cytolytic activity against infected target cells. *J. Exp. Med.* 164: 363–364.
7. Ladel, C. H., I. E. F. Flesch, J. Arnoldi, and S. H. E. Kaufmann. 1994. Studies with MHC-deficient knock-out mice reveal impact of both MHC-I and MHC-II dependent T cell responses on *Listeria monocytogenes* infection. *The J. Immunol.* 153: 3116–3122.
8. Berg, R. E., E. Crossley, S. Murray, and J. Forman. 2003. Memory $CD8^+$ T cells provide innate immune protection against *Listeria monocytogenes* in the absence of cognate antigen. *J. Exp. Med.* 198: 1583–1593.
9. Kambayashi, T., E. Assarsson, A. E. Lukacher, H.-G. Ljunggren, and P. E. Jensen. 2003. Memory $CD8^+$ T cells provide an early source of IFN-γ. *J. Immunol.* 170: 2399–2408.
10. Lertmemongkolchai, G., G. Cai, C. A. Hunter, and G. Bancroft. 2001. Bystander activation of $CD8^+$ T cells contributes to the rapid production of IFN-γ in response to bacterial pathogens. *J. Immunol.* 166: 1097–1105.
11. Berg, R. E., C. J. Cordes, and J. Forman. 2002. Contribution of $CD8^+$ T cells to innate immunity: IFN-γ secretion induced by IL-12 and IL-18. *Eur. J. Immunol.* 32: 2807–2816.
12. Berg, R. E., E. Crossley, S. Murray, and J. Forman. 2005. Relative contributions of NK and CD8 T cells to IFN-γ mediated innate immune protection against *Listeria monocytogenes*. *J. Immunol.* 175: 1751–1757.
13. Jones, S., and D. A. Portnoy. 1994. Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. *Infect. Immun.* 62: 5608–5613.
14. Starnbach, M. N., M. J. Bevan, and M. F. Lampe. 1994. Protective cytotoxic T lymphocytes are induced during murine infection with *Chlamydia trachomatis*. *J. Immunol.* 153: 5183–5189.
15. Frehel, C., C. d. Chastellier, T. Lang, and N. Rastogi. 1986. Evidence for inhibition of fusion of lysosomal and prelysosomal compartments with phagosomes in macrophages infected with pathogenic *Mycobacterium avium*. *Infect. Immun.* 52: 252–262.
16. Nomura, T., I. Kawamura, K. Tsuchiya, C. Kohda, H. Baba, Y. Ito, T. Kimoto, I. Watanabe, and M. Mitsuyama. 2002. Essential role of interleukin-12 (IL-12) and IL-18 for γ interferon production induced by listeriolysin O in mouse spleen cells. *Infect. Immun.* 70: 1049–1055.
17. Xiong, H., I. Kawamura, T. Nishibori, and M. Mitsuyama. 1994. Cytokine gene expression in mice at an early stage of infection with various strains of *Listeria* spp. differing in virulence. *Infect. Immun.* 62: 3649–3654.
18. Higgins, D. E., N. Shastri, and D. A. Portnoy. 1999. Delivery of protein to the cytosol of macrophages using *Escherichia coli* K-12. *Mol. Microbiol.* 31: 1631–1641.
19. Radford, K. J., D. E. Higgins, S. Pasquini, E. J. Cheadle, L. Carta, A. M. Jackson, N. R. Lemoine, and G. Vassaux. 2002. A recombinant *E. coli* vaccine to promote MHC class I-dependent antigen presentation: application to cancer immunotherapy. *Gene Ther.* 9: 1455–1463.
20. Hu, P. Q., R. J. Tuma-Warrino, M. A. Bryan, K. G. Mitchell, D. E. Higgins, S. C. Watkins, and R. D. Salter. 2004. *Escherichia coli* expressing recombinant antigen and listeriolysin O stimulate class I-restricted $CD8^+$ T cells following uptake by human APC. *J. Immunol.* 172: 1595–154.
21. Cheers, C., and I. F. C. McKenzie. 1978. Resistance and susceptibility of mice to bacterial infection: genetics of listeriosis. *Infect. Immun.* 19: 755–762.
22. Bhardwaj, V., O. Kanagawa, P. E. Swanson, and E. R. Unanue. 1998. Chronic *Listeria* infection in SCID mice: requirements for the carrier state and the dual role of T cells in transferring protection or suppression. *J. Immunol.* 160: 376–384.
23. Mielke, M. E. A., C. Peters, and H. Hahn. 1997. Cytokines in the induction and expression of T cell-mediated granuloma formation and protection in the murine model of listeriosis. *Immunol. Rev.* 158: 79–93.
24. Gaillard, J. L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti. 1987. In vitro model of penetration and intracellular growth of *L. monocytogenes* in human enterocyte-like cell line Caco-2. *Infect. Immun.* 55: 2822–2829.
25. Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs. 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *J. Exp. Med.* 167: 1459–1471.
26. Ito, Y., I. Kawamura, C. Kohda, H. Baba, T. Nomura, T. Kimoto, I. Watanabe, and M. Mitsuyama. 2003. Seeligeriolysin O, a cholesterol-dependent cytolysin of *Listeria seeligeri*, induces γ interferon from spleen cells of mice. *Infect. Immun.* 71: 234–241.
27. Tsuchiya, K., I. Kawamura, A. Takahashi, T. Nomura, C. Kohda, and M. Mitsuyama. 2005. Listeriolysin O-induced membrane permeation mediates persistent interleukin-6 production in Caco-2 cells during *Listeria monocytogenes* infection in vitro. *Infect. Immun.* 73: 3869–3877.
28. Repp, H., Z. Pamukci, A. Koschinski, E. Domann, A. Darji, J. Birringer, D. Brockmeier, T. Chakraborty, and F. Dreyer. 2002. Listeriolysin O of *Listeria monocytogenes* forms Ca^{2+}-permeable pores leading to intracellular Ca^{2+} oscillations. *Cell. Microbiol.* 4: 483–491.
29. Beauregard, K. E., K. D. Lee, R. J. Collier, and J. A. Swanson. 1997. pH-dependent perforation of macrophage phagosomes by listeriolysin O from *Listeria monocytogenes*. *J. Exp. Med.* 186: 1159–1163.

30. Glomski, I. J., M. M. Gedde, A. W. Tsang, J. A. Swanson, and D. A. Portnoy. 2002. The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. *J. Cell Biol.* 156: 1029–1038.
31. Baba, H., I. Kawamura, C. Kohda, T. Nomura, Y. Ito, T. Kimoto, I. Watanabe, S. Ichiyama, and M. Mitsuyama. 2002. Induction of γ interferon and nitric oxide by truncated pneumolysin that lacks pore-forming activity. *Infect. Immun.* 70: 107–113.
32. Kohda, C., I. Kawamura, H. Baba, T. Nomura, Y. Ito, T. Kimoto, I. Watanabe, and M. Mitsuyama. 2002. Dissociated linkage of cytokine-inducing activity and cytotoxicity to different domains of listeriolysin O from *Listeria monocytogenes*. *Infect. Immun.* 70: 1334–1341.
33. Gekara, N. O., T. Jacobs, T. Chakraborty, and S. Weiss. 2005. The cholesterol-dependent cytolysin listeriolysin O aggregates rafts via oligomerization. *Cell. Microbiol.* 7: 1345–1356.
34. McCaffrey, R. L., P. Fawcett, M. O'Riordan, K.-D. Lee, E. A. Havell, P. O. Brown, and D. A. Portnoy. 2004. A specific gene expression program triggered by Gram-positive bacteria in the cytosol. *Proc. Natl. Acad. Sci. USA* 101: 11386–11391.
35. O'Riordan, M., C. H. Yi, R. Gonzales, K.-D. Lee, and D. A. Portnoy. 2002. Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. *Proc. Natl. Acad. Sci. USA* 99: 13861–13866.
36. Stetson, D. B., and R. Medzhitov. 2006. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 24: 93–103.
37. Stockinger, S., B. Reutterer, B. Schaljo, C. Schellack, S. Brunner, T. Materna, M. Yamamoto, S. Akira, T. Taniguchi, P. J. Murray, et al. 2004. IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by TLR and Nod2-independent mechanism. *J. Immunol.* 173: 7416–7425.
38. Auerbach, V., D. G. Brockstedt, N. Meyer-Morse, M. O'Riordan, and D. A. Portnoy. 2004. Mice lacking the type I interferon receptor are resistant to *Listeria monocytogenes*. *J. Exp. Med.* 200: 527–533.
39. O'Connell, R. M., S. K. Saha, S. A. Vaidya, K. W. Bruhn, G. A. Miranda, B. Zarnegar, A. K. Perry, B. O. Nguyen, T. F. Lane, T. Taniguchi, et al. 2004. Type I interferon production enhances susceptibility to *Listeria monocytogenes* infection. *J. Exp. Med.* 200: 437–445.
40. Portnoy, D. A. 2005. Manipulation of innate immunity by bacterial pathogens. *Curr. Opin. Immunol.* 17: 25–28.
41. Curtsinger, J. M., D. C. Lins, C. M. Johnson, and M. F. Mescher. 2005. Signal 3 tolerant CD8 T cells degranulate in response to antigen but lack granzyme B to mediate cytolysis. *J. Immunol.* 175: 4392–4399.
42. Biron, C. A. 2001. Interferons α and β as immune regulators—a new look. *Immunity* 14: 661–664.
43. Park, J. M., V. H. Ng, S. Maeda, R. F. Rest, and M. Karin. 2004. Anthrolysin O and other Gram-positive cytolysins are Toll-like receptor 4 agonists. *J. Exp. Med.* 200: 1647–1655.
44. Stevens, M. P., M. W. Wood, L. A. Taylor, P. Monaghan, P. Hawes, P. W. Jones, T. S. Wallis, and E. E. Galyov. 2002. An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. *Mol. Microbiol.* 46: 649–659.
45. Liu, T., H. Nishimura, T. Matsuguchi, and Y. Yoshikai. 2000. Differences in interleukin-12 and -15 production by dendritic cells at the early stage of *Listeria monocytogenes* infection between BALB/c and C57BL/6 mice. *Cell. Immunol.* 202: 31–40.